Tumor-Driven Paracrine Platelet-Derived Growth Factor Receptor α Signaling Is a Key Determinant of Stromal Cell Recruitment in a Model of Human Lung Carcinoma

Max L. Tejada, Lanlan Yu, Jianying Dong, Kenneth Jung, Gloria Meng, Franklin V. Peale, Gretchen D. Frantz, Linda Hall, XiaoHuan Liang, Hans-Peter Gerber, and Napoleone Ferrara

Abstract

Activated fibroblasts are thought to play important roles in the progression of many solid tumors, but little is known about the mechanisms responsible for the recruitment of fibroblasts in tumors. Using several methods, we identified platelet-derived growth factor A (PDGFA) as the major fibroblast chemoattractant and mitogen from conditioned medium generated by the Calu-6 lung carcinoma cell line. In addition, we showed that Calu-6 tumors express significant levels of PDGFC, and that the levels of expression of these two PDGFRα ligands correlate strongly with the degree of stromal fibroblast infiltration into the tumor mass. The most intense expression of PDGFRα was observed in fibroblasts in the tumor outer rim. We subsequently showed that disrupting PDGFRα-mediated signaling results in significant inhibition of tumor growth in vivo. Furthermore, analysis of a compendium of microarray data revealed significant expression of PDGFA, PDGFC, and PDGFRα in human lung tumors. We propose that therapies targeting this stromal cell type may be effective in treating certain types of solid tumors.

It has become apparent that the microenvironment in which tumor cells develop profoundly influences many steps of tumor progression. The tumor microenvironment consists of a stroma, which is composed of immune and inflammatory cells, endothelial cells and fibroblasts, a variety of growth factors and cytokines, and an insoluble extracellular matrix. In various experimental tumor models, each of the stromal cell types has been shown to influence the efficiency of tumor formation, the rate of tumor growth, the extent of invasiveness, and the ability of the tumor cells to metastasize (1–3).

Much attention has been focused on the role of endothelial cells in tumor angiogenesis; however, there is accumulating evidence that in many solid tumor types, the influence of the microenvironment is mediated in large part by paracrine signaling between the tumor epithelium and neighboring stromal fibroblasts (4). Fibroblasts are the major constituent of the stroma in many solid tumors, and in some cases, the fibroblast stroma comprises >90% of the tumor mass. These tumor-associated fibroblasts often exist in a constitutively activated state and often exhibit biological markers consistent with those found at the sites of normal wound healing (3).

In the normal wound repair process, fibroblasts are recruited from the surrounding tissue. However, cancer cells are capable of recruiting fibroblasts from the surrounding environment and inducing their activation to the myofibroblast phenotype directly (3). In addition to their presence in the tumor mass, myofibroblasts are often found at the invasive fronts of a number of solid tumors, where they have been shown to affect the growth, invasion, and metastasis of the tumors through their increased synthesis of extracellular matrix components and the increased production of proteases, growth, and angiogenic factors (4).

The growth of solid tumors requires the formation of new blood vessels through angiogenesis, the sprouting of new blood vessels from preexisting ones, although more recent evidence indicates that recruitment of bone marrow–derived angiogenic cells contributes to the development of a neovascular supply (5, 6). Although a number of angiogenic factors have been identified, vascular endothelial growth factor A (VEGF-A) is probably the most ubiquitous, and much experimental evidence implicates this factor in the regulation of both physiologic and pathologic angiogenesis (7, 8). Administration of an anti-VEGF-A humanized monoclonal antibody (mAb; bevacizumab) in combination with chemotherapy results in increased survival relative to chemotherapy alone in patients with previously untreated metastatic colorectal cancer (9). In several experimental systems, inactivation of the VEGF-A gene indicates that tumor-derived VEGF is important for tumor angiogenesis and growth (10, 11). However, other studies have detected significant levels of VEGF in the stromal compartment, suggesting a significant role for stromal fibroblast-derived VEGF (12, 13).

Members of the platelet-derived growth factor (PDGF) family have been shown to be potent mitogens and chemoattractants...
for a wide variety of cell types of mesenchymal origin (14). In both human and mouse, the PDGF family consists of four members (PDGFA, PDGFB, PDGFC, and PDGFD), which exert their biological effects by binding to two receptor tyrosine kinases (PDGFRα and PDGFRβ). PDGFAA, PDGFB, PDGFB, and PDGFC dimers bind to PDGFRα with high affinity, whereas PDGFB and PDGFD dimers bind PDGFRβ preferentially. PDGFR signaling is critical for proper embryonic development, whereas in the adult, it plays a role in wound healing and in the control of interstitial fluid pressure (14).

Ablent PDGFR signaling is a hallmark of a number of solid tumors, and studies of breast, lung, and colon cancers have shown a tight correlation between deregulated paracrine PDGFR signaling and cancer progression (15–17). Furthermore, in gliomas, fibrosarcomas, and osteosarcomas, coexpression of the PDGF ligands and their cognate receptors by the tumor cells leads to an autocrine mechanism that drives carcinogenesis (18–20). Unlike the other members of its family, PDGFA exhibits a weak transforming activity, and thus far, most of the studies have focused on its role in embryonic development (21).

Many studies have focused upon the role of tumor-derived factors, such as PDGFB, basic fibroblast growth factor, and transforming growth factor β-1 in the induction of the myofibroblast phenotype and the angiogenic process (22–24). However, attempts to identify bona fide tumor-derived stromal fibroblast recruitment factors are rare, despite the fact that intuitively, this precedes fibroblast activation (25–27). In addition, using a dominant-negative strategy, Shao et al. showed that the expression of several PDGF family members was responsible for a desmoplastic response in mammary gland tumor xenografts (28).

Recently, using fibrosarcomas derived from VEGF null mouse embryonic fibroblasts, Dong et al. showed a crucial role for PDGFA in the recruitment of a stroma capable of mediating tumor angiogenesis in the absence of tumor-derived VEGF (29).

