Cellular Source and Amount of Vascular Endothelial Growth Factor and Platelet-Derived Growth Factor in Tumors Determine Response to Angiogenesis Inhibitors

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Abstract

Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and their receptors are important targets in cancer therapy based on angiogenesis inhibition. However, it is unclear whether inhibition of VEGF and PDGF together is more effective than inhibition of either one alone. Here, we used two contrasting tumor models to compare the effects of inhibiting VEGF or PDGF alone, by adenovirally generated soluble receptors, to the effects of inhibiting both together. In RIP-Tag2 tumors, VEGF and PDGF inhibition together reduced tumor vascularity and abundance of pericytes. However, VEGF inhibition reduced tumor vascularity without decreasing pericyte density, and PDGF inhibition reduced pericytes without reducing tumor vascularity. By contrast, in Lewis lung carcinomas (LLC), inhibition of VEGF or PDGF reduced blood vessels and pericytes to the same extent as did inhibition of both together. Similar results were obtained using tyrosine kinase inhibitors AG-013736 and imatinib. In LLC, VEGF expression was largely restricted to pericytes and PDGF was largely restricted to endothelial cells, but, in RIP-Tag2 tumors, expression of both growth factors was more widespread and significantly greater than in LLC. From these findings suggest that inhibition of PDGF in LLC reduced pericytes, and then tumor vessels regressed because pericytes were the main source of VEGF. The vasculature of RIP-Tag2 tumors, in which most VEGF is from tumor cells, was more resistant to PDGF inhibition. The findings emphasize the interdependence of pericytes and endothelial cells in tumors and the importance of tumor phenotype in determining the cellular effects of VEGF and PDGF inhibitors on tumor vessels.

Introduction

Angiogenesis is a pivotal process in the growth, invasion, and spread of tumors (1–3) and is used as a therapeutic target in several types of cancer based on the abnormalities of tumor blood vessels (4–6). Endothelial cells of tumor vessels are disorganized, loosely connected, branched, sprouting, and form a defective cellular lining of the vessel wall (7). Pericytes, which play a key role in vascular development, stabilization, maturation, and remodeling (8–10), are present on tumor vessels but have multiple abnormalities, including loose association with the vessel wall, impaired support of endothelial function, and altered protein expression (11, 12).

Endothelial cells and pericytes interact through vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), and their receptors are important targets in cancer therapy based on angiogenesis inhibition. However, it is unclear whether inhibition of VEGF and PDGF together is more effective than inhibition of either one alone. Here, we used two contrasting tumor models to compare the effects of inhibiting VEGF or PDGF alone, by adenovirally generated soluble receptors, to the effects of inhibiting both together. In RIP-Tag2 tumors, VEGF and PDGF inhibition together reduced tumor vascularity and abundance of pericytes. However, VEGF inhibition reduced tumor vascularity without decreasing pericyte density, and PDGF inhibition reduced pericytes without reducing tumor vascularity. By contrast, in Lewis lung carcinomas (LLC), inhibition of VEGF or PDGF reduced blood vessels and pericytes to the same extent as did inhibition of both together. Similar results were obtained using tyrosine kinase inhibitors AG-013736 and imatinib. In LLC, VEGF expression was largely restricted to pericytes and PDGF was largely restricted to endothelial cells, but, in RIP-Tag2 tumors, expression of both growth factors was more widespread and significantly greater than in LLC. These findings suggest that inhibition of PDGF in LLC reduced pericytes, and then tumor vessels regressed because pericytes were the main source of VEGF. The vasculature of RIP-Tag2 tumors, in which most VEGF is from tumor cells, was more resistant to PDGF inhibition. The findings emphasize the interdependence of pericytes and endothelial cells in tumors and the importance of tumor phenotype in determining the cellular effects of VEGF and PDGF inhibitors on tumor vessels.

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administering the two agents together. Similar results were obtained when we targeted VEGF and PDGF signaling using the tyrosine kinase inhibitor AG-013736, which blocks VEGFRs (35, 36), and imatinib, which blocks PDGFRs (37). The contrasting responses of the two tumors were consistent with differences in the amount and cellular location of VEGF and PDGF in the tumors.

