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Cyclooxygenase-2-Dependent Expression of Angiogenic CXC Chemokines ENA-78/CXC Ligand (CXCL) 5 and Interleukin-8/CXCL8 in Human Non-Small Cell Lung Cancer

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ABSTRACT

Elevated tumor cyclooxygenase (COX)-2 activity plays a multifaceted role in non-small cell lung cancer (NSCLC). To elucidate the role of COX-2 in the *in vitro* and *in vivo* expression of two known NSCLC angiogenic peptides, CXC ligand (CXCL) 8 and CXCL5, we studied two COX-2 gene-modified NSCLC cell lines, A549 and H157. COX-2 overexpression enhanced the *in vitro* expression of both CXCL8 and CXCL5. In contrast, specific COX-2 inhibition decreased the production of both peptides as well as nuclear translocation of nuclear factor κB. In a severe combined immunodeficient mouse model of human NSCLC, the enhanced tumor growth of COX-2-overexpressing tumors was inhibited by neutralizing anti-CXCL5 and anti-CXCL8 antisera. We conclude that COX-2 contributes to the progression of NSCLC tumorigenesis by enhancing the expression of angiogenic chemokines CXCL8 and CXCL5.

INTRODUCTION

Cyclooxygenase (COX) is the rate-limiting enzyme for the production of prostaglandins (PGs) and thromboxanes from free arachidonic acid (1). The enzyme is bifunctional, with fatty acid COX and PG hydroperoxidase activities. Two forms of COX have been described: (*a*) a constitutively expressed enzyme, COX-1, present in most cells and tissues; and (*b*) an inducible isoenzyme, COX-2, expressed in response to cytokines, growth factors, and other stimuli (2, 3). We and others have reported that COX-2 is frequently constitutively elevated in human non-small cell lung cancer [NSCLC (4–7)]. High levels of COX-2 mRNA and protein expression correlate with a poor prognosis in this disease (8, 9). In agreement with these clinical data, lung tumors with high COX-2 expression metastasize at high frequency (10), promote angiogenesis (11–15), and are more resistant to apoptosis by various stimuli (16–19). Our previous studies documented that COX-2 expression in NSCLC promotes immune suppression and tumor invasion (4, 20–22).

Tumor progression is dependent on angiogenesis. Net tumor-induced angiogenesis is due to an imbalance in the overexpression of angiogenic factors as compared with angiostatic factors (23). Previous studies have documented the role of two CXC family chemokines, CXC ligand (CXCL) 8 and CXCL5, in human NSCLC (24–33). The CXC chemokine family consists of a number of structurally related peptides that are either angiogenic or angiostatic. All angiogenic members of this family, including CXCL8 and CXCL5, possess a 3-amino acid motif, the glutamic acid-leucine-arginine (ELR) motif, which immediately precedes the first cysteine in their CXC motif located in their NH_2 terminus (34). The angiogenic activity of ELR+CXC chemokines is mediated via the CXC motif ligand receptor 2 [CXCR2 (35)]. CXCR2 has been shown to bind all ELR+CXC chemokines, including CXCL8 and CXCL5, with high affinity (36–39). The expression of CXCL8 and CXCL5 is up-regulated by nuclear factor (NF)-κB (40–43). NF-κB, a heteromeric transcription factor, is activated in response to many stress signals. Factors activating NF-κB-related gene expression initiate degradation of the cytoplasmic NF-κB/IκB complex and subsequent translocation of NF-κB into the nucleus, where it activates gene expression (40, 44, 45).

The current study defines a role for COX-2 in the expression of NSCLC angiogenic peptides CXCL8 and CXCL5. We have found that COX-2 up-regulates both the *in vitro* and *in vivo* expression of CXCL8 and CXCL5 in NSCLC cells. Our data suggest that COX-2 up-regulates the expression of these chemokines by activating NF-κB nuclear translocation. *In vivo*, the COX-2-enhanced expression of CXCL8 and CXCL5 was associated with enhanced NSCLC tumor growth and angiogenesis.

MATERIALS AND METHODS

Cell Lines. The NSCLC cell lines used in this study have been described previously by us in detail (21, 22). Briefly, A549 (human lung adenocarcinoma) and H157 (squamous cell carcinoma) were obtained from American Type Culture Collection (Manassas, VA) and National Cancer Institute, NIH, respectively. A 2.0-kb cDNA fragment of human COX-2 (generously provided by Dr. Harvey Herschman, University of California, Los Angeles) was expressed in these cell lines in sense and antisense orientation. The following cell line terminology is used in the text: (*a*) A549-S and H157-S are the cell lines transfected with COX-2 in sense orientation; (*b*) A549-AS and H157-AS are the cell lines transfected with COX-2 in antisense orientation; and (*c*) A549-V and H157-V are the cells transfected with the expression vector pLNCX only.

Reagents. SC58236 (provided by Pharmacia, Peapack, NJ) was dissolved in 100% ethanol at 100 mM; BAY-11-7082 [(E)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (Calbiochem, La Jolla, CA)] was dissolved in DMSO at 50 mM, and interleukin (IL)-1β (BD Biosciences, Palo Alto, CA) was dissolved in PBS at 2,000 units/ml. Anti-PGE_2 [monoclonal antibody (mAB) 2B5] and isotype-matched control mouse IgG1 (MOPC21) were provided by Pharmacia and dissolved in PBS at 1 mg/ml. Polyclonal goat antimurine CXCR2 was produced by the immunization of a goat with a peptide containing the ligand-binding sequence MGEFKVDKFNIQDFFSG of CXCR2. This neutralizing polyclonal anti-CXCR2 antibody blocks mouse CXCR2 and detects CXCR2 by Western blot and fluorescence-activated cell-sorting analysis of neutrophils *in vivo* (46, 47). Neutralizing antihuman CXCL5 and anti-human CXCL8 sera used for *in vivo* experiments are highly specific neutralizing antisera described in our previous studies (24, 25). They were produced by immunization of rabbits or goats with CXCL5 or CXCL8 (R&D Systems, Minneapolis, MN) in multiple intradermal sites with complete Freund's adjuvant. Direct ELISA was used to evaluate antisera titers, and sera were used for Western blot, ELISA, and neutralization assays when titers had reached >1/1,000,000. Furthermore,

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in a sandwich ELISA, these antibodies are specific for either CXCL5 or CXCL8 without cross-reactivity to a panel of 12 other recombinant human cytokines or the murine chemokines CXCL1, CXCL2, and CXCL3.

Growth Conditions. The A549 and H157 cell lines were plated in standard 6-well plates (Corning Inc., Corning, NY) at 20×10^3 and 40×10^3 cells/well, respectively. The cells were grown for 24 h, at which point SC58236 at 12.5 μM was added into the plating medium. Seventy-two h later, supernatants were harvested, and cells were counted by hemacytometer. Cells were grown in duplicates for all conditions. For purification of total RNA, the experiment was scaled up in 10-cm Petri dishes (Becton Dickinson, Franklin Lakes, NJ). In experiments with BAY-11-7082, the cells were plated in 6-well plates as described above and grown for 72–96 h, at which time-point the wasted medium was removed and replaced with fresh 10% FBS-RPMI 1640 containing BAY-11-7082. The cells were then grown for an additional 24 h, trypsinized, and counted, and the supernatants were analyzed for CXCL8 and CXCL5.

Blocking PGE_2 with Neutralizing Anti-PGE_2 Monoclonal Antibody. H157-S, H157-V, A549-S, and A549-V were plated as described above and grown for 96 h in 10% FBS-RPMI 1640, at which time-point fresh medium was added for the final 24 h of incubation. For the final 24 h, cells were divided into four treatment groups: (*a*) control mAB; (*b*) neutralizing anti-PGE_2 mAB; (*c*) control mAB with IL-1β at 200 units/ml; and (*d*) anti-PGE_2 mAB with IL-1β at 200 units/ml. All groups were grown in duplicates. Our previous studies show that IL-1β is a potent inducer of both COX-2 and PGE_2 in A549-S, A549-V, H157-S, and H157-V cells. In contrast, the A549-AS and H157-AS cells produce very low levels of PGE_2, and they were therefore excluded from the anti-PGE_2 mAB experiments (21, 22).