However, the relevance of these findings to human tumors remained unknown. To investigate the mechanisms of stromal recruitment in a model of human lung carcinoma, we used the Calu-6 cell line that is characterized by its ability to elicit a significant host stromal response in vivo. Using chromatographic and immunologic methods, we identified PDGFA as the major chemotactic and mitogenic activity whose levels correlate positively with the Calu-6 tumor’s ability to elicit a significant stromal response in vivo. Adenoviral delivery of selective antagonists of PDGFR signaling showed that disrupting PDGFRα signaling inhibits tumor growth in vivo. To validate these findings, we analyzed the GeneExpress compendium of microarray data and confirmed the significant expression of PDGFA and PDGFC as well as their cognate receptor PDGFRs in human lung tumor samples. Thus, inhibition of signaling through PDGFRα may prove to be an effective method of affecting the growth and development of certain human solid tumors by targeting recruitment of the stromal fibroblasts.

Materials and Methods

Cell lines. The human lung carcinoma cell line Calu-6 cell line and the mouse fibroblast cell line NIH3T3 (American Type Culture Collection, Manassas, VA) were cultured routinely in growth medium [Ham’s F12 50%, low-glucose DMEM 50% supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/streptomycin, 2 mmol/L L-glutamine, and 1 μg/mL Fungizone (Invitrogen, Carlsbad, CA)]. Cells were incubated at 37°C in an atmosphere of 95% air/5% CO2. The human embryonic kidney 293 cell line (American Type Culture Collection), used to generate the adenovirus used in these experiments, was typically grown using high-glucose DMEM supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/streptomycin, 2 mmol/L L-glutamine, and 1 μg/mL Fungizone (Invitrogen).

Generation and fractionation of conditioned medium. Calu-6 cells were allowed to grow to confluency in the presence of growth medium on gelatin-coated 500-cm2 dishes. The medium was then replaced with serum-free DMEM/Ham’s F12 medium, and the cells were incubated for an additional 72 hours, at which time the media (total of 6 liters) were harvested and concentrated 5-fold using a Filtertron Ultrasette tangential flow device with 10K membrane (Filtertron Technology Corp., Northborough, MA). All fractionation procedures described were done using an AKTA Explorer chromatography system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Cation-exchange column chromatography. The concentrated medium was dialyzed against 25 mmol/L sodium phosphate (pH 6) and applied on a 5-ml HiTrap S Sepharose column (Amersham Pharmacia Biotech), which had been equilibrated with the same buffer. After a low salt wash, the column was eluted with 1 mol/L NaCl.

Size exclusion column chromatography. Bioactive 1 mol/L NaCl fractions generated using cation-exchange chromatography were pooled, and 5-ml aliquots were loaded onto a TSK3000 column (21.5 × 30 cm; Tosoh Biosep LC, Montgomeryville, PA) that had been equilibrated in 20 mmol/L Tris (pH 7.5), 2 mol/L NaCl, and 0.02% Tween 20. The flow rate was 3 ml/min, 3-ml fractions were collected, and aliquots were tested for bioactivity.

Reverse-phase column chromatography. Bioactive fractions generated using size exclusion chromatography were pooled, diluted 5-fold in water containing 0.1% trifluoroacetic acid and loaded onto a C4 4000A column (4.6 × 100 mm). The column was eluted using a linear gradient of 15% to 50% acetonitrile (120 minutes) in 0.1% (v/v) trifluoroacetic acid at the flow rate of 0.6 ml/min. Fractions of 0.6 ml were collected and tested for bioactivity.

Proliferation assays. NIH3T3 cells were seeded on 96-well plates (BD Biosciences, Bedford, MA) at a density of 3,000 in a 100-μl final volume. Following overnight incubation, the medium was replaced with starvation media (0.1% v/v bovine serum albumin in DMEM/Ham’s F12 medium) supplemented with 2 to 10 μl of each fraction to be tested, to a final volume of 100 μl. Following overnight incubation, 1 μCi of [3H]-methyl thymidine was added to each well, and the cells incubated for 6 hours before harvesting. Cellular DNA was harvested using a Filtermate 196 Harvester (Perkin Elmer, Wellesley, MA) and Unifilter-96, GF/C plates (part no. 6005174; Perkin-Elmer, Wellesley, MA). Incorporation of [3H]-methyl thymidine was detected using a TopCount Microplate Scintillation Counter (Packard BioScience). The data were plotted as total cpm of incorporated over the fraction number. Inhibition assays were done as described above, except that soluble PDGFβ was included in the assay mixtures at a final concentration of 1 μg/mL (>30 nmol/L). The samples were then processed as described. To rule out the possibility that the effects of the soluble PDGFβ were nonspecific, we tested the ability of such soluble receptors to inhibit the effects of a variety of unrelated growth factors, including basic fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, PDGFA, and PDGFB, on fibroblast proliferation and found no inhibitory effects at the concentrations used to affect the bioactivity present in the Calu-6 conditioned medium.

To assess Calu-6 proliferation, the Calu-6 cells were seeded in a final volume of 1 ml in growth medium at a density of 3,000 per well in a 24-well plate (BD Biosciences, Bedford, MA). Following overnight incubation, the medium was replaced with assay medium (0.5% v/v fetal bovine serum in DMEM/Ham’s F12 medium) that included either soluble PDGFβ or PDGFB at concentrations of 1 or 5 μg/mL (30 and 150 nmol/L, respectively). As controls, the cells were grown in assay media supplemented with basic fibroblast growth factor, epidermal
growth factor, PDGFAA, or PDGFBB at final concentrations of 20 μg/mL or in the presence of complete 50:50 media (10% fetal bovine serum). Following a 5-day incubation, the cells were trypsinized and counted using a Coulter Counter (Beckman Coulter, Miami, FL). The results are presented as total cell numbers over treatment.