Materials and Methods

Animals. Tumor-bearing RIP-Tag2 transgenic mice (C57BL/6 background; ref. 34) were studied at 10 wk of age (36). For the implantation of LLC tumors, we used two different approaches as described in Supplementary data. All experiments were done in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of California, San Francisco. In each experimental group, 5 to 10 mice were included.

Construction and use of adenoviral vectors. The Ad-VEGFR1 construct encoding the murine VEGFR-1 ectodomain, the Ad-PDGFRβ construct encoding the murine PDGFR-β ectodomain, and Ad-Fc encoding the constant Fc region of human immunoglobulin G were prepared as previously described (32, 33, 38). Both soluble ectodomains of VEGFR-1 and PDGFR-β had a COOH-terminal 6×His epitope tag (32, 33, 38).

Mice were injected i.v. on day 0 with (a) Ad-PDGFRβ at a dose of 4 × 10⁹ plaque-forming units (pfu); (b) Ad-VEGFR1 at a dose of 1 × 10⁹ pfu (32); (c) combination of Ad-PDGFRβ and Ad-VEGFR1 at a dose of 4 × 10⁸ and 1 × 10⁹ pfu, respectively; or (d) Ad-Fc, as a control, at a dose of 1 × 10⁹ or 4 × 10⁹ pfu. To analyze the adenoviral expression of the appropriate transgene, plasma samples were collected after anesthesia from the tail vein of mice 7 d after adenoviral injection, and 1 μl of plasma was analyzed by Western blot with rabbit anti-His antibody (Santa Cruz Biotechnology). Blots were developed with rabbit-anti-horseradish peroxidase conjugates (New England Pharmacy and administered at a dose of 50 mg/kg body weight in a volume of 5 mg of total RNA using the cDNA synthesis kit (Roche). AG-013736 (Axitinib), a potent small-molecule inhibitor of VEGF, was supplied by Pfizer Global Research and Development. AG-013736 was administered at a dose of 10 mg/kg body weight in a volume of 5 ml/g twice daily by gavage for 7 d (39). Imatinib (Gleevec, Novartis Pharma), an inhibitor of several receptor tyrosine kinases, including PDGFR-α, PDGFR-β, v-Abi, and c-Kit, was purchased from the UCSF Pharmacy and administered at a dose of 50 mg/kg body weight in a volume of 5 ml/g twice daily by gavage for 7 d. Same dosages of AG-013736 and imatinib were used for the combination treatment.

Estimate of tumor size. At the end of the treatments, the weight (in milligrams) of LLC tumors was recorded. In RIP-Tag2 mice, the treatment effect on tumor size after treatment was assessed in 80-μm-thick cryostat sections stained for CD31 immunoactivity. Digital fluorescence microscopic images of all tumors visible in any of three sections cut at different levels of each pancreas were captured (5× objective, 1× Optovar, tissue region 1,920 by 2,560 μm), and then the sectional area of each tumor (8–20 tumors per mouse) was measured with ImageJ. Tumors too large to fit into a single image were recorded as multiple images and the data combined (36).

Fixation by vascular perfusion and immunohistochemistry. Following treatment, mice were fixed by vascular perfusion and the tumors were processed for and stained by immunohistochemistry as previously described (36). Endothelial cells were labeled with rat monoclonal anti-mouse CD31 (platelet/endothelial cell adhesion molecule 1; clone MECA 13.3, Pharmingen; 1:500) or hamster monoclonal anti-mouse CD31 (clone 2B8, Chemicon; 1:500). Pericytes were stained with Cy3-conjugated mouse monoclonal anti-α-smooth muscle actin (α-SMA; clone 1A4, Sigma; 1:1,000), rabbit polyclonal anti-chicken desmin (A0611, DAKO; 1:2,000), rabbit polyclonal anti-mouse NG2 proteoglycan (AB5320, Chemicon; 1:2,000), or rat monoclonal anti–PDGFR-β (clone APB5, e-Bioscience; 1:2,000). VEGF was stained with goat polyclonal anti-mouse VEGF antibody (1:400, R&D Systems, Inc.) that recognizes mouse VEGF₁₆₅ and VEGF₁₄₅. PDGF was stained with goat polyclonal anti-PDGF antibody (Upstate Biotechnology; 1:500) that recognizes mouse PDGF-AA, PDGF-AB, and PDGF-BB. Macrophages were stained with rat monoclonal anti-mouse F4/80 antibody (Serotec; 1:500) and rabbit polyclonal anti-mouse Iba1 antibody (Wako; 1:1,000).