PGE_2 Enzyme Immunoassay. Cells were stimulated with IL-1β (200 units/ml; Genzyme, Cambridge, MA) for 24 h. PGE_2 concentration in each group (with or without IL-1β stimulation) was measured by enzyme immunoassay using a PGE_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) as reported previously (21). All measurements were made in duplicates.

CXCL8 and CXCL5 ELISA. The concentrations of CXCL8 and CXCL5 were determined as described previously (24, 25). The antibody pairs and standards were purchased from R&D Systems. For CXCL8 ELISA, MAB208 was used as a coating antibody, and BAF208 was used as a biotinylated antibody. For CXCL5 ELISA, MAB254 was used as a coating antibody, and BAF254 was used as a biotinylated antibody. The 96-well MaxiSorp ELISA plates (Nunc, Rochester, NY) were coated with a capture antibody overnight, washed three to four times in PBS/0.5% Tween 20, and blocked with PBS/2% BSA for 1–2 h. After washing the plates as described above, the wells were incubated with supernatants (50 μl) for 1–3 h. The biotinylated antibody was added after washing the plates, and cells were incubated for 1 h. Horseradish peroxidase-conjugated streptavidin (Jackson Laboratory, Bar Harbor, ME) was added after washing the plates, and plates were incubated for 30–45 min, followed by three to four washes. Peroxidase and tetramethylbenzidine peroxidase substrate (Kierkegaard and Perry Laboratories, Gaithersburg, MD) mix was added for 5–10 min, and the reaction was stopped by 1 M phosphoric acid. The $A_{450\ nm}$ was determined by Bio-Lab Benchmark Microplate reader (Bio-Rad, Hercules, CA). Each plate contained appropriate standards for the standard curve.

Northern Blots. Total RNA was purified using the RNeasy Midi kit from Qiagen (Valencia, CA). Ten μg of total RNA from each cell line were fractionated in formaldehyde gel, blotted onto nylon membrane, and hybridized under high-stringency conditions with CXCL8- and CXCL5-specific probes. The CXCL8- and CXCL5-specific probes were generated by PCR using [α-^{32}P]dCTP (specific activity, 3000 Ci/mmol; Blue/Neg/513H; Perkin-Elmer, Franklin Lakes, NJ). The unlabeled, specific CXCL8 and CXCL5 templates for PCR labeling were generated by PCR using the total cDNA from parental A549 cells. The initial 5-min denaturation period at 95°C was followed by 40 cycles of amplification, each consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s. The following primers were used: CXCL8 template, CXCL8FOR1 (5′-ACC-TTT-CCA-CCC-CAA-ATT-TAT-CAA-A -3′) and CXCL8REV (5′-TAA-CCA-GGA-ATC-TTG-TAT-TGC-ATC-T-3′); and CXCL5, CXCL5FOR1 (5′-ACC-ACG-CAG-GGA-GTT-CAT-CCC-AAA-A-3′) and CXCL5REV (5′-GCT-AAC-TAC-TGG-GGA-AAC-CAA-ATA-AA-3′). The resulting CXCL8 and CXCL5 fragments were purified from agarose gel, and small amounts of them were used in the second round of PCR to generate the [α-^{32}P]dCTP-labeled specific CXCL8 and CXCL5 probes. The forward primer for labeling the CXCL8 probe was CXCL8FOR2 (5′-TTG-AAG-AGG-GCT-GAG-AAT-TCA-TAA-A-3′), and the forward primer for labeling the CXCL5 probe was CXCL5FOR2 (5′-CTG-AAG-AAC-GGG-AAG-GAA-ATT-TGT-C-3′). The reverse primers used for labeling were as described above, CXCL8REV and CXCL5REV. The labeling was carried out in standard PCR buffer in a total volume of 20 μl at low nucleotide concentration. The final concentration for each of the cold nucleotides (dATP, dTTP, and dGTP) was 3 μM, and the concentration of [α-^{32}P]dCTP was ~2 μM. We used ~10 μl of radioactive dCTP per 20 μl of PCR labeling mix. The same cycling regimen as described above was used for PCR labeling, except that a total of 45 cycles of PCR was carried out. The β-actin probe was amplified from total cDNA from parental A549 cells using the same PCR regimen as described above. The following primers were used: forward primer, 5′-CCA-TCG-AGC-ACG-GCA-TCG-TC-3′; and reverse primer, 5′-TCC-AGA-CGC-AGG-ATG-GCA-TG-3′.

NSCLC Tumor Growth in Severe Combined Immunodeficient (SCID) Mice. Pathogen-free SCID Beige CB17 (8–12 weeks of age) mice were obtained from the University of California, Los Angeles animal facility and maintained in the West Los Angeles VA Animal Research *vivarium*. All studies were approved by the institution's animal studies review board. Five million cells of each NSCLC cell line were implanted s.c. in the suprascapular area of BALB/C SCID mice. Tumor growth was assessed three times each week after tumor implantation. Two bisecting diameters of each tumor were measured with calipers, and the volume was calculated using the formula $0.4 \times ab^2$, where *a* represents the larger diameter, and *b* represents the smaller diameter. Each group consisted of eight animals. At the conclusion of the experiment (day 50), the animals were sacrificed, and tumors were removed and homogenized in antiprotease buffer. These tumor lysates were cleared of insoluble debris by centrifugation and frozen at –80°C. In three separate series of experiments, the tumor growth of NSCLC lines was monitored. In the first series, the tumor growth of untreated NSCLC cell lines was monitored. In the second series, the tumor growth of both SC58236-treated parental NSCLC cell lines was compared with that of their respective diluent-treated control. SC58236 was injected i.p. at 3 mg/kg body weight, 3×/week. In the third series, the tumor growth of antihuman CXCL8 and antihuman CXCL5 (see above) antisera-treated NSCLC cell lines was compared with that of untreated control NSCLC cell lines. Both CXC antisera and the control antiserum were injected at 0.5 ml, i.p., 3×/week. The tumor volume was determined as described above.

Rat Corneal Micropocket Assay of Angiogenesis. Equal volumes of lyophilized supernatants normalized to total protein were combined with sterile Hydron (IFN Sciences Inc., New Brunswick, NJ) casting solution. The anti-CXCR2 (see above) or control antibody (goat polyclonal) was mixed at a 1:100 dilution with the specimen to be tested and then added to the Hydron. Aliquots (5 μl) were pipetted onto an inverted sterile polypropylene specimen container and polymerized overnight in a laminar flow hood under UV light. Before implantation, pellets were rehydrated with normal saline. Animals were given i.p. ketamine (150 mg/kg) and atropine (250 μg/kg) for anesthesia. Corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution followed by implantation of the Hydron pellet into an intracorneal pocket. Six days after implantation, animals received heparin (1,000 units) and ketamine (150 mg/kg) i.p., followed by a 10-ml perfusion of colloidal carbon via the left ventricle. Corneas were harvested and photographed. Positive neovascularization responses were defined as sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant. Negative responses were defined as either no growth or only an occasional sprout or hairpin loop displaying no evidence of sustained growth. For quantitative analysis of angiogenesis in the cornea, corneas were further microscopically analyzed using NIH Image 1.55 software to determine vascular density of the ingrowth of the vessels.