Migration assays. A modified Boyden chamber migration assay was done using 24 transwell fluoroblock migration plates (HTS Fluoroblock Multiwell Insert System, BD Clontech, Mountain View, CA). The plates (8 μm pore size) were precoated with 0.1% w/v gelatin. A 700-μl final volume of starving media incorporating 10 to 20 μl samples of the fractions to be tested were placed on the lower chamber of the chemotaxis plates. Alternatively, a negative buffer-only control and controls containing either PDGFAA or PDGFBB at a final concentration of 20 ng/mL was used. Subsequently, 40,000 NIH3T3 cells were plated in a final volume of 200 μl onto the upper chamber. Following overnight incubation at 37°C in an atmosphere of 95% air/5% CO2, the medium in the both upper and lower chambers was aspirated, and the cells were fixed in cold methanol for 20 minutes at 4°C. The methanol was removed, and the plates were dried at room temperature before staining in a final concentration of 1 mmol/L YoPro-1 (Invitrogen, Eugene, OR) in PBS. Migration was assessed fluorimetrically using a CytoFluor Multiwell Plate Reader and CytoFluor Software (Invitrogen, Eugene, OR) in PBS. Before staining in a final concentration of 1 mmol/L YoPro-1, the medium in both upper and lower chambers was aspirated, and the plates were dried at room temperature before staining in a final concentration of 1 mmol/L YoPro-1. Methanol was removed, and the plates were dried at room temperature before staining in a final concentration of 1 mmol/L YoPro-1.

Expression of members of the PDGF family was analyzed in GeneExpress (Genetech, Gaithersburg, MD), a compendium of microarray gene expression data from thousands of clinical samples. Using this database, 138 normal human lung and 159 human lung tumor samples were examined using probes specific for each member of the PDGF family. Signal intensity (arbitrary units) was computed using Affymetrix MAS 5 software (Affymetrix, Inc., Santa Clara, CA). Normal samples are shown in green above the respective lines for each PDGF, whereas tumor samples are shown in red; t statistics and their corresponding two-tailed Ps were calculated using the R program for statistical computing (http://www.r-project.org), assuming unequal variance in the normal and tumor groups. t < 0.0001 was considered statistically significant.

Generation of adenoviral constructs. cDNA encoding the β-galactosidase gene, a region encoding the first three immunoglobulin-like domains of murine VEGFR1 fused to the Fc portion of murine IgG1 (mFlt(1-3)-IgG), and the extracellular domain that includes the first immunoglobulin-like domains of murine PDGFRα (PDGFRα-Ig) were cloned into the cytomegalovirus shuttle vector using NotI and HindIII sites, and recombinant virus was produced using the AdEasy system according to the manufacturer’s directions (Stratagene, La Jolla, CA). Virus was purified by using the Megakit from Virapur (BD Clontech) and titered by conventional methods.

Mouse xenograft experiments. Briefly, Calu-6 cells suspended at a concentration of 5 × 10⁶ per mL Matrigel were injected s.c. into the dorsal flank region of beige nude XID mice (Harlan Sprague-Dawley, Indianapolis, IN). Five days after tumor cell inoculation, when the xenografts were established and had reached a volume of 50 to 100 mm³, i.p. treatment with mAb A.4.6.1 (30) was initiated, at a dose of 10 mg/kg. Thereafter, the mice were treated twice weekly. mFlt(1-3)-IgG, also designated as mFlt-IgG, was administered i.p. every day at a dose of 25 mg/kg. Tumor volumes were calculated every second day using the ellipsoid volume formula (6 × L × W × H, where L = length, W = width).
Table 1. Quantitative RT-PCR primer sequences used in these experiments

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Orientation</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>187486</td>
<td>5'-CTTTTCCTGGACATGGA-3</td>
<td>Forward</td>
<td>PDGFA</td>
</tr>
<tr>
<td>187487</td>
<td>5'-GCACACTGCAATAAAAGCA-3</td>
<td>Reverse</td>
<td>PDGFA</td>
</tr>
<tr>
<td>187488</td>
<td>5'-TACATGGCCTTCATCTCAAGAT-3</td>
<td>Probe</td>
<td>PDGFA</td>
</tr>
<tr>
<td>168036</td>
<td>5'-CGATCCGCTCTTTGATGAT-3</td>
<td>Forward</td>
<td>PDGFB</td>
</tr>
<tr>
<td>168037</td>
<td>5'-TCCAACTGCCCACATCT-3</td>
<td>Reverse</td>
<td>PDGFB</td>
</tr>
<tr>
<td>168038</td>
<td>5'-CTGCAGGAGCCCGAGGAGG-3</td>
<td>Reverse</td>
<td>PDGFC</td>
</tr>
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</tr>
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<td>5'-TTACTACGTTGATCGCC-3</td>
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<td>PDGFC</td>
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<td>PDGFC</td>
</tr>
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<td>5'-CAGTAAAGCTCCACTTGATT-3</td>
<td>Forward</td>
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<td>VEGF</td>
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<tr>
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<td>5'-AGGTTTGATTCCGCATGATC-3</td>
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<td>Probe</td>
<td>RPL19</td>
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</tbody>
</table>

width, and $H =$ height; ref. 31). For statistical analysis of differences between groups, a one-way ANOVA followed by a Tukey HSD pairwise analysis was done using JMP software (SAS Institute, Inc., Cary, NC). $P < 0.001$ was considered significant.