Imaging and analysis. Specimens were examined with a Zeiss Axioptophot fluorescence microscope and a Zeiss LSM 510 laser scanning confocal microscope. Area densities were calculated from digital fluorescence microscopic images using an empirically determined threshold value of 30 to 50 as previously described (36). In each experiment, five mice per group were analyzed.

Reverse transcription-PCR methods. Total RNA was isolated from ~50 mg of tumor tissue using the RNeasy extraction kit (Qiagen). RNA yield and purity were determined by spectrophotometry. cDNA synthesis was done with 1 μg of total RNA using the cDNA synthesis kit (Roche). Quantitative reverse transcription-PCR (RT-PCR) was done using SYBR GreenER qPCR Supermix (Invitrogen) using a Bio-Rad Myq detection system. Expression of each target gene was normalized to the expression of the control gene β-actin. Primer sequences are available on request.

Statistical analysis. Values are expressed as means ± SE (minimum of n = 5 mice per group). The significance of differences among groups was assessed using ANOVA followed by the Bonferroni-Dunn or Fisher test for multiple comparisons. P < 0.05 was considered significant.

Results

Greater effect on RIP-Tag2 tumor area and vasculature by inhibiting VEGF and PDGF together than inhibiting either one alone. Systemic production of the sVEGFR-1 and sPDGFR-β was confirmed by the presence of a strong band for His-Tag in the plasma of RIP-Tag2 tumors (Supplementary Fig. S1). His-Tag sVEGFR-1 and PDGFR-β had a robust signal after injection of Ad-VEGFR1 and Ad-PDGFRβ but was not present after control virus (Ad-Fc; Supplementary Fig. S1).

In RIP-Tag2 mice, treatment with combination of Ad-VEGFR1 and Ad-PDGFRβ resulted in a 63% decrease in tumor areas compared with vehicle-treated mice (0.68 ± 0.14 versus 1.85 ± 0.19 mm²). After Ad-VEGFR1 treatment, the tumor sectional areas were reduced by 37% (1.16 ± 0.07 versus 1.85 ± 0.19 mm²). No reduction was observed after Ad-PDGFRβ treatment for 7 days (Fig. 1A).

RIP-Tag2 tumors were densely vascular under baseline conditions (Fig. 1B-i). After treatment with Ad-VEGFR1 and Ad-PDGFRβ for 7 days, tumor vascularity was reduced by 75%, as reflected by confocal microscopic images (Fig. 1B-ii) and measurements of CD31-positive endothelial cells (Fig. 1B-iii). A similar reduction in CD31 area density was observed after treatment with Ad-VEGFR1 alone (75% reduction), whereas treatment with Ad-PDGFRβ did not change the overall vascularity of RIP-Tag2 tumors (Fig. 1B-iii).

Next we analyzed the effects of blocking VEGF and PDGF signaling by using the tyrosine kinase inhibitors AG-013736 and imatinib. Combination treatment of AG-013736 and imatinib for 7 days reduced the vasculature of RIP-Tag2 tumors by 70%, whereas AG-013736 alone caused a 53% reduction. Imatinib did not decrease the blood vessels density of RIP-Tag2 tumors (Fig. 1D-i).

Pericytes identified by α-SMA immunoreactivity were abundant in untreated RIP-Tag2 tumors (Fig. 1C-i). Ad-PDGFRβ and Ad-VEGFR1 administered together caused a 75% decrease in α-SMA-positive pericytes (Fig. 1C-ii and C-iii). This reduction was greater than that observed after treatment with Ad-PDGFRβ (40% reduction) or Ad-VEGFR1 (50% reduction) alone (Fig. 1C-iii).