Preparation of Nuclear Extracts. Cells (1×10^7) were washed twice with cold PBS, and the cell pellet was suspended in 40 μl of cell lysis buffer for 10 min on ice. Nuclei were extracted by sedimentation (microcentrifugation at 6,500 rpm) for 10 min at 4°C. The resulting nuclear pellet was then suspended in 15 μl of extraction buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] and incubated for 10 min at 4°C with brief intermittent mixing. The mixture was microcentrifuged (14,000 rpm for 10 min at 4°C), and the nuclear protein was resuspended in 60 μl of extraction buffer D [20

mM HEPES (pH 7.9), 25% glycerol, 50 mM NaCl, 1.5 mM KCl, 0.2 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride].

Electrophoretic Mobility Shift Assay. Ten μg of nuclear extract were preincubated in 20 μl (total reaction volume) of binding buffer containing 20 mM HEPES (pH 7.9) 80 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 8% glycerol, and 2 μg of poly(deoxyinosinic-deoxycytidylic acid) (Amersham Pharmacia Biotech) for 15 min at 4°C. The reaction mixture was then incubated with 1×10^5 cpm of [γ-^{32}P]ATP (3000 Ci/mM)-labeled double-stranded NF-κB consensus binding oligonucleotide (AGT-TGA-GGG-GAC-TTT-CCC-AGG-G; Santa Cruz Biotechnology, Santa Cruz, CA) for 20 min at room temperature. The samples were then resolved by nondenaturing 6% PAGE in 0.5× Tris-borate EDTA buffer, dried, and analyzed by autoradiography.

Statistics. Student's *t* test assuming equal variance was used in statistical analysis.

RESULTS

COX-2 Determines the Expression of CXCL8 and CXCL5 in NSCLC. To assess whether COX-2 regulated the expression of ELR+CXC chemokines CXCL5 and CXCL8, NSCLC cell lines transfected with COX-2-S, COX-2-AS, or pLNCX (vector control) were examined for the expression of CXCL5 and CXCL8 (Fig. 1). The overexpression of COX-2 in NSCLC cell lines significantly increased expression of both CXCL8 and CXCL5. Conversely, genetic ablation of COX-2 expression significantly reduced the expression of CXCL8 and CXCL5. In fact, the mRNA and protein expression of CXCL8 and CXCL5 in H157 cells was completely suppressed in H157-AS (Fig. 1). Furthermore, a COX-2 inhibitor drug, SC58236, inhibited the expression of CXCL8 in A549-S and H157-S cells (data not shown).

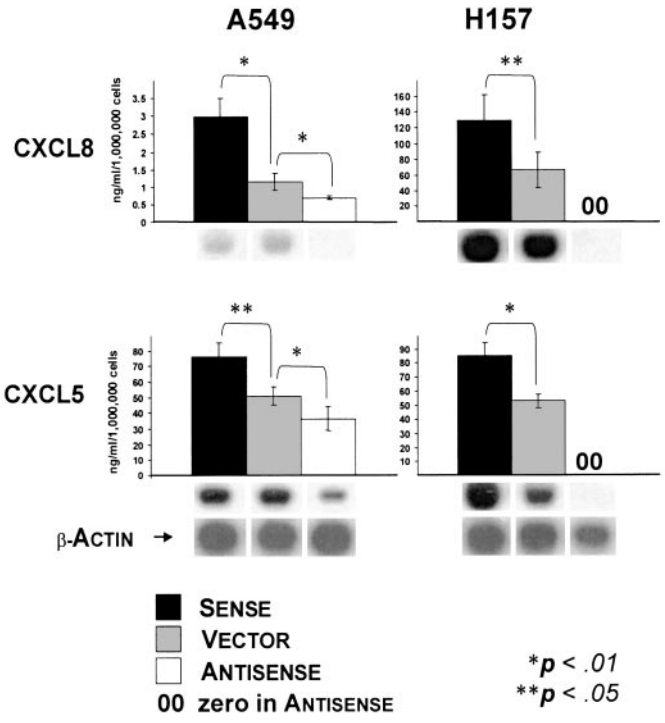

Fig. 1. Cyclooxygenase-2-dependent expression of CXC ligand (CXCL) 8 and CXCL5 in non-small lung cancer. CXCL8 and CXCL5 concentrations in cell supernatants were determined by ELISA. *Bar graphs* show the values of CXCL8 and CXCL5 protein expression, adjusted for cell number. The *photographs* under the bar graphs demonstrate CXCL8 and CXCL5 mRNA expression. β-Actin mRNA expression is depicted below the CXCL5 mRNA expression data (see "Materials and Methods"). Each column represents the mean ± SE of four to five independent experiments.

COX-2 Determines NSCLC Tumor Growth and CXCL8 and CXCL5 Content in a SCID Mouse Model for Human NSCLC. To determine whether COX-2 modulates NSCLC tumor growth and the *in vivo* expression of CXCL8 and CXCL5, we used a SCID mouse model for human NSCLC tumorigenesis. As depicted in Fig. 2, overexpression of COX-2 in H157 cells increased tumor growth. Conversely, the genetic inhibition of COX-2 suppressed the tumor growth of H157 cells (Fig. 2*A*). In addition, in a separate experiment, SC58236 suppressed the tumor growth of the parental H157 cells in SCID mice (Fig. 2*B*). Similar to the results with H157 cells, the genetic or pharmacological inhibition of COX-2 in A549 cells resulted in reduced tumor growth (data not shown). Consistent with our *in vitro* data, the NSCLC tumor content of CXCL8 and CXCL5 in SCID mice was COX-2 dependent in that both genetic and pharmacological inhibition of COX-2 lowered the *in vivo* CXCL8 and CXCL5 content (Table 1). Also, the overexpression of COX-2 in NSCLC cells increased the tumor content of CXCL5 (Table 1). The importance of CXCL5 and CXCL8 in COX-2-dependent NSCLC tumor growth was confirmed by the effect of neutralizing antihuman CXCL8 and antihuman CXCL5 antisera. Statistically significant reduction in H157-S tumor growth was observed in response to treatment with either neutralizing anti-CXCL5 or anti-CXCL8 (Fig. 2*C*).

COX-2-Mediated Angiogenic Capacity of NSCLC Cells Is Dependent on CXCL8 and CXCL5. To determine whether COX-2-dependent angiogenic activity was mediated via CXCL5 and CXCL8, conditioned medium from H157-S, H157-AS, and H157-V was assessed in an *in vivo* angiogenesis assay. Compared with H157-AS, the supernatants of H157-S and H157-V exhibited a distinctly higher angiogenic capacity in a rat corneal pocket model (Fig. 3). Because all ELR+CXC chemokines bind to CXCR2 to mediate their angiogenic activity (35), the angiogenic activity of the conditioned medium from H157-S, H157-AS, and H157-V was assessed in the presence of specific anti-CXCR2 or control antibodies. Conditioned media from H157-S and H157-V demonstrated constitutive angiogenic activity (Fig. 3). However, when the conditioned media of these cells were assessed in the presence of anti-CXCR2 antibodies, the angiogenic activity was significantly reduced, to a level comparable with the angiogenic activity of H157-AS cells (Fig. 3). The capacity of anti-CXCR2 to block the COX-2-dependent angiogenesis supports the hypothesis that CXCL8 and CXCL5 mediate a significant portion of COX-2-dependent angiogenesis in NSCLC.

Specific Genetic Inhibition of COX-2 in NSCLC Cells Decreases NF-κB Nuclear Translocation. As shown in earlier studies, NF-κB activity regulates the expression of CXCL5 and CXCL8 and angiogenesis (43, 48–52). In addition, nonsteroidal anti-inflammatory drugs modulate NF-κB activity (53). Thus, we sought to elucidate whether the expression level of COX-2 modulates the nuclear translocation of NF-κB in NSCLC cells, and whether the expression of CXCL8 and CXCL5 in NSCLC cells is NF-κB dependent. Consistent with previous reports, pharmacological inhibition of NF-κB by BAY-11-7082 inhibited the expression of CXCL8 (41), as well as CXCL5 (Fig. 4*A*). Also, genetic inhibition of COX-2 clearly down-regulated the nuclear translocation of NF-κB in both sets of NSCLC cells (Fig. 4*B*). These findings suggest that NF-κB nuclear translocation plays an important role in the COX-2-regulated production of CXCL8 and CXCL5.