Calu-6 cells were resuspended in Matrigel (BD Clonetics) at a concentration of $1 \times 10^8$ cells/mL and injected (100 µL/mouse) s.c. into the dorsal flank regions of Beige XID mice (Harlan Sprague-Dawley) using a 28-gauge needle and 0.5-mL tuberculin syringe. Following a 24-hour incubation, the mice were separated into groups of six, and the tumor volumes were determined by measurement along their length × width × height using vernier calipers (32). The mice were then injected i.t. with $1 \times 10^6$ plaque-forming units of each adenovirus in 100 µL volume. Treatment was done once per week for a period of 4 weeks, at which time the tumors were measured, excised, and weighed, and portions were snap-frozen in liquid nitrogen for RNA extraction or fixed in 10% (v/v) formalin and paraffin embedded. Sections 5-µm thick were deparaffinized, deproteinated in 4 A and portions were snap-frozen in liquid nitrogen for RNA extraction or fixed in 10% (v/v) formalin for subsequent analysis. Data are presented as mean ± SE of six mice per group. For statistical analysis of differences between groups, a one-way ANOVA followed by a Tukey HSD pairwise analysis was done using JMP software (SAS Institute). $P < 0.001$ was considered significant.

Generation of tumor lysates. Tumor lysates were prepared by homogenization in cold modified radiolymphonuclease assay buffer [50 mmol/L Tris-HCl (pH 7.2), 100 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% w/v Triton X-100, 0.5% w/v sodium deoxycholic acid, 0.1% w/v SDS, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride] supplemented with 1 Complete protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN) per 50 mL of buffer. Tissue and cell debris was removed by centrifugation. Protein concentration was determined with a modified bicinchoninic acid protein assay (Pierce Chemical) as per the manufacturer’s instructions.

In situ analyses of xenograft tumor samples. All tissues were fixed in 10% (v/v) formalin and paraffin embedded. Sections 5-µm thick were deparaffinized, deproteinized in 4 µg/mL of proteinase K for 30 minutes at 37°C, and further processed for in situ hybridization as previously described (33, 34). $^{35}$P-UTP labeled sense and antisense probes were hybridized to the sections at 55°C overnight. Unhybridized probe was removed by incubation in 20 µg/mL RNase A for 30 minutes at 37°C followed by a high stringency wash at 55°C in 0.1 × SSC for 2 hours and dehydration through graded ethanol. The slides were dipped in NBT2 nuclear track emulsion (Eastman Kodak, Rochester, NY), exposed in sealed plastic slide boxes containing desiccant for 4 to 6 weeks at 4°C, developed, and counterstained with H&E. The following probe templates were PCR amplified using the primers described below. Upper primers and lower primers for murine $VEGF$ exon 3, murine $PDGFA$, and murine $PDGFB$ had 27 nucleotide extensions appended to the 5′ ends encoding T7 RNA polymerase and T3 RNA polymerase promoters, respectively, for generation of sense and antisense transcripts. $mVEGF$ exon 3 probe: 193 nucleotides corresponding to nucleotides 202-394 of NM_005505, upper primer, 5′-TAGCAAGTTCATGCAGAGCAACATCAC-3′ and lower primer, 5′-ATGGTGAATGTTGCTGCTGCTGCT-3′. Murine $PDGFA$ probe: 571 nucleotides corresponding to nucleotides 192-762 of NM_008808, upper primer, 5′-TGCGCTTCCCGCTCGCTGCTTCCT-3′ and lower primer, 5′-CTGCTCTCTCCTCGGTGCTGCT-3′. Murine $PDGFB$ probe: 653 nucleotides corresponding to nucleotides 1082-1734 of NM_011057, upper primer, 5′-CACCTCATCGCCCTTTGTGA-3′ and lower primer, 5′-AAATACCCTGCCCCACATCTTG-3′. Two templates were generated for murine $PDGFR\alpha$, $PDGFR\beta$, human $PDGFA$, and human $PDGFB$ with T7 RNA polymerase promoters appended to the 5′ end of either the upper primer for sense transcripts or the lower primer for antisense transcripts. $mPDGFR\alpha$ probe: length 799 nucleotides corresponding to nucleotides 268-1066 of NM_001058, upper primer, 5′-TATCCCTCATCCATCTGGAGAC-3′ and lower primer, 5′-GGGCGACATCATCCTACTGCC-3′. $mPDGFR\beta$ probe: 742 nucleotides corresponding to nucleotides 574-1315 of NM_008809, upper primer, 5′-ATGGTGAATGTTGCTGCTGCTGCT-3′ and lower primer, 5′-GGGCGACATCATCCTACTGCC-3′. Human $PDGFA$ probe: 578 nucleotides corresponding to nucleotides 844-1421 of NM_002607, upper primer, 5′-GACCTTGCTGCCTCGCTGCTCCTGCC-3′ and lower primer, 5′-ATGGTGAATGTTGCTGCTGCTGCT-3′. Human $PDGFB$ probe: 621 nucleotides corresponding to nucleotides 1037-1657 of NM_002608, upper primer, 5′-CTGCTCTGCTCTCTGCTGCTCCTGCC-3′ and lower primer, 5′-CTGCTCTGCTCTCTGCTGCTCCTGCC-3′.
**Immunohistochemical analyses of xenografted tumor samples.** Tumors were fixed in neutral-buffered formalin for 24 hours before paraffin embedding. H&E staining and immunohistochemistry were done as described previously (33). Immunohistochemical staining was done using the antigen retrieval systems and appropriate antibodies listed in Table 2. Each was detected sequentially using the appropriate biotinylated secondary antibody, Vectastain Avidin-Biotin Complex Peroxidase Elite and Vectastain Elite, or streptavidin-alkaline phosphatase (Vector Laboratories, Burlingame, CA). Reaction product was generated using metal-enhanced 3,3′-diaminobenzidine (Pierce Chemical) or Vector Blue (Vector Laboratories) as appropriate. Sections were lightly counterstained with hematoxylin, dehydrated, and coverslipped.

