

Anti-PIGF Inhibits Growth of VEGF(R)-Inhibitor-Resistant Tumors without Affecting Healthy Vessels

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SUMMARY

Novel antiangiogenic strategies with complementary mechanisms are needed to maximize efficacy and minimize resistance to current angiogenesis inhibitors. We explored the therapeutic potential and mechanisms of α PIGF, an antibody against placental growth factor (PIGF), a VEGF homolog, which regulates the angiogenic switch in disease, but not in health. α PIGF inhibited growth and metastasis of various tumors, including those resistant to VEGF(R) inhibitors (VEGFR^RIs), and enhanced the efficacy of chemotherapy and VEGFR^RIs. α PIGF inhibited angiogenesis, lymphangiogenesis, and tumor cell motility. Distinct from VEGFR^RIs, α PIGF prevented infiltration of angiogenic macrophages and severe tumor hypoxia, and thus, did not switch on the angiogenic rescue program responsible for resistance to VEGFR^RIs. Moreover, it did not cause or enhance VEGFR^RI-related side effects. The efficacy and safety of α PIGF, its pleiotropic and complementary mechanism to VEGFR^RIs, and the negligible induction of an angiogenic rescue program suggest that α PIGF may constitute a novel approach for cancer treatment.

INTRODUCTION

Antiangiogenic agents prolong the survival of cancer patients, however, without cure and at the expense of side effects (Hurwitz et al., 2004; Kramer and Lipp, 2007). The role of VEGF and VEGFR-2 in tumor growth is well

established, but other angiogenic factors switch on during cancer progression and induce resistance to VEGFR^RI monotherapy. By reducing tumor angiogenesis and increasing hypoxia, VEGFR^RIs rescue angiogenesis via other angiogenic factors. Combination therapy of antiangiogenic agents with complementary mechanisms could reduce resistance but might increase toxicity.

PIGF is a pleiotropic cytokine that stimulates endothelial cell (EC) growth, migration, and survival; chemoattracts angiocompetent macrophages and bone marrow progenitors; and determines the metastatic niche. Unlike VEGF, PIGF selectively binds VEGFR-1 and its coreceptors neuropilin-1 and -2. Besides indirect effects (Park et al., 1994), PIGF signals directly via VEGFR-1, thus, acting independently of VEGF in ECs, macrophages, bone marrow progenitors, and tumor cells, which primarily express VEGFR-1 (Clauss et al., 1996; Hattori et al., 2002; Kaplan et al., 2005).

PIGF stimulates angiogenesis, leukocyte infiltration, tumor growth and stromal cell migration (Luttun et al., 2002a; Marcellini et al., 2006; Roy et al., 2005), and revascularization of ischemic tissues (Luttun et al., 2002b), indicating that PIGF has biological activity in vivo. An anti-PIGF antiserum inhibits tumor cell invasion in vitro (Taylor and Goldenberg, 2007) and vascular leakage in vivo (Carmeliet et al., 2001), while PIGF overexpressing tumors grow less in VEGFR-1/TK^{-/-} mice (Hiratsuka et al., 2001). However, genetic studies show that PIGF is redundant for vascular development and maintenance, but contributes to the angiogenic switch in disease (Carmeliet et al., 2001; Rakic et al., 2003). This raised the question whether PIGF inhibitors might reduce pathological angiogenesis but, unlike VEGFR^RIs, without affecting healthy blood vessels, and thus provide an attractive drug with a better safety profile.

PIGF levels in plasma and tumors correlate with tumor stage, vascularity, recurrence, metastasis, and survival in various tumors (Chen et al., 2004; Ho et al., 2007;