Endogenously Produced PGE_2 Modulates the *in Vitro* Expression of CXCL8. To assess whether PGE_2 modulates the expression of CXCL5 and CXCL8 in NSCLC cell lines, we cultured the A549 and H157 cell lines in presence of specific neutralizing anti-PGE_2 mAB or isotype-matched control mAB. Our previous studies show that IL-1β up-regulates both COX-2 and PGE_2 in H157 and A549

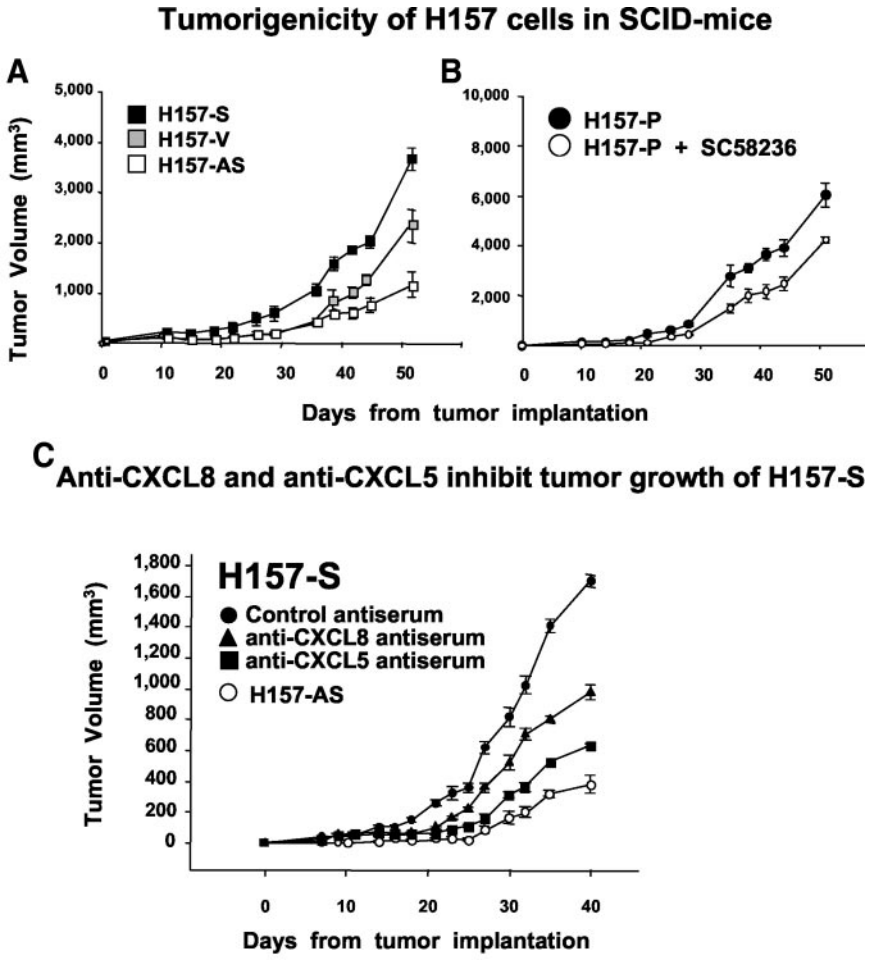

Fig. 2. Cyclooxygenase-2 inhibition limits non-small lung cancer tumor growth in a CXC ligand (CXCL) 5- and CXCL8-dependent manner. Five million cells of each cell line were implanted s.c. in severe combined immunodeficient Beige/CB17 mice (see "Materials and Methods"). *A*, tumor growth of H157-S, H157-V, and H157-AS. *B*, SC58236 treatment (3 mg/kg, 3 times/week) reduced tumor growth compared to diluent-treated controls. *C*, tumor growth of the anti-CXCL5- anti-CXCL8-treated H157-S *versus* control antiserum-treated H157-S. The H157-tumor bearing mice were treated i.p. 3×/week, and tumor volumes were quantified as described above. Tumor growth of H157-AS was significantly reduced as compared to that of H157-S ($P < 0.001$). Anti-CXCL8 and anti-CXCL5 antisera significantly inhibited the tumor growth of H157-S by day 18 of the experiment ($P < 0.05$).

cells (21, 54). Therefore, we tested whether neutralization of IL-1β-induced PGE_2 in H157 and A549 culture supernatants modulates the production of CXCL8 and CXCL5. We expected IL-1β to increase the production of CXCL8 and CXCL5, as well as PGE_2, and the anti-PGE_2 mAB to decrease this IL-1β-stimulated CXCL8 and CXCL5 production. Consistent with our previous data, IL-1β up-regulated PGE_2 in H157 and A549 cells (21) and was accompanied by sharply elevated levels of CXCL5 and CXCL8, as compared to the control cells (Fig. 5). The anti-PGE_2 mAB, however, significantly down-regulated only the CXCL8 production of H157-S (Fig. 5), whereas the production of CXCL5 remained unchanged (data not shown). Thus, our results suggest a partial role for PGE_2 in ELR+CXC chemokine expression.

DISCUSSION

Lung cancer accounts for more than 28% of all cancer deaths each year and is the leading cause of cancer-related mortality in the United States (55). Despite focused research in conventional therapies, the 5-year survival rate remains 14% and has improved only minimally in the past 25 years. Newly discovered molecular mechanisms in the pathogenesis of lung cancer provide novel opportunities for targeted therapies of NSCLC (56). These investigations in the molecular pathogenesis of lung cancer have presented translational researchers with new targets that may specifically impact carcinogenesis (57). COX-2 is one of the novel targets under evaluation for lung cancer therapy and chemoprevention (58).

Table 1 *In vivo expression of CXCL5 and CXCL8 in a SCID mouse model of NSCLC*

The NSCLC chemokine content was determined in the tumors resulting from tumor growth experiments (see also Fig. 2 and "Materials and Methods").

A. *In vivo* expression of CXCL5 and CXCL8 (pg/ml/mg protein)

	A549-S	A549-AS	A549-V	H157-S	H157-AS	H157-V
CXCL5	22.1 ± 1.1[a]	.02 ± .001	16.9 ± 0.1	38.4 ± 0.4[a]	0	26.6 ± 1.1
CXCL8	550 ± 130[b]	220 ± 10	640 ± 20	540 ± 70[b]	300 ± 20	620 ± 90

B. SC58236 inhibits *in vivo* expression of CXCL8 and CXCL5

	A549-P	A549-P + SC58236	H157-P	H157-P + SC58236
CXCL5	15.5 ± 0.2[c]	7.9 ± 1.4	29.6 ± 0.8[c]	19.4 ± 1.5
CXCL8	650 ± 110	400 ± 100	630 ± 60	540 ± 60

[a] $P < 0.00001$, sense *versus* antisense.
[b] $P < 0.05$ sense *versus* antisense.
[c] $P < 0.01$, untreated parental *versus* SC58236-treated parental cells.

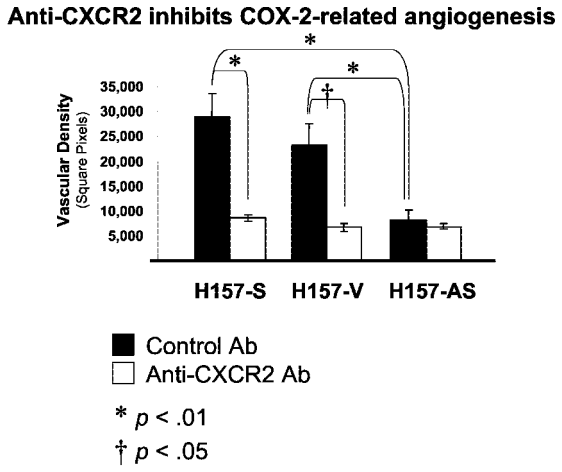

Fig. 3. Angiogenicity of H157 supernatants in a rat corneal pocket model. Corneas were captured using an Olympus BH-2 microscope (New Hyde Park, NY) coupled to a Sony 3CCD camera (Tokyo, Japan) and computer. The vascular density was quantified using NIH Image 1.55 software. Results were expressed as square pixels at ×20 magnification. Only one of five H157-AS supernatants was able to induce a sustained ingrowth of new blood vessels, whereas all five of the H157-S and H157-V supernatants did so. This figure shows that predominant angiogenic activity of H157 supernatants is mediated via the common receptor for ELR+CXC chemokines, CXCR2, because anti-CXCR2 efficiently blocked the H157-S- and H157-V-induced angiogenesis.