**Results**

**Stromal cell recruitment correlates with the resistance to anti-human VEGF therapy in a xenograft model.** To examine the influence of the stroma on the response of these tumors to anti-VEGF therapy, we made use of a human tumor cell line, Calu-6, which is characterized by its ability to induce a strong host stromal response in vivo. In several experiments, Calu-6 tumor growth was minimally inhibited by treatment with mAb A4.6.1, an antibody recognizing human VEGF (30). Figure 1A illustrates a representative experiment in which only 5% inhibition was achieved. However, treatment of these tumors with mFlt(1-3)-IgG, a soluble form of VEGFR1 that binds and sequesters both tumor- and host-derived VEGF, resulted in growth inhibition of >90% (35). In contrast, the A673 tumors, which was used as a stromal fibroblast cell model because it has proven to be a more robust cell type than primary stromal cell cultures (data not shown). This was important given the harsh nature of some of the buffers used in the purification protocol. Calu-6 conditioned medium was subjected to cation-exchange chromatography, and fractions 4 to 8 were found to contain potent mitogenic and chemotactic activities (Fig. 2A and B, respectively). These fractions were pooled and subjected to size exclusion chromatography. Fractions 30 to 32, corresponding to an Mr of 20 to 45 kDa, contained a potent mitogenic (Fig. 2C) and chemotactic (Fig. 2D) activity. These fractions were pooled and purified further by reverse-phase chromatography. Again, prominent mitogenic and chemotactic activities (Fig. 2E and F, respectively) coeluted in fractions 15 to 22, which corresponded to 25% to 35% acetonitrile.

**Calu-6 cells secrete a potent fibroblast bioactive factor.** The extensive incorporation of stromal fibroblasts into the Calu-6 tumor mass (Fig. 1B) suggested that these tumor cells could be a source of stromal mitogens and chemoattractants. Therefore, Calu-6 conditioned medium was generated and subjected to column chromatography. Both mitogenic and chemotactic activities were monitored using the NIH3T3 fibroblast cell line, which was used as a stromal fibroblast cell model because it has proven to be a more robust cell type than primary stromal cell cultures (data not shown). This was important given the harsh nature of some of the buffers used in the purification protocol. Calu-6 conditioned medium was subjected to cation-exchange chromatography, and fractions 4 to 8 were found to contain potent mitogenic and chemotactic activities (Fig. 2A and B, respectively). These fractions were pooled and subjected to size exclusion chromatography. Fractions 30 to 32, corresponding to an Mr of 20 to 45 kDa, contained a potent mitogenic (Fig. 2C) and chemotactic (Fig. 2D) activity. These fractions were pooled and purified further by reverse-phase chromatography. Again, prominent mitogenic and chemotactic activities (Fig. 2E and F, respectively) coeluted in fractions 15 to 22, which corresponded to 25% to 35% acetonitrile.

**Calu-6 cells express significant levels of PDGFA and PDGFC.** Previous studies aimed at characterizing novel fibroblast chemoattractants identified fibronectin as the major bioactive factor (25–27). Therefore, a number of size-excluded fractions ranging in size from 15 to 670 kDa were examined for the presence of fibronectin by Western blotting. Fibronectin is present in the fractions of molecular weight of >158 kDa (Fig. 3A), which represent a minor portion of the total chemotactic activity in Calu-6 conditioned medium and were subsequently excluded from further purification. A candidate approach was then taken to try to identify the factors present in the major peaks of bioactivity. Because members of the PDGF family are the most potent fibroblast mitogens and chemoattractants, the fractions were initially tested for PDGFRB or PDGFRB, and their levels were determined to be below the limits of detection by ELISA (<31.25 pg/mL). In contrast, significant amounts of PDGFAA (>30 ng/mL per fraction) were found to coelute with the major peak of activity in the size-excluded fractions (Fig. 3B). The bioactive fractions generated

### Table 2. Summary of antibodies and reagents

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<th>Antibody</th>
<th>Source</th>
<th>Primary antibody</th>
<th>Primary antibody (µg/mL)</th>
<th>Antigen retrieval</th>
<th>Secondary antibody</th>
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<td>Goat polyclonal AF1062</td>
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<td>Target, High pH Trilogy</td>
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<td>Goat polyclonal AF1062</td>
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<td>MECA-32 (PVLAP endothelial antigen)</td>
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<td>Rabbit anti-rat biotinylated</td>
<td>2.5</td>
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</table>
by cation-exchange or reverse-phase column chromatography were also tested and found to contain considerable amounts of PDGFAA (data not shown).

We also tested for the presence of PDGFAA and PDGFBB in the conditioned medium generated from a variety of human cancer cell lines whose respective tumors are associated with substantial amounts of stroma (Table 3). The results show considerable expression of PDGFAA by these tumor cell lines. In contrast, PDGFBB was undetectable. Because there is a paucity of effective antibody reagents to test for the presence of either PDGFCC or PDGFD, we used quantitative RT-PCR to assess the levels of the various PDGF family members in RNA isolated from Calu-6 cells using human PDGF-specific primers. Calu-6 cells express considerable levels of PDGFA and C, whereas PDGFBB and PDGFD are virtually undetectable (Fig. 3C).

To assess the physiologic relevance of the results derived using the Calu-6 tumor model, we examined RNA isolated from tumors derived from a human pancreatic adenocarcinoma cell line HPAC, which is associated with a significant stromal response in vivo (37). Similarly to the Calu-6 tumors, HPAC tumors expressed significant levels of PDGFA and PDGFCC, whereas the levels of PDGFBB or PDGFD were barely detectable (Fig. 3D). ELISA done on the conditioned medium of this cell line indicated the presence of PDGFAA (0.608 ng/mL) at levels that were comparable with the levels detected in the Calu-6 conditioned medium (0.893 ng/mL). In contrast, A673 conditioned medium contains little PDGFAA (0.062 ng/mL). In all conditioned media, PDGFBB was undetectable (Table 3).