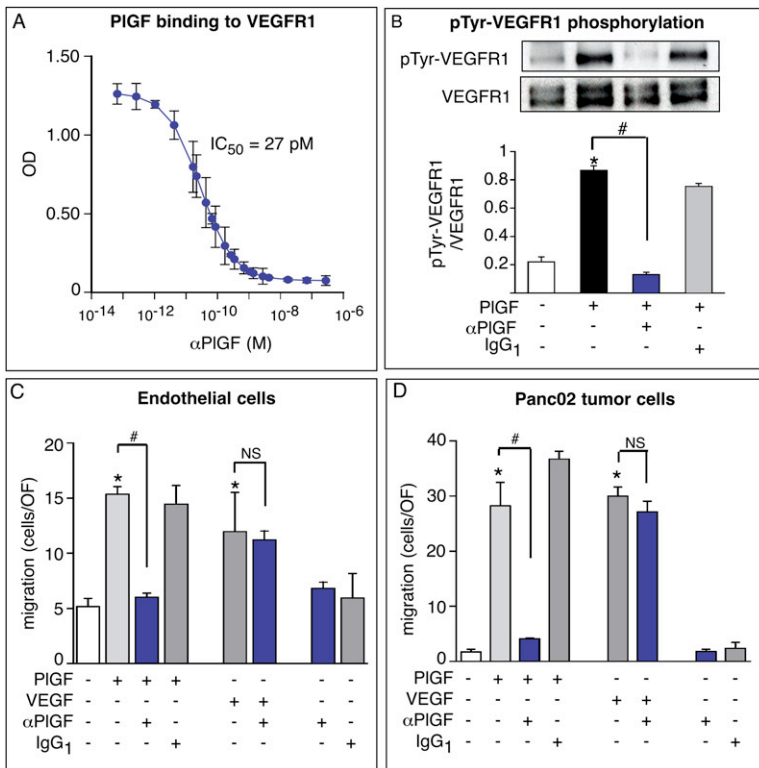


Figure 1. Characterization of α PIGF

(A) α PIGF inhibits binding of PIGF to VEGFR-1 (ELISA; n = 8).

(B) Inhibition of PIGF-induced VEGFR-1 tyrosine phosphorylation in 293T cells by α PIGF.

*p < 0.05 versus control; #p < 0.05 versus PIGF.

(C and D) Migration of PIGF^{-/-} endothelial (C) or Panc02 tumor cells (D) in response to PIGF or VEGF (each at 50 ng/ml), assessed in the presence or absence of a 100-fold molar excess of α PIGF or IgG₁.

The number of migrated cells per high-power optical field (OF) are shown (n = 5; *p < 0.05 versus control [0.1% FBS]); #p < 0.05 versus PIGF. Error bars represent mean \pm SEM.

Matsumoto et al., 2003; Parr et al., 2005; Wei et al., 2005). Notably, PIGF is upregulated in cancer patients treated with VEGFR^{R1} therapy (Motzer et al., 2006; Rosen et al., 2007; Willett et al., 2005), as well as in human tumors after radio-immunotherapy (Taylor et al., 2003), suggesting a key role of PIGF in the angiogenic rescue. Here, we generated a neutralizing α PIGF and evaluated its therapeutic potential and mechanism of action in the inhibition of solid tumor growth.

RESULTS

Characterization of an Anti-PIGF Antibody

We developed a neutralizing murine anti-PIGF monoclonal antibody (clone 5D11D4; referred to as α PIGF) that specifically recognizes mouse PIGF-2 (all mouse proteins denoted without prefix; human proteins are preceded with “h”) with a high affinity ($K_D = 7.0 \times 10^{-10}$ M). α PIGF inhibited the binding of PIGF to VEGFR-1 (IC_{50} : 27 pM) and neuropilin-1, tyrosine phosphorylation of VEGFR-1 in response to PIGF, and the response of endothelial and tumor cells to PIGF, without, however, inhibiting the binding or activity of VEGF or other related factors (Figures 1A–1D; Figures S1A–S1F in the Supplemental Data available with this article online).

Effect of α PIGF on Tumor Growth and Metastasis

PIGF was undetectable in most healthy tissues but induced by hypoxia, abundant in all tumors analyzed and expressed by both tumor and stromal cells (Figures S1G

and S1H; data not shown). When injecting B16 melanoma cells subcutaneously or pancreatic Panc02 tumor cells orthotopically, α PIGF dose-dependently inhibited tumor growth (Figures 2A–2D) and reduced the incidence of local tumor invasion, bile duct stenosis and hemorrhagic ascites (n = 65–71; Table S1; for pharmacokinetics and rationale of dosing, see Note S1). α PIGF dose-dependently inhibited metastasis of Panc02 tumors to regional and distant lymph nodes, independent of primary tumor size (Figures 2E–2G; Note S2). Metastasis to lymph nodes occurred in >78% of control mice, while only in 29% of α PIGF-treated mice (n = 18; p < 0.05; Table S1). Overall, α PIGF inhibited the growth and/or metastasis of 12 different tumor models tested, mostly by 55% to 66% (Figures 2H and 2I; Table S2), including human xenograft tumors (data not shown). α PIGF also suppressed the growth of established B16 tumors (by 35%) and the lodging of intravenously injected B16 tumor cells (by 50%; data not shown).