Expansion and maintenance of a functional vascular network serving the tumor are required for propagation, invasion, and subsequent metastasis. Thus, angiogenesis is requisite for tumor growth (23) and has been specifically implicated in the pathogenesis and prognosis of lung cancer (59–64). Several growth factors and cytokines have been implicated in tumor-related angiogenesis in lung cancer including vascular endothelial growth factor, transforming growth factors α and β, basic fibroblast growth factor, platelet-derived endothelial cell growth factor, macrophage migration inhibitory factor, and ELR+CXC family chemokines such as CXCL8 and CXCL5 (24–33, 65–68). As shown recently, a high level coexpression of vascular endothelial growth factor, macrophage migration inhibitory factor, and CXC family chemokines in NSCLC tumor samples is associated with high risk of NSCLC recurrence after resection (33). Thus, it could be concluded that multiple angiogenic factors are simultaneously up-regulated in NSCLC.

Here we demonstrate the importance of COX-2-dependent regulation of ELR+CXC family chemokines. Genetic and pharmacological inhibition of COX-2 significantly decreased these chemokines in lung cancer cells *in vitro*. Similarly, *in vivo*, COX-2 inhibition also reduced the production of CXCL5 and CXCL8. Most importantly, anti-CXCL5 and anti-CXCL8 antisera significantly inhibited the enhanced tumor growth of COX-2-overexpressing lung cancer cells. Thus, COX-2-dependent regulation of these ELR+CXC family chemokines is important in NSCLC tumor growth (Figs. 1 and 2; Table 1) but may not be the only activity that modulates NSCLC tumor growth. As shown by Hida *et al.* (69), inhibition of COX-2 may directly reduce the viability of tumor cells. Therefore, the COX-2-antisense-mediated inhibition of NSCLC tumor growth (Fig. 2, *A* and *C*) should be interpreted as resulting from the net effect of COX-2 inhibition, in which down-regulation of CXCL5/CXCL8 plays a prominent role (Figs. 1–3).

Our data agree with earlier studies demonstrating that COX-2 inhibitors limit the growth of tumors expressing COX-2. Previously, we have shown that both SC58236 and NS398 inhibit the growth of Lewis lung carcinoma (20). Likewise, Masferrer *et al.* (13) found that a COX-2 inhibitor drug, Celecoxib, reduced the growth of both Lewis lung carcinoma and HT-29 colon carcinoma cells, whereas Williams *et al.* (70) showed that Celecoxib inhibits the tumor growth of HCA-7 colon carcinoma and Lewis lung carcinoma cells (71). In keeping with these studies, Howe *et al.* (72) demonstrated that Celecoxib decreased the number of breast tumors in mouse mammary tumor virus/neu mice. A recent study also indicates that COX-2 inhibition reduces tumor growth in transgenic mice that spontaneously develop lung cancer (73). Although a variety of antitumor mechanisms may be operative during COX-2 inhibition, it has been suggested that inhibition of angiogenesis is one of the important pathways (11–15, 72). Our study indicates that the ELR+CXC chemokines are critically important in COX-2-dependent NSCLC angiogenesis as determined by a rat micropocket model for angiogenesis (Fig. 3). Specific antibody-mediated blockade of CXCR2 reduced the angiogenic capacity of the supernatants of H157-S and H157-V (Fig. 3). The effect of anti-CXCR2 was comparable with that of genetic inhibition of COX-2 (see Fig. 3, H157-AS). Thus, this finding supports our earlier data demonstrating that the majority, if not all, angiogenic activity of ELR+CXC chemokines is mediated through CXCR2 (35).

As shown in previous studies, the following regulatory events occur in NSCLC and the other types of tumors: (*a*) NF-κB nuclear translo-

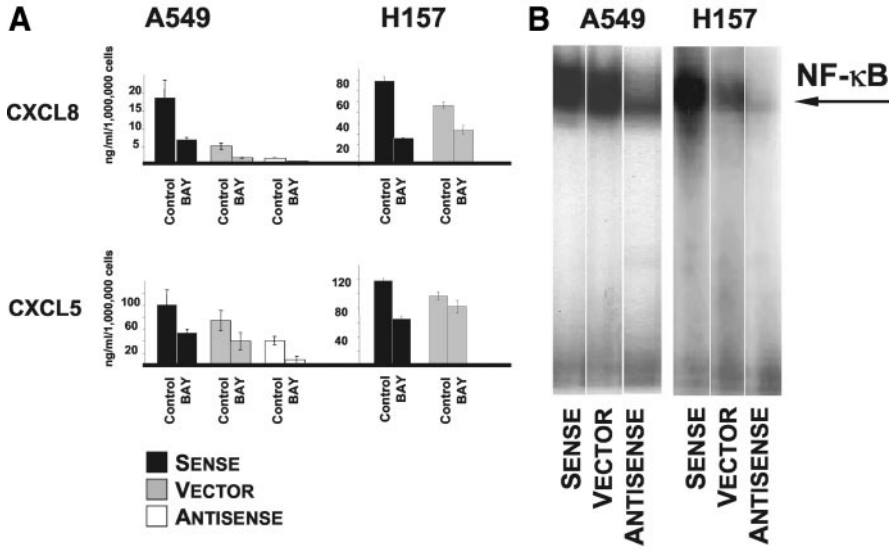

Fig. 4. Cyclooxygenase-2 drives the nuclear facor (NF)-κB-dependent expression of CXC ligand (CXCL) 8 and CXCL5. *A*, cells were plated at 20,000–40,000 cells/well in standard 6-well plates and grown for 72–96 h. At this time point, the wasted medium was removed and replaced with fresh complete medium containing a specific NF-κB inhibitor drug, BAY-11-7082 (3 μM). Cell supernatants were harvested, and cells were counted 24 h later. Supernatants were analyzed for CXCL8 and CXCL5. The H157-AS supernatants were not included because they contain no detectable CXCL8 and CXCL5. *B*, NF-κB nuclear translocation in non-small lung cancer cells. Electrophoretic mobility shift assay (see "Materials and Methods").