We also assessed the relative expression of each human PDGF in the Calu-6 and A673 tumors using quantitative RT-PCR. The results show significant expression of PDGFA and PDGFCC by the Calu-6 tumors in contrast to the considerably lower levels detected in the A673 tumors. In both tumors, the levels of PDGFBB were significantly lower, whereas the levels of PDGFD were barely detectable (Fig. 3E). ELISA was used to confirm the relative levels of PDGFAA and PDGFBB in Calu-6 tumor lysates. Although Calu-6 tumors contain significant levels of PDGFAA [267 ± 55 pg/mg total protein (SE)], the levels of PDGFBB are ∼3-fold lower [93.7 ± 24.9 pg/mg total protein (SE)]. Similar results were obtained in an independent experiment (data not shown).

To complement these studies, we analyzed the expression of the PDGFs and their cognate receptors using GeneExpress, a compendium of microarray gene expression data containing thousands of clinical samples. This analysis, which included 138 normal samples and 159 tumor samples, showed considerable PDGFA expression in the normal lung and the tumor samples. However, a subset of lung tumor samples (20 of 159), classified predominantly as adenocarcinomas, contained significantly higher levels of PDGFA (Fig. 3F). Interestingly, the overall levels of PDGFCC were found to be higher in the tumor samples compared with the normal samples (mean signal intensities of 1,486 and 1,174 arbitrary units, respectively; P < 0.0001). In contrast, both PDGFBB and PDGFD are expressed at lower levels overall, and no differences in expression were found in the normal compared with the tumor samples. The considerable expression of both PDGFRα and PDGFRβ detected in both normal and tumor samples coupled with the predominant expression of PDGFA and C suggests that this axis of PDGF signaling may be an important part in the development of lung cancer.
**PDGF/PDGFR signaling is critical to tumor growth in vivo.**

To discern whether the activities in the various fractions exerted their effects by signaling through PDGFRα or PDGFRβ, the size-excluded fractions were retained in the presence of neutralizing recombinant human PDGFR receptors. Soluble PDGFRα, which primarily inhibits PDGFA and PDGFCC signaling, abrogated the mitogenic (Fig. 4A) and chemotactic (Fig. 4B) activities detected in the size-excluded fractions. In contrast, the presence of soluble PDGFRβ, which primarily inhibits PDGFB and PDGFD signaling, did not affect either activity.

To further characterize the different bioactivities, we used an reverse-phase column chromatography. Once again, the prominent peak of mitogenic and chemotactic activity was found to coincide with the elution of PDGFA (data not shown).

Given the significant levels of PDGFA and PDGFCC expressed by the tumors and the effects of blocking PDGFα signaling in vitro, we examined the effects of inhibiting PDGFα signaling on tumor growth in vivo. A xenograft experiment was done, in which Calu-6 cells were injected s.c. into the rear flanks of immune-deficient mice. Beginning 24 hours later, the mice were treated i.t. with adenovirus encoding LacZ (Ad-LacZ), Flt-IG (Ad-mFlt-IG), or soluble forms of PDGFRα (Ad-PDFGRα-IgG) or PDGFRβ (Ad-PDGFRβ-IgG), and their effect on tumor growth was assessed. Compared with the Ad-LacZ-treated samples, all of the treatments significantly affected the rate (P < 0.01; Fig. 4C) and the extent (P < 0.001; Fig. 4D) of tumor growth. Notably, Ad-PDGFRα treatment was as effective as treatment with Ad-mFlt-IG (Fig. 4C and D). In representative histologic sections of treated tumors (n = 6 or 7 per group in each of two separate experiments), the areas of tumor necrosis, estimated visually, was similar in control and PDGFRα-IgG groups (36 ± 26%, n = 12; P = 0.055 versus controls) and Ad-PDGFRα-IgG-treated tumors (43 ± 24%, n = 12; P = 0.006 versus controls). The extent of necrosis was not significantly different between Ad-Flt- and Ad-PDGFRα-IgG-treated groups (P = 0.47). We also tested the effects of the adenoviral constructs on the growth of established tumors. As single agents, the soluble PDGFR receptors and mFlt-IG effectively inhibited the growth of established tumors of an average size of approximately 150 mm³ (data not shown).

PDGFRα autocrine signaling has been described in gliomas and sarcomas but not in carcinomas (18, 38). However, it was important to show that the antitumor effects observed resulted from effects upon the host stromal cells rather than anti-proliferative effects on the tumor cells caused by disruption of an existing autocrine loop. Therefore, we tested the effects of the soluble PDGFR receptors on Calu-6 proliferation in vitro. Calu-6 cell proliferation was unaffected by either receptor (Fig. 4E), even at levels 5-fold greater than those used to inhibit NIH3T3 fibroblast proliferation and migration (Fig. 4A and B).

Additionally, Calu-6 cells failed to respond to several recombinant growth factors tested, including PDGFA and BB; yet, they proliferated readily in complete medium (10% fetal bovine serum).