To determine whether the reduction of α-SMA-positive pericytes corresponded to a reduction in pericyte number or to a
down-regulation of marker expression, we analyzed three different pericyte markers: desmin, NG2 proteoglycan (NG2), and PDGFR-β (23). Desmin-, NG2-, and PDGFR-β-positive pericytes were abundant in the vehicle-treated RIP-Tag2 tumors (Fig. 2A). The combination treatment reduced desmin immunoreactivity by 70% (Fig. 2B and D-iii), NG2 immunoreactivity by 50% (Fig. 2B and D-iii), and PDGFR-β immunoreactivity by 50% (Fig. 2D-iii). Surprisingly, treatment with Ad-VEGFR1 alone had only small effects on desmin (20% reduction), NG2 (4% reduction), and PDGFR-β (10% reduction; Fig. 2C). These results suggested that in RIP-Tag2 tumors, inhibition of the VEGF signaling pathway did not significantly affect the number of pericytes. As expected, Ad-PDGFRβ treatment alone significantly reduced pericyte number (Fig. 2D), with reductions ranging from 40% to 53%, depending on the marker (Fig. 2D-iii).

Similar effects were observed after treatment with AG-013736 and imatinib. Indeed, combination of AG-013736 and imatinib reduced all the four pericyte markers analyzed by 50% to 70% (Fig. 1D-ii; Supplementary Fig. S2A). These data confirmed that in RIP-Tag2 tumors, inhibition of the VEGF signaling pathway did not significantly affect the number of pericytes.

Equal effect on LLC tumor size and vasculature by inhibiting VEGF or PDGF alone as inhibiting both together.

We then investigated the effect of monotherapy and combination therapy in LLC tumors. Systemic production of sVEGFR-1 and sPDGFR-β after treatment with Ad-VEGFR1 and Ad-PDGFRβ was confirmed by Western blot (data not shown).

In LLC tumors, treatment with Ad-VEGFR1 and Ad-PDGFRβ alone or in combination reduced the tumor size to a similar extent (40% reduction; Fig. 3A).

Figure 1. Reduction in RIP-Tag2 tumor area and vasculature by inhibition of VEGF and PDGF together. Combination of Ad-VEGFR1 and Ad-PDGFRβ for 7 d reduced RIP-Tag2 tumor area significantly more than did Ad-VEGFR1 or Ad-PDGFRβ treatment alone (A). Confocal micrographs compare the dense vasculature (CD31 immunoreactivity) of RIP-Tag2 tumor under baseline conditions (B-i) and pruned vasculature after combination of Ad-VEGFR1 and Ad-PDGFRβ for 7 d (B-ii). Analysis of CD31 area density shows that combination treatment reduced blood vessels to the same extent as did Ad-VEGFR1 alone and that Ad-PDGFRβ alone did not affect the tumor vasculature in RIP-Tag2 tumors (B-iii). The combined viral vectors reduced α-SMA–positive pericytes in RIP-Tag2 tumors (C-i and C-ii). Measurement revealed that all treatments reduced the area density of α-SMA, but the combination of Ad-VEGFR1 and Ad-PDGFRβ caused the greatest decrease (C-iii). Combination of AG-013736 and imatinib decreased the CD31 area density more than did AG-013736 treatment alone whereas imatinib did not change the blood vessels in RIP-Tag2 tumors (D-i). AG-013736 and imatinib combined reduced α-SMA–positive pericytes significantly more than did AG-013736 or imatinib alone (D-ii). *, P < 0.05, compared with Ad-Fc or vehicle. †, P < 0.05, compared with Ad-VEGFR1 or Ad-PDGFRβ. #, P < 0.05, compared with AG-013736 or imatinib. Scale bar in C-ii applies to all images: 115 μm.
The combined viral vectors Ad-VEGFR1 and Ad-PDGFR\(\beta\) caused a 65% reduction in vascularity of LLC tumors (Fig. 3B). Ad-VEGFR1 alone reduced blood vessels to a similar extent (50%; Fig. 3B-iii). Interestingly, in LLC, in contrast to RIP-Tag2 tumors, treatment with Ad-PDGFR\(\beta\) alone was sufficient to cause a 60% reduction in vascularity (Fig. 3B-iii).

Combination of AG-013736 and imatinib decreased the blood vessel density of LLC tumors by 60% (Fig. 3D-i). A similar reduction was induced by AG-013736 or imatinib treatment alone (Fig. 3D-i).

In LLC tumors, \(\alpha\)-SMA–positive pericytes (Fig. 3C-i) were severely reduced after combination of Ad-VEGFR1 and Ad-PDGFR\(\beta\) (78% reduction; Fig. 3C-ii and iii). Treatment with Ad-VEGFR1 reduced \(\alpha\)-SMA by 60% and treatment with Ad-PDGFR\(\beta\) decreased \(\alpha\)-SMA immunoreactivity by 70% (Fig. 3C-iii).

The analysis of desmin, NG2, and PDGFR-\(\beta\) immunoreactivities revealed that in LLC tumors, combination of Ad-VEGFR1 and Ad-PDGFR\(\beta\) reduced them by roughly the same extent (75% reduction; Fig. 4B and D-iii). In LLC, in contrast to RIP-Tag2 tumors, Ad-VEGFR1 significantly decreased all the markers, ranging in amount from 48% to 59% (Fig. 4C and D-iii), suggesting a decrease in terms of pericyte numbers. As expected, pericyte numbers were also severely reduced after Ad-PDGFR\(\beta\) (Fig. 4D), reflecting 75% to 78% reductions in immunoreactivities of the three markers (Fig. 4D-iii).

These findings were confirmed by inhibition of VEGF and PDGF signaling using AG-013736 and imatinib. Indeed, AG-013736 and imatinib either alone or combined significantly reduced all four pericyte markers (Fig. 4D-ii; Supplementary Fig. S2B).

**Figure 2.** Different effects on pericytes in RIP-Tag2 tumors by inhibiting VEGF and/or PDGF. Confocal microscopic images of Ad-Fc–treated RIP-Tag2 tumors stained for CD31 (green) and desmin (A-i) or NG2 (A-ii, red). After treatment with combination of Ad-VEGFR1 and Ad-PDGFR\(\beta\), a few desmin-positive (B-i) or NG2-positive (B-ii) pericytes remained. The area density measurements revealed that desmin was reduced by 70%, PDGFR\(\beta\) by 50%, and NG2 by 50% (D-ii). Treatment with Ad-VEGFR1 for 7 d significantly decreased desmin-positive (C-i), but not NG2- and PDGFR\(\beta\)-positive (C-ii and D-iii), pericytes. Ad-PDGFR\(\beta\) for 7 d reduced the immunoreactivities of desmin by 45% (D-i and D-ii), NG2 by 52% (D-ii and D-iii), and PDGFR\(\beta\) by 40% (D-ii). *, \(P < 0.05\), compared with Ad-Fc. Scale bar in D-ii applies to all images: 115 \(\mu m\).
understand why RIP-Tag2 tumors were less sensitive to monotherapy compared with LLC tumors, we analyzed the level and localization of VEGF and PDGF in the two tumor models.

In RIP-Tag2 tumors, the overall VEGF expression was higher than in LLC tumors. Indeed VEGF immunoreactivity was present in and around the tumor cells (Fig. 5A-i). In contrast, in LLC tumors, most VEGF-positive cells were located near endothelial cells (Fig. 5A-ii). The analysis of VEGF area density revealed that in RIP-Tag2 tumor, VEGF immunoreactivity was 10-fold higher compared with LLC (Fig. 5A-iii). This result was confirmed by VEGF mRNA analysis. The level of VEGF-A mRNA was more than 100 times higher in RIP-Tag2 tumors than in LLC (Fig. 5A-iv). Immunostaining experiments revealed that the VEGF-positive cells expressed the three pericyte markers α-SMA (Fig. 5B-i), PDGFR-β, and NG2 (Supplementary Fig. S3A), suggesting that pericytes are the main source of VEGF in LLC tumors.

Macrophages are a well-described source of VEGF (40); to assess if macrophage expressed VEGF in LLC tumors, we stained tumor sections with two different macrophage markers, F4/80 and Iba1. The staining revealed that very little VEGF immunoreactivity was present in F4/80- and Iba1-positive cells (Supplementary Fig. S3B).

To confirm our hypothesis that the different effects of VEGF and PDGF inhibition depend on the level and distribution of VEGF expression, we analyzed the effects of inhibiting VEGF and PDGF together or alone on a different clone of LLC tumor (LLCx) previously described to express high amounts of VEGF (33). Indeed, in LLCx, VEGF immunoreactivity was significantly higher compared with LLC tumors (Fig. 5B-ii and iii). By mRNA analysis, we found that the level of VEGF-A mRNA was more than 10 times higher in LLCx than in LLC tumors (Fig. 5C-i). Considering this difference, we asked whether LLCx had a different sensitivity to monotherapy and combined therapy compared with LLC tumors.

![Figure 3](image-url)

Figure 3. Combination of VEGF and PDGF inhibition reduced tumor size, vascularity, and α-SMA-positive pericytes in LLC tumors. Treatment with Ad-VEGFR1 and Ad-PDGFRβ alone or combined reduced the weight of LLC tumors by 45% (A). Vascularity was more sparse in LLC tumors (B-i) than in RIP-Tag2 tumors under baseline conditions and was reduced after Ad-VEGFR1 in combination with Ad-PDGFRβ (B-ii). Bar graph shows comparable reduction in CD31 immunoreactivities after combination, Ad-VEGFR1, or Ad-PDGFRβ treatment for 7 d (B-iii). Similarly, α-SMA-positive pericytes were significantly decreased after combination treatment (C-i and iii). The measurements of α-SMA area density showed that the combination, Ad-PDGFRβ, or Ad-VEGFR1 treatment caused a similar reduction (C-iii). AG-013736 and imatinib treatment combined or alone reduced the CD31 area density and α-SMA area density to similar extent (D). *P < 0.05, compared with Ad-Fc or vehicle. Scale bar in C-ii applies to all images: 110 μm.
In LLCx, treatment with combination induced a greater reduction of tumor blood vessels (82%) compared with single treatment (Fig. 5C-ii). Indeed, in LLCx, Ad-VEGFR1 reduced the tumor vessel density by 70% (Fig. 5C-ii). Moreover, in LLCx, treatment with Ad-PDGFRβ alone caused a significantly smaller reduction of the tumor vasculature (30% reduction) compared with that observed in LLC (60% reduction; Fig. 5C-ii). These results confirmed that PDGF inhibition had less effect on tumor vessels in tumors with high VEGF expression.

We then investigated the expression level and localization of PDGF. The overall level of PDGF immunoreactivity was higher in RIP-Tag2 tumors than in LLC. In RIP-Tag2 tumors, PDGF immunoreactivity was widely distributed in tumor cells (Fig. 5D-i) with some PDGF-positive blood vessels (Fig. 5D-i, arrows). In contrast, PDGF immunoreactivity was mainly restricted to endothelial cells in LLC tumors (Fig. 5D-ii). Measurements of PDGF area density revealed that PDGF immunoreactivity was 3-fold higher in RIP-Tag2 tumors than in LLC (Fig. 5D-iii). Different isoforms of PDGF are frequently present in tumors (41). Because the antibody used for the staining recognized PDGF-AA, PDGF-AB, and PDGF-BB, by quantitative RT-PCR, we analyzed the mRNA expression levels of PDGF-A and PDGF-B in both tumor types. Our data showed that PDGF-A and PDGF-B mRNA levels were 2.5- and 7.0-fold higher, respectively, in RIP-Tag2 tumors compared with LLC tumors (Fig. 5D-iv; Supplementary Fig. S3C).

**Different plasticity of VEGF and PDGF in RIP-Tag2 and LLC tumors.** We then tested our hypothesis that the differences in the responsiveness of RIP-Tag2 tumors and LLC tumors to monotherapy and combination therapy were due to differences in the level and localization of VEGF and PDGF expression. Levels of VEGF and PDGF were analyzed in both tumor types after inhibition of VEGF or PDGF signaling.

In RIP-Tag2 tumors, the widespread VEGF immunoreactivity (area density, 87 ± 1%) was reduced by 78% after Ad-VEGFR1
treatment (area density, 19 ± 2%) but it was not noticeably altered after Ad-PDGFRβ treatment (Fig. 6A). By comparison, in LLC tumors, the sparsely distributed VEGF immunoreactivity (area density, 9 ± 1%) was significantly reduced by ~70% after either Ad-VEGFR1 or Ad-PDGFRβ treatment (Fig. 6B). The number of VEGF-positive cells (identified as pericytes) was conspicuously reduced in LLC tumors after Ad-VEGFR1 or Ad-PDGFRβ treatment (Fig. 6B).

In RIP-Tag2 tumors, the extensive PDGF immunoreactivity was reduced by 88% after PDGF inhibition but was not significantly affected by VEGF inhibition (Fig. 6C). However, in LLC, sequestration of VEGF decreased by 60% the level of PDGF. The number of PDGF-positive cells (identified as endothelial cells) was drastically reduced after Ad-sVEGFR1 or Ad-PDGFRβ treatment (Fig. 6D).

**Discussion**

The present study sought to dissect the effects of combined blockage of VEGF and PDGF signaling on tumor blood vessels in two mouse tumor models, RIP-Tag2 tumors and LLC tumors. We found that combination of Ad-VEGFR1 and Ad-PDGFRβ strongly reduced tumor size, blood vessels, and pericytes in both tumor models. In RIP-Tag2 tumors, single Ad-VEGFR1 or Ad-PDGFRβ treatment caused a lower reduction in tumor area, blood vessels, and pericytes compared with the combination treatment. In contrast, in LLC tumors, blocking VEGF or PDGF signaling alone reduced tumor size, pericytes, and endothelial cell density to the same extent as did the combination treatment. Similar results were observed by inhibition of VEGF and PDGF signaling using the tyrosine kinase inhibitors AG-013736 and imatinib.

Pericytes in tumors are loosely associated with endothelial cells and have cytoplasmic processes that extend away from the vessel wall (12, 42). Pericytes express different markers in different organs and tumors (12, 43, 44). The lack of a single unique marker for all pericytes presents a challenge for their identification. The absence of a marker could reflect absence of expression by pericytes or absence of pericytes. In the present study, to assess the presence of...
pericytes, we used four markers: α-SMA, desmin, NG2, and PDGFR-β (23). Pericytes in RIP-Tag2 tumors and LLC tumors expressed all four markers, with minor differences in cellular localization due to the association of α-SMA and desmin with the cytoskeleton and of NG2 and PDGFR-β with the plasma membrane. By looking at these four markers, we found that Ad-VEGFR1 as well as AG-013736 did not affect the overall number of pericytes in RIP-Tag2 tumors but reduced the amount of α-SMA and desmin immunoreactivity. Because many blood vessels regressed in these tumors after VEGF signaling inhibition, many pericytes were left without endothelial cells. The reduction in the two cytoskeletal markers may be a consequence of the reorganization of the pericyte cytoskeleton, which could occur after loss of contact with endothelial cells. Treatment with Ad-PDGFR-β led to pericyte loss in both RIP-Tag2 tumors and LLC tumors, but the reduction was much greater in LLC tumors. In RIP-Tag2 tumors, the combined viral vectors had a complex effect on pericytes, involving loss of about half of the pericyte population and change in phenotype of the remaining pericytes, as reflected by decreased expression of α-SMA. LLC tumors differed in this regard from RIP-Tag2 tumors. In LLC tumors, the combined viral vectors reduced by 75% pericyte density detected by all four markers, a reduction comparable to the one obtained by treatment with Ad-VEGFR1 or Ad-PDGFR-β alone. After combination of Ad-PDGFR-β and Ad-VEGFR1, almost all the surviving blood vessels were associated with pericytes. It has been reported that in RIP-Tag2 tumors, after blocking PDGFR and VEGFR with the tyrosine kinase inhibitors SU5416 and imatinib or SU5416 and SU6668, the remaining blood vessels were covered by few pericytes (24). These differences could be attributed to the following reasons: the different specificities of the inhibitors used, the duration of the treatment, and the stage of the tumors analyzed. The difference in responsiveness between RIP-Tag2 tumors and LLC tumors could be attributed to the level and/or localization of

**Figure 6.** Greater VEGF and PDGF-plasticity in LLC tumors than in RIP-Tag2 tumors. Confocal images and measurements of RIP-Tag2 tumors show that the strong VEGF immunoreactivity (red) after Ad-Fc (A-i) was reduced after Ad-VEGFR1 (A-ii), whereas after Ad-PDGFR-β it was unchanged (A-iii and A-iv). By comparison, VEGF immunoreactive cells were much less abundant in control LLC tumors (Ad-Fc) and even sparser after Ad-VEGFR1 or Ad-PDGFR-β (B). PDGF immunoreactivity (red) was widespread in RIP-Tag2 tumors and was equally strong after Ad-Fc (C-i) and Ad-VEGFR1 (C-ii) but was reduced by Ad-PDGFR-β treatment (C-iii and C-iv). LLC tumors had fewer PDGF-positive cells at baseline (D-i) and even less after Ad-VEGFR1 (D-ii) or Ad-PDGFR-β treatment (D-iii, D-iv). Scale bar in D-iii applies to all images: 120 μm in A; 60 μm in B and C; and 40 μm in D.
VEGF and PDGF expression. Indeed, RIP-Tag2 tumors had significantly higher VEGF and PDGF transcript levels and immunoreactivity compared with LLC tumors. By analyzing the distribution of VEGF within tumors, we found that both tumor cells and pericytes produced VEGF in RIP-Tag2 tumors. Those results are in line with previous studies showing that VEGF is highly expressed in the tumor cells of tumorigenic islets as well as in the normal islets in RIP-Tag2 mice (45–47). Our data suggest that in RIP-Tag2 tumors, the cellular sources targeted by the Ad-VEGFR1 treatment are mainly the tumor cells, and that these are responsible for the reduction of the endothelial cells. This hypothesis is confirmed by the fact that the elimination of pericytes by Ad-PDGFRβ did not lead to reduction of tumor blood vessels.

In contrast, in LLC tumor, VEGF immunoreactivity was located in cells close to blood vessels, and most of these cells were immunoreactive to pericyte markers. VEGF production has been previously described in the ovary pericytes (48), and pericytes isolated from RIP-Tag2 tumors have high VEGF transcription level (25). In addition, previous studies have shown that pericytes facilitate the maintenance of endothelial cells by secreting growth factors (10, 13, 16) such as VEGF (16). Macrophages have been described to be an important source of VEGF (42). In LLC tumors, double staining with VEGF and macrophage markers showed that only few macrophages were positive for VEGF. Similarly, VEGF immunoreactivity was not observed in LLC tumor cells. We cannot rule out that VEGF was not expressed in these cells, but the fact that the amount was too little to be detectable by immunohistochemistry suggests that in LLC tumor, pericytes are the main source of VEGF.

Interestingly, when we analyzed the effects of VEGF and PDGF inhibition in a LLC tumor line expressing high amount of VEGF, LLCx tumors, we found that combination therapy had a greater effect compared with single treatment. Moreover, PDGF blockage induced only a small reduction of the blood vessel density. These findings supported our hypothesis that the amount and distribution of VEGF determine the effects of PDGF inhibition on tumor blood vessels.

Endothelial cells in RIP-Tag2 tumors express genes for PDGF-A and PDGF-B (24). In our experiments, both tumor cells and endothelial cells produced PDGF in RIP-Tag2 tumors whereas only endothelial cells produced PDGF in LLC tumors. These findings suggest that VEGF and PDGF inhibitors affect blood vessels differently depending on the amount and cellular distribution of VEGF and PDGF within the tumor. Our model is that in LLC tumors, PDGF inhibition affects blood vessels by first eliminating pericytes, the major source of VEGF; similarly, VEGF inhibition reduces the number of pericytes by targeting and eliminating endothelial cells, the major source of PDGF. This model is consistent with our results showing that in LLC tumors, inhibition of VEGF strongly reduced PDGF immunoreactivity and inhibition of PDGF strongly reduced VEGF immunoreactivity.

In RIP-Tag2 tumors, where tumor cells produce both VEGF and PDGF, targeting endothelial cells via VEGF inhibition did not lead to a reduction in pericytes because PDGF was unaffected. Similarly, targeting pericytes by PDGF inhibition did not affect endothelial cells because VEGF was unaffected. Our results illustrated that PDGF inhibitors can have effects on tumor vessels similar to those of VEGF inhibitors but only in tumors where pericytes are the main source of VEGF. Future experiments in other tumor models will broaden our findings.

In conclusion, the present study highlights the importance of VEGF and PDGF signaling in sustaining the tumor vasculature. Our results also illustrate that tumor vasculature can respond to VEGF and/or PDGF inhibition differently depending on the cellular source and amount of growth factors within the tumor. These findings emphasize the importance of the tumor phenotype in the responsiveness to inhibitors of VEGF and PDGF. A better understanding of the interaction of factors from endothelial cells, pericytes, and other tumor compartments is required to design the most effective antitumor therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cellular Source and Amount of Vascular Endothelial Growth Factor and Platelet-Derived Growth Factor in Tumors Determine Response to Angiogenesis Inhibitors

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