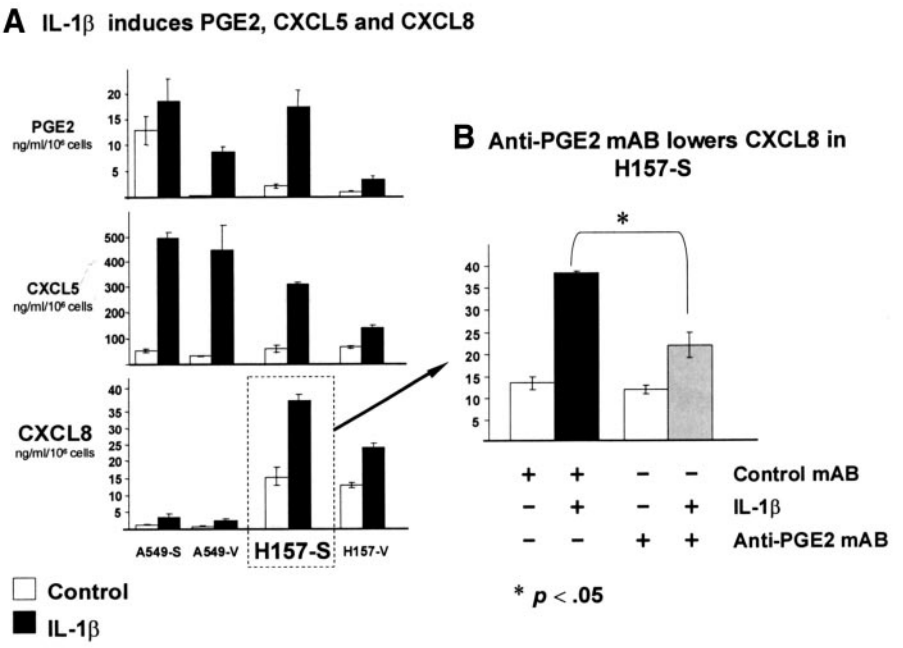

Fig. 5. *A*, interleukin 1β increases the expression of prostaglandin (PG) E_2, CXC ligand (CXCL) 5, and CXCL8 in non-small lung cancer cells. *B*, anti-PGE_2 monoclonal antibody antagonizes the CXCL8 production of H157-S cells (mean ± SE of two independent experiments). As a control for anti-PGE_2 monoclonal antibody, an isotype-matched control antibody was used (see "Materials and Methods").

cation up-regulates CXCL8, CXCL5, and COX-2; (*b*) pharmacological and genetic inhibition of NF-κB nuclear translocation decreases the expression of CXCL8 and CXCL5; (*c*) both COX-2 and ELR+CXC chemokines promote angiogenesis; and (*d*) nonsteroidal anti-inflammatory drugs inhibit the activity of NF-κB (24, 25, 27, 28, 33, 41–43, 48–53). Here, we explored the relationship between the expression of COX-2, NF-κB nuclear translocation, and ELR+CXC chemokine production in NSCLC. The current study implicates NF-κB as a downstream mediator of COX-2 in the COX-2-dependent enhancement of tumor growth and angiogenesis. The apparent COX-2-driven expression of NF-κB-dependent genes such as CXCL8 and CXCL5 in NSCLC cells gave rise to a more malignant NSCLC phenotype *in vivo* as demonstrated by a SCID mouse model for NSCLC, as well as a rat corneal pocket model for angiogenesis (Figs. 2 and 3). Our data suggesting that COX-2 drives the level of NF-κB-dependent CXCL8 and CXCL5 expression are in agreement with earlier studies showing that nonsteroidal anti-inflammatory drugs inhibit the activity of NF-κB (53). Also, our results for NSCLC tumor growth and angiogenesis are compatible with the recent clinical studies demonstrating that high levels of COX-2 mRNA and protein expression (8, 9) as well as angiogenic chemokines including CXCL8, CXCL5, and CXCL1 (27–31) in NSCLC tumor samples are associated with a poor prognosis.

The current study suggests a role for PGE_2 in the expression of ELR+CXC chemokines in NSCLC cells (Fig. 5). We interpret the fact that only the CXCL8 expression of H157-S was modulated by anti-PGE_2 as an indication that COX-2-enhanced NSCLC angiogenesis and tumor growth may have two distinct mechanistic pathways: (*a*) a PGE_2-dependent pathway, as also described in previous studies (20, 74–76); and (*b*) a PGE_2-independent pathway. Our findings implicate both of these pathways in COX-2-dependent ELR+CXC chemokine expression in H157 cells. It is possible that a unique repertoire of E-prostanoid cell surface receptors is required for PGE_2 to modulate the expression of individual ELR+CXC chemokines. Additional studies, however, are needed to address this issue.

In conclusion, NSCLC COX-2 expression appears to be an important determinant for ELR+CXC chemokine expression. As the current study shows, a high NSCLC ELR+CXC chemokine content is associated with enhanced tumor growth, which can be reduced by inhibition of COX-2. Thus, our study confirms that COX-2 is a potential target for NSCLC chemoprevention or therapy, and it also implies that CXCL8 and CXCL5 could be used as markers of COX-2 inhibitor effects in clinical trials. Furthermore, our data suggest that CXCL8, CXCL5, and their receptors could be used as direct targets in future therapies of NSCLC.

REFERENCES

1. Herschman, H. Review: prostaglandin synthase 2. Biochim. Biophys. Acta, *1299:* 125–140, 1996.
2. DuBois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van De Putte, L. B., and Lipsky, P. E. Cyclooxygenase in biology and disease. FASEB J., *12:* 1063–1073, 1998.
3. Smith, W. L., DeWitt, D. L., and Garavito, R. M. Cyclooxygenases: structural, cellular and molecular biology. Annu. Rev. Biochem., *69:* 145–182, 2000.
4. Huang, M., Stolina, M., Sharma, S., Mao, J., Zhu, L., Miller, P., Wollman, J., Herschman, H., and Dubinett, S. M. Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin-10 and down-regulation of interleukin-12 production. Cancer Res., *58:* 1208–1216, 1998.
5. Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozaki, K., Nakamura, S., Ogawa, M., Mitsudomi, T., Sugiura, T., and Takahashi, T. Increased expression of cyclooxygenase-2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res., *58:* 3761–3764, 1998.
6. Wolff, H., Saukkonen, K., Anttila, S., Karjalainen, A., Vainio, H., and Ristimaki, A. Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res., *58:* 4997–5001, 1998.
7. Hosomi, Y., Yokose, T., Hirose, Y., Nakajima, R., Nagai, K., Nishiwaki, Y., and Ochiai, A. Increased cyclooxygenase-2 expression occurs frequently in precursor lesions of human adenocarcinoma of the lung. Lung Cancer, *30:* 73–81, 2000.
8. Khuri, F., Wu, H., Lee, J., Kemp, B., Lotan, R., Lippman, S., Feng, L., Hong, W., and Xu, X-C. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin. Cancer Res., *7:* 861–867, 2001.
9. West, H., Yaziji, H., Giarrita, S., Livingston, R., Vallieres, E., Wood, D., and Gown, A. Cyclooxygenase-2 (COX-2) overexpression by immunohistochemistry (IHC) is associated with more aggressive biologic behavior of non-small cell lung cancer (NSCLC) tumors. Proc. Am. Soc. Clin. Oncol., *21:* 1235a, 2002.
10. Tsujii, M., Kawano, S., and DuBois, R. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc. Natl. Acad. Sci. USA, *94:* 3336–3340, 1997.
11. Liu, X., Kirschenbaum, A., Yao, S., Lee, R., Holland, J., and Levine, A. Inhibition of cyclooxygenase-2 suppresses angiogenesis and the growth of prostate cancer *in vivo*. J. Urol., *164:* 820–825, 2000.
12. Leahy, K., Koki, A., and Masferrer, J. Role of cyclooxygenases in angiogenesis. Curr. Med. Chem., *7:* 1163–1170, 2000.
13. Masferrer, J. L., Leahy, K. M., Koki, A. T., Zweifel, B. S., Settle, S. L., Woerner, B. M., Edwards, D. A., Flickinger, A. G., Moore, R. J., and Seibert, K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res., *60:* 1306–1311, 2000.