**Expression of PDGFRα is highest in fibroblasts in the tumor outer rim.**

To dissect the mechanisms underlying the antitumor effects observed, in situ hybridization analysis was done on Calu-6 tumor sections using probes for human and murine PDGFA and PDGFB and the murine PDGFRα and PDGFRβ (Fig. 5). As the adenoviral treatments did not affect host expression of any of the genes examined, only the Ad-LacZ-treated controls are provided. Human PDGFA is abundantly expressed throughout the tumor mass with particularly intense regions around areas of necrosis (Fig. 5A). In contrast, no signal was detected in the sense control. PDGFB was more weakly expressed throughout the tumor mass, with modest increases in intensity around necrotic regions. To assess the host's contribution of these factors, probes specific to murine PDGFA and PDGFB were used. PDGFA signal was weak and scattered throughout the tumor mass, whereas the PDGFB signal detected was associated with blood vessels (Fig. 5B). These results suggested that the majority of the PDGFA were produced by the tumor epithelium. To confirm these results, quantitative RT-PCR was done using primers that recognize both human and murine forms of PDGFA. When compared with the results obtained using primers specific for human PDGFA the results, normalized to glyceraldehyde-3-phosphate dehydrogenase, indicated that the preponderance of PDGFA in the tumors is produced by the tumor cells themselves and not host cells (data not shown). In situ hybridization revealed that the most intense expression of PDGFα was localized to stromal fibroblasts at the tumor periphery (Fig. 5C). Alternatively, PDGFB displayed a punctuate pattern of expression, associated with discrete stromal cell clusters throughout the tumor. Because members of the PDGF family have been shown to affect the angiogenic process, there was a possibility that Ad-PDGFR-IgG treatment affected the expression of VEGF.

A VEGF-specific probe displays a modest signal from the fibroblasts within the stroma, with the highest intensity associated with the tumor cells at the periphery of the necrotic regions (Fig. 5D). The significant cross-reactivity of the probe for the human and murine VEGF transcripts made it difficult to discern any significant changes in VEGF expression resulting from the adenoviral treatments. Taqman analysis of tumor RNA using primers specific for murine VEGF-A indicated that treatment with Ad-PDGFRα resulted in an ~40% reduction in expression relative to Ad-LacZ (Supplementary Fig. S1).

**Calu-6 tumors recruit PDGFRα+ stromal fibroblasts.**

To verify these results, immunohistochemistry of the tumor samples was done using antibodies to murine PDGFRα, PDGFRβ, αSMA, and MECA-32, an endothelial PLVAP-specific antibody (Fig. 6). The most intense PDGFRα-specific staining localized to stromal fibroblasts present at the periphery of the tumor (Fig. 6B). Within the tumor mass, this stromal cell type seemed to exhibit...
a decreased expression of PDGFRα. In the surrounding tissue, expression of PDGFRα localized predominantly to the vasculature (Fig. 6E). In addition, a low level of expression was associated with a portion of the fibroblast cells. Within the tumor mass, PDGFRα expression also localized to blood vessels but was equivalently expressed in a major portion of the stromal fibroblasts. MECA-32 staining was done to more clearly identify blood vessels in the tumor periphery and in the tumor mass (Fig. 6C-E). Staining for αSMA in the normal stroma was strongly associated with vascular smooth muscle cells in a manner similar to the results of PDGFRα staining (Fig. 6K). Within the tumor mass, αSMA expression remained vascular, but in addition, localized to nonvascular stromal cells in a pattern that was most consistent with that observed for PDGFRα expression (Fig. 6L).

**Discussion**

In previous studies, Dong et al. showed a VEGF-null murine tumor’s ability to overcome the need for VEGF by recruiting the host stroma, a significant contributor to tumor angiogenesis and growth, in a PDGFAA-dependent manner (29). Here, we show that this is also true of human tumor cells.

We initially focused on the response of Calu-6 tumors to treatment using an anti-human VEGF antibody mAb A4.6.1. These studies revealed a significant incorporation of host stromal cells, displaying the spindle-like fibroblast phenotype, into the Calu-6 tumor mass. The degree of stromal cell incorporation correlated strongly with the host-stromal contribution of VEGF and the ability of these tumors to resist treatment with mAb 4.6.1. In contrast, tumors without a substantial host-derived stromal component, such as the A673 rhabdomyosarcoma, are profoundly inhibited by mAb 4.6.1. These results suggest that recruitment of stromal fibroblasts might play an important role in tumor angiogenesis, by providing additional VEGF. Alternatively, recent studies indicate that tumor fibroblasts may contribute to angiogenesis through VEGF-independent pathways. Orimo et al. have reported that cancer-associated fibroblasts produce SDF-1, which in turn leads to recruitment of bone marrow-derived endothelial progenitor cells by the tumor vasculature (39). Thus, the elucidation of the mechanisms of tumor stroma recruitment is potentially important for our understanding of the development of resistance to anti-VEGF therapies.

Surprisingly, few studies have attempted to identify tumor-derived chemoattractants for stromal cells. Using conditioned medium from a colon carcinoma cell line, Morimoto et al. identified fibronectin fragments of 210 and 185 kDa as the major fibroblast chemoattractants (17, 18, 40, 41). Although fibronectin was detected in the Calu-6 conditioned medium and size-excluded fractions, it was determined to represent a relatively minor portion of the chemotactic activity.
In contrast, we detected significant levels of PDGFAA protein in the bioactive fractions generated from Calu-6 conditioned medium and, additionally, in the conditioned medium of a variety of cell lines whose tumors are associated with a significant stromal response in vivo. The absence of detectable levels of PDGFBB in these conditioned media was notable given that it is the most potent and tumorigenic of the PDGF family members (42, 43). Using quantitative RT-PCR and

<table>
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<th>Tumor cell line</th>
<th>PDGFAA (ng/mL)</th>
<th>PDGFBB (ng/mL)</th>
<th>Description</th>
<th>Tissue source</th>
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<td>Anaplastic carcinoma</td>
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<td>ND</td>
<td>Rhabdomyosarcoma</td>
<td>Muscle</td>
</tr>
</tbody>
</table>

NOTE: Below the limits of detection of the ELISA (<31.2 pg/mL).
Abbreviation: ND, not detected.

Fig. 4. Antagonizing PDGFR-signaling inhibits Calu-6 tumor growth in vivo. A, size-excluded fractions were tested for mitogenic activity alone and in the presence of recombinant soluble PDGFRα or PDGFRβ. B, in a similar manner, a migration assay was done with these fractions alone and in the presence of recombinant soluble PDGFRα or PDGFRβ. C, Calu-6 derived tumors were treated with adeno virus encoding β-galactosidase (LacZ), mFlt(1-3)-IgG, PDGFRα-IgG, or PDGFRβ-IgG. Tumor volume (mm³) over time in days for each of the treatment groups. Columns, mean of six mice per group; bars, SE. Differences between groups were assessed by one-way ANOVA followed by a Tukey HSD pairwise analysis. *P < 0.001 was considered significant. D, net weights of the Calu-6 tumors at the end of the experiment are presented as wet tumor weight (g) over adeno viral treatment. E, effects of soluble PDGFRα and PDGFRβ on the proliferation Calu-6 cell were examined in vitro. Total cell number over treatment.


PDGFRα Signaling and Human Lung Carcinoma
ELISA, we showed that Calu-6 tumors express significant levels of PDGFAA protein and PDGFC transcripts. In contrast, these tumors contained comparably lower levels of PDGFB. We showed that soluble PDGFRα but not PDGFRβ abrogates the chemotactic and mitogenic activities in the size-excluded fractions, supporting the presence of PDGFAA. However, given the lack of suitable antibody reagents, we are presently unable to confirm the presence of PDGFC in either the Calu-6 conditioned medium or the Calu-6 tumors. To address the physiologic relevance of the results derived using the Calu-6 tumor model, we showed that tumors derived from a human pancreatic adenocarcinoma cell line also express significant levels of PDGFA and PDGFC. Similar to the Calu-6 tumors, these pancreatic tumors are characterized by their capacity to induce a significant stromal infiltration in vivo (37).

Interestingly, an earlier study (27) reported that PDGFA is important to initiate a desmoplastic response in a ras-transformed breast carcinoma cell line, in agreement with our findings in a lung carcinoma model. However, the functional significance of stromal recruitment was not explored in that study.

Studies done in vitro and in vivo have shown that, like PDGFA, PDGFC dimers signal primarily through PDGFRα to stimulate fibroblast proliferation, collagen deposition, and angiogenesis (44, 45). Our studies, along with those of others, have shown that PDGFC is expressed in a wide variety of tumor cell lines, indicating an active role for PDGFC in tumorigenesis (46). Coupled with the data showing that expression of PDGFC is significantly up-regulated in human lung tumors, our results implicate PDGFRα signaling in the development or progression of such types of solid tumors.

The high levels of PDGFA and PDGFC in the Calu-6 tumors prompted us to examine the effect of antagonizing the PDGFRα signaling pathway on tumor growth. Commonly used small-molecule receptor tyrosine kinase inhibitors of PDGF display characteristic nonspecific cross-reactivity with other receptor tyrosine kinases, making it difficult to distinguish the effects of targeting a single type of receptor on an individual class of cells from targeting several receptor classes on various different cell types (47). Therefore, we used adenovirus to maintain a sustained and robust expression of neutralizing forms of the PDGF receptors in vivo. We showed that disrupting PDGFRα signaling inhibits tumor growth as efficiently as blocking VEGF, and that this did not result from antiproliferative effects on the tumor cells themselves. This latter result was consistent with in vivo evidence showing the existence of PDGFRα autocrine signaling in gliomas and sarcomas but not in carcinomas (18, 38). The significant antitumor effects of Ad-PDGFRβ are consistent with antivascular effects, such as inhibition of pericyte recruitment. Furthermore, the localization of PDGFRβ in fibroblasts within the tumor mass suggests that inhibition of stromal recruitment may also contribute to the effect.

In situ hybridization of tumor samples revealed particularly significant PDGFRβ expression in the stromal fibroblasts at the periphery of the tumors, and this was confirmed by immunohistochemistry, which also indicated a lower level of PDGFRα expression in the cells that had become incorporated into the tumor. This localization of PDGFRβ is intriguing, considering that several recent studies have emphasized that the tumor periphery is a particularly dynamic area. Tumor growth and resistance to vascular targeting agents are mediated by tumor cell proliferation starting at the outer rim (48). Furthermore, recruitment of bone marrow–derived endothelial progenitor
cells first occurs at the tumor periphery (49). Studies are ongoing to further characterize such PDGFRα-expressing cells.

The finding that Ad-PDGFRα administration resulted in a reduced expression of mVEGF is consistent with the view that decreased stromal-derived angiogenesis is, at least in part, responsible for the antitumor effects.

Studies in a variety of solid tumors have shown that the overexpression of PDGFAA and constitutively activated forms of PDGFRα are associated with a poor prognosis (50–52). Our results suggest that blocking the tumor-directed recruitment of PDGFRα+ fibroblasts into the tumor mass underlies the significant antitumor effects observed in our xenograft experiments and may have implications for the treatment of these types of tumors. Our in situ hybridization and immunohistochemistry analyses revealed the appositional expression of PDGFB and PDGFRβ in the vasculature, consistent with a role for stromal cell paracrine signaling in pericyte recruitment and vessel maturation. This interaction has also been shown to be critical to tumor growth (53–55). Given the shown expression of PDGFRα and PDGFRβ by the stromal fibroblasts and pericytes, it is likely that the PDGF signaling pathways mediated by PDGFRα and PDGFRβ play distinct and complementary roles in the tumorigenic process.

Our studies highlight the critical role played by PDGFRα signaling and underscore the effect of targeting stromal fibroblasts, in addition to the endothelial cells and pericytes.

Acknowledgments

We thank Joe Kowalski for his helpful assistance, Kyu Hong and John Gutierrez for development of the murine VEGF ELISA, and Dr. Luc Desnoyer and Raji Kaul for the HPAC tumor samples used in these studies.

References

Human Cancer Biology


Tumor-Driven Paracrine Platelet-Derived Growth Factor Receptor α Signaling Is a Key Determinant of Stromal Cell Recruitment in a Model of Human Lung Carcinoma

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