To evaluate how efficiently α PIGF inhibited tumor growth as compared to established VEGFR^{R1}s, we used an anti-VEGFR-2 antibody (α VEGFR-2). Compared to α VEGFR-2, α PIGF comparably inhibited the growth of B16 tumors (Figure 2J) or was less effective in inhibiting growth of Panc02 tumors, a VEGFR^{R1}-sensitive model (by $62 \pm 3\%$ versus $82 \pm 2\%$; n = 45; p < 0.05). Interestingly, however, α PIGF was more effective in suppressing growth of CT26 tumors, a model relatively resistant to VEGFR^{R1}s (by $56 \pm 3\%$ versus $31 \pm 4\%$; n = 90; p < 0.05; see also Figure 3D). Similar results were obtained when using other

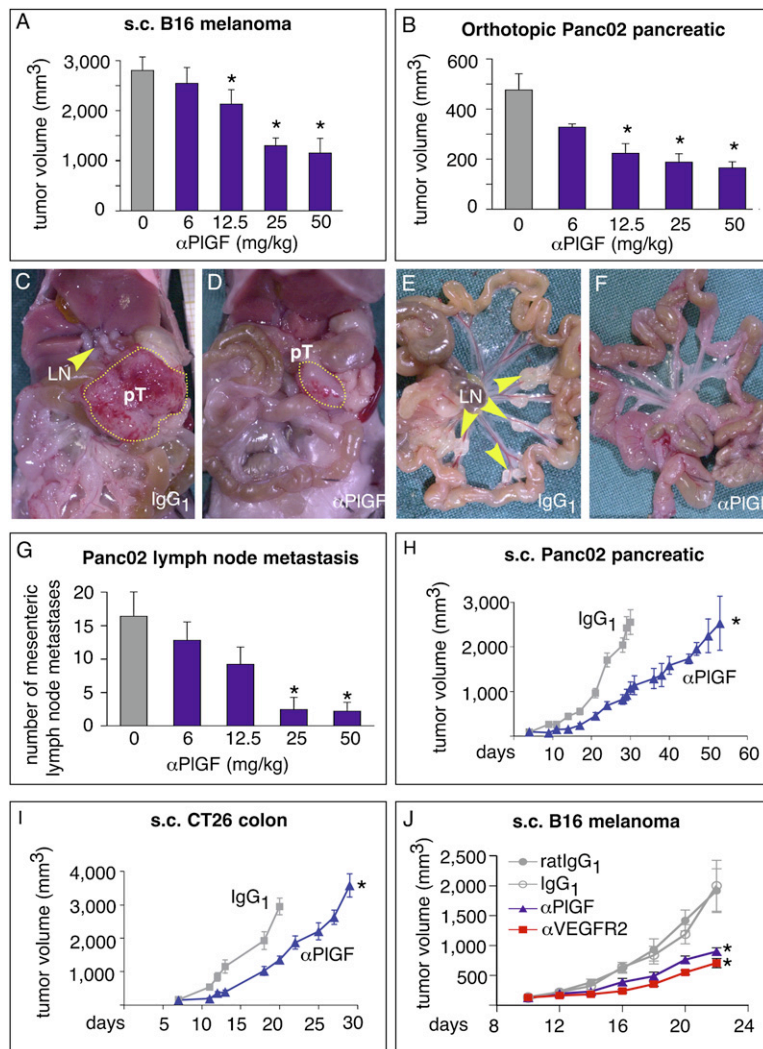


Figure 2. α PIGF Inhibits Tumor Growth and Metastasis of Syngeneic Tumors

(A–G) α PIGF dose dependently inhibits s.c. B16 (N = 15) (A), and orthotopic Panc02 tumor growth (n = 10) (B–D) and lymphatic metastasis (n = 9–11) (G). Compared to control (C and E), α PIGF inhibits growth of primary Panc02 tumors (pT) (D) and metastasis of celiac (D) and mesenteric (F) lymph nodes (LN; arrows). (H and I) α PIGF inhibits growth of syngeneic s.c. Panc02 (H) and CT26 (I) tumors (n = 10). (J) α PIGF and α VEGFR-2 comparably inhibit B16 tumor growth (n = 15). *p < 0.05 versus control. Error bars represent mean \pm SEM.

VEGFRs (see below). Thus, α PIGF inhibited the growth of VEGFR^R-sensitive but also of VEGFR^R-resistant tumors.

α PIGF