14. Uefuji, K., Ichikura, T., and Mochizuki, H. Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer. Clin. Cancer Res., *6:* 135–138, 2000.
15. Gately, S. The contributions of cyclooxygenase-2 to tumor angiogenesis. Cancer Metastasis Rev., *19:* 19–27, 2000.
16. Tsujii, M., and DuBois, R. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2. Cell, *83:* 493–501, 1995.
17. Liu, X. H., Yao, S., Kirschenbaum, A., and Levine, A. C. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. Cancer Res., *58:* 4245–4249, 1998.
18. Hsu, A. L., Ching, T. T., Wang, D. S., Song, X., Rangnekar, V. M., and Chen, C. S. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. J. Biol. Chem., *275:* 11397–11403, 2000.
19. Sheng, H., Shao, J., Morrow, J., Beauchamp, J. R., and DuBois, R. Modulation of apoptosis and Bcl-2 expression by prostaglandin E_2 in human colon cancer cells. Cancer Res., *58:* 362–366, 1998.
20. Stolina, M., Sharma, S., Lin, Y., Dohadwala, M., Gardner, B., Luo, J., Zhu, L., Kronenberg, M., Miller, P. W., Portanova, J., and Dubinett, S. M. Specific inhibition of cyclooxygenase-2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J. Immunol., *164:* 361–370, 2000.
21. Dohadwala, M., Luo, J., Zhu, L., Lin, Y., Dougherty, G. J., Sharma, S., Huang, M., Pold, M., Batra, R. K., and Dubinett. S. M. Non-small cell lung cancer cyclooxygenase-2-dependent invasion is mediated by CD44. J. Biol. Chem., *276:* 20809–20812, 2001.
22. Dohadwala, M., Batra, R. K., Luo, J., Lin, Y., Krysan, K., Pold, M., Sharma, S., and Dubinett, S. M. Autocrine/paracrine prostaglandin E_2 production by non-small cell lung cancer cells regulates matrix metalloproteinase-2 and CD44 in cyclooxygenase-2-dependent invasion. J. Biol. Chem., *277:* 50828–50833, 2002.
23. Folkman, J. Angiogenesis-dependent diseases. Semin. Oncol., *28:* 536–542, 2001.
24. Arenberg, D. A., Keane, M. P., DiGiovine, B., Kunkel, S. L., Morris, S. B., Xue, Y. Y., Burdick, M. D., Glass, M. C., Iannettoni, M. D., and Strieter, R. M. Epithelial-neutrophil activating peptide (ENA-78) is an important angiogenic factor in non-small cell lung cancer. J. Clin. Investig., *102:* 465–472, 1998.
25. Arenberg, D. A., Kunkel, S. L., Polverini, P. J., Glass, M., Burdick, M. D., and Strieter, R. M. Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. J. Clin. Investig., *97:* 2792–2802, 1996.
26. Anderson, I. C., Mari, S. E., Broderick, R. J., Mari, B. P., and Shipp, M. A. The angiogenic factor interleukin 8 is induced in non-small cell lung cancer/pulmonary fibroblast cocultures. Cancer Res., *60:* 269–272, 2000.
27. Colasante, A., Mascetra, N., Brunetti, M., Lattanzio, G., Diodoro, M., Caltagirone, S., Musiani, P., and Aiello, F. B. Transforming growth factor β1, interleukin-8 and interleukin-1, in non-small-cell lung tumors. Am. J. Respir. Crit. Care Med., *156:* 968–973, 1997.
28. Smith, D. R., Polverini, P. J., Kunkel, S. L., Orringer, M. B., Whyte, R. I., Burdick, M. D., Wilke, C. A., and Strieter, R. M. Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. J. Exp. Med., *179:* 1409–1415, 1994.
29. Orditura, M., De Vita, F., Catalano, G., Infusino, S., Lieto, E., Martinelli, E., Morgillo, F., Castellano, P., Pignatelli, C., and Galizia, G. J. Elevated serum levels of interleukin-8 in advanced non-small cell lung cancer patients: relationship with prognosis. J. Interferon Cytokine Res., *22:* 1129–1135, 2002.
30. Yuan, A., Yang, P. C., Yu, C. J., Chen, W. J., Lin, F. Y., Kuo, S. H., and Luh, K. T. Interleukin-8 messenger ribonucleic acid expression correlates with tumor progression, tumor angiogenesis, patient survival, and timing of relapse in non-small-cell lung cancer. Am. J. Respir. Crit. Care Med., *162:* 1957–1963, 2000.
31. Masuya, D., Huang, C., Liu, D., Kameyama, K., Hayashi, E., Yamauchi, A., Kobayashi, S., Haba, R., and Yokomise, H. The intratumoral expression of vascular endothelial growth factor and interleukin-8 associated with angiogenesis in non-small cell lung carcinoma patients. Cancer (Phila.), *92:* 2628–2638, 2001.
32. Yuan, A., Yu, C. J., Luh, K. T., Kuo, S. H., Lee, Y. C., and Yang, P. C. Aberrant p53 expression correlates with expression of vascular endothelial growth factor mRNA and interleukin-8 mRNA and neoangiogenesis in non-small-cell lung cancer. J. Clin. Oncol., *20:* 900–910, 2002.
33. White, E. S., Flaherty, K. R., Carskadon, S., Brant, A., Iannettoni, M. D., Yee, J., Orringer, M. B., and Arenberg, D. A. Macrophage migration inhibitory factor and CXC chemokine expression in non-small cell lung cancer: role in angiogenesis and prognosis. Clin. Cancer Res., *9:* 853–860, 2003.
34. Strieter, R. M., Polverini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, V. A., Marriott, D., Chan, S-Y., Roczniak, S., and Shanafelt, A. B. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. J. Biol. Chem., *270:* 27348–27357, 1995.
35. Addison, C. L., Daniel, T. O., Burdick, M. D., Liu, H., Ehlert, J. E., Xue, Y. Y., Buechi, L., Walz, A., Richmond, A., and Strieter, R. M. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. J. Immunol., *165:* 5269–5277, 2000.
36. Murphy, P. M. The molecular biology of leukocyte chemoattractant receptors. Annu. Rev. Immunol., *12:* 593–633, 1994.
37. Murphy, P. M., and Tiffany, H. L. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. Science (Wash. DC), *253:* 1280–1283, 1991.
38. Ahuja, S. K., and Murphy, P. M. The CXC chemokines growth-regulated oncogene (GRO)α, GROβ, GROγ, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. J. Biol. Chem., *271:* 20545–20550, 1996.
39. Lee, J., Horuk, R., Rice, G. C., Bennett, G. L., Camerato, T., and Wood, W. I. Characterization of two high affinity human interleukin-8 receptors. J. Biol. Chem., *267:* 16283–16287, 1992.
40. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. NF-κB in cancer: from innocent bystander to major culprit. Nat. Rev. Cancer, *2:* 301–310, 2002.
41. Remacle-Bonnet, M. M., Garrouste, F. L., Heller, S., Andre, F., Marvaldi, J. L., and Pommier, G. J. Insulin-like growth factor-I protects colon cancer cells from death factor-induced apoptosis by potentiating tumor necrosis factor α-induced mitogen-activated protein kinase and nuclear factor κB signaling pathways. Cancer Res., *60:* 2007–2017, 2000.
42. Chandrasekar, B., Melby, P. C., Sarau, H. M., Raveendran, M., Perla, R. P., Marelli-Berg, F. M., Dulin, N. O., and Singh, I. S. Chemokine-cytokine cross-talk. The ELR+ CXC chemokine LIX (CXCL5) amplifies a proinflammatory cytokine response via a phosphatidylinositol 3-kinase-NF-κB pathway. J. Biol. Chem., *278:* 4675–4686, 2003.
43. Chandrasekar, B., Smith, J. B., and Freeman, G. L. Ischemia-reperfusion of rat myocardium activates nuclear factor-κB and induces neutrophil infiltration via lipopolysaccharide-induced CXC chemokine. Circulation, *103:* 2296–2302, 2001.
44. Haddad, J. Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. Cell. Signal., *14:* 879–897, 2002.
45. Harris, A. L. Related hypoxia: a key regulatory factor in tumour growth. Nat. Rev. Cancer, *2:* 38–47, 2002.
46. Belperio, J. A., Keane, M. P., Burdick, M. D., Londhe, V., Xue, Y. Y., Li, K., Phillips, R. J., and Strieter, R. M. Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. J. Clin. Investig., *110:* 1703–1716, 2002.
47. Mehrad, B., Strieter, R. M., Moore, T. A., Tsai, W. C., Lira, S. A., and Standiford, T. J. CXC chemokine receptor-2 ligands are necessary components of neutrophil-mediated host defense in invasive pulmonary aspergillosis. J. Immunol., *163:* 6086–6094, 1999.
48. Wolf, J. S., Chen, Z., Dong, G., Sunwoo, J. B., Bancroft, C. C., Capo, D. E., Yeh, N. T., Mukaida, N., and Van Waes, C. IL (interleukin)-1α promotes nuclear factor-κB and AP-1-induced CXCL8 expression, cell survival, and proliferation in head and neck squamous cell carcinomas. Clin. Cancer Res., *7:* 1812–1820, 2001.
49. Huang, S., DeGuzman, A., Bucana, C. D., and Fidler, I. J. Nuclear factor-κB activity correlates with growth, angiogenesis, and metastasis of human melanoma cells in nude mice. Clin. Cancer Res., *6:* 2573–2581, 2000.
50. Huang, S., Robinson, J. B., Deguzman, A., Bucana, C. D., and Fidler, I. J. Blockade of nuclear factor-κB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin-8. Cancer Res., *60:* 5334–5339, 2000.
51. Huang, S., Pettaway, C. A., Uehara, H., Bucana, C. D., and Fidler, I. J. Blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Oncogene, *20:* 4188–4197, 2001.
52. Le, X., Shi, Q., Wang, B., Xiong, Q., Qian, C., Peng, Z., Li, X. C., Tang, H., Abbruzzese, J. L., and Xie, K. Molecular regulation of constitutive expression of interleukin-8 in human pancreatic adenocarcinoma. J. Interferon Cytokine Res., *20:* 935–946, 2000.
53. Yamamoto, Y., and Gaynor, R. B. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J. Clin. Investig., *107:* 135–142, 2001.
54. Huang, M., Sharma, S., Mao, J. T., and Dubinett, S. M. Non-small cell lung cancer-derived soluble mediators and prostaglandin E_2 enhance peripheral blood lymphocyte IL-10 transcription and protein production. J. Immunol., *157:* 5512–5520, 1996.
55. Greenlee, R. T., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics, 2000. CA Cancer J. Clin., *50:* 7–33, 2000.
56. Dy, G. K., and Adjei, A. A. Novel targets for lung cancer therapy: part I. J. Clin. Oncol., *20:* 2881–2894, 2002.
57. Hirsch, F. R., Franklin, W. A., and Bunn, P. A., Jr. Expression of target molecules in lung cancer: challenge for a new treatment paradigm. Semin. Oncol., *29:* 2–8, 2002.
58. Subbaramaiah, K., and Dannenberg, A. J. Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol. Sci., *24:* 96–102, 2003.
59. Harpole, D. H., Jr., Richards, W. G., Herndon, J. E., II, and Sugarbaker, D. J. Angiogenesis and molecular biologic sub-staging in patients with stage I non-small cell lung cancer. Ann. Thorac. Surg., *61:* 1470–1476, 1996.
60. Macchiarini, P., Fontanini, G., Hardin, M. J., Squartini, F., and Angeletti, C. A. Relation of neovascularization to metastasis of non-small cell lung cancer. Lancet, *340:* 145–146, 1992.
61. Duarte, I. G., Bufkin, B. L., Pennington, M. F., Gal, A. A., Cohen, C., Kosinski, A. S., Mansour, K. A., and Miller, J. I. Angiogenesis as a predictor of survival after surgical resection for stage I non-small cell lung cancer. J. Thorac. Cardiovasc. Surg., *115:* 652–659, 1998.
62. Giatromanolaki, A., Koukourakis, M., O'Byrne, K., Fox, S., Whitehouse, R., Talbot, D. C., Harris, A. L., and Gatter, K. C. Prognostic value of angiogenesis in operable non-small cell lung cancer. J. Pathol., *179:* 80–88, 1996.
63. Fontanini, G., Bigini, D., Vignati, S., Basolo, F., Mussi, A., Lucchi, M., Chine, S., Angeletti, C. A., Harris, A. L., and Bevilacqua, G. Microvessel count predicts metastatic disease and survival in non-small cell lung cancer. J. Pathol., *177:* 57–63, 1995.
64. Dazzi, C., Cariello, A., Maioli, P., Solaini, L., Scarpi, E., Rosti, G., Lanzanova, G., and Marangolo, M. Prognostic and predictive value of intratumoral microvessel density in operable non-small cell lung cancer. Lung Cancer, *24:* 81–88, 1999.
65. Ohta, Y., Shridhar, V., Bright, R. K., Kalemkerian, G. P., Du, W., Carbone, M., Watanabe, Y., and Pass, H. I. VEGF and VEGF type C play an important role in

angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. Br. J. Cancer, *81:* 54–61, 1999.
66. Wikstrom, P., Stattin, P., Franck-Lissbrant, I., Damber, J. E., and Bergh, A. Transforming growth factor-β1 is associated with angiogenesis, metastasis and poor clinical outcome in prostate cancer. Prostate, *37:* 19–29, 1998.
67. Guddo, F., Fontanini, G., Reina, C., Vignola, A. M., Angeletti, A., and Bonsignore, G. The expression of basic fibroblast growth factor in tumor-associated stromal cells and vessels is inversely correlated with non-small cell lung cancer progression. Hum. Pathol., *30:* 788–794, 1999.
68. Koukourakis, M. I., Giatromanolaki, A., O'Byrne, K. J., Comley, M., Whitehouse, R. M., Talbot, D. C., Gatter, K. C., and Harris, A. L. Platelet-derived endothelial cell growth factor expression correlates with tumour angiogenesis and prognosis in non-small cell lung cancer. Br. J. Cancer, *75:* 477–481, 1997.
69. Hida, T., Kozaki, K., Muramatsu, H., Masuda, A., Shimizu, S., Mitsudomi, T., Sugiura, T., Ogawa, M., and Takahashi, T. Cyclooxygenase-2 inhibitor induces apoptosis and enhances cytotoxicity of various anticancer agents in non-small cell lung cancer cell lines. Clin. Cancer Res., *6:* 2006–2011, 2000.
70. Williams, C. S., Watson, A. J., Sheng, H., Helou, R., Shao, J., and DuBois, R. N. Celecoxib prevents tumor growth *in vivo* without toxicity to normal gut: lack of correlation between *in vitro* and *in vivo* models. Cancer Res., *60:* 6045–6051, 2000.
71. Williams, C. S., Tsujii, M., Reese, J., Dey, S. K., and DuBois, R. N. Host cyclooxygenase-2 modulates carcinoma growth. J. Clin. Investig., *105:* 1589–1594, 2000.
72. Howe, L. R., Subbaramaiah, K., Patel, J., Masferrer, J. L., Deora, A., Hudis, C., Thaler, H. T., Muller, W. J., Du, B., Brown, A. M., and Dannenberg, A. J. Celecoxib, a selective cyclooxygenase 2 inhibitor, protects against human epidermal growth factor receptor 2 (HER-2)/neu-induced breast cancer. Cancer Res., *62:* 5405–5407, 2002.
73. Yang, S-C., Hillinger, S., Zhu, L., Huang, M., Batra, R. K., Strieter, R. M., Sharma, S., and Dubinett, S. M. Systemic COX-2 inhibition limits tumor progression in a murine model of spontaneous lung cancer. Proc. Am. Assoc. Cancer Res., *44:* 924–925, 2003.
74. Form, D. M., and Auerbach, R. PGE_2 and angiogenesis. Proc. Soc. Exp. Biol. Med., *172:* 214–218, 1983.
75. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:* 353–364, 1996.
76. Zweifel, B. S., Davis, T. W., Ornberg, R. L., and Masferrer, J. L. Direct evidence for a role of cyclooxygenase 2-derived prostaglandin E_2 in human head and neck xenograft tumors. Cancer Res., *62:* 6706–6711, 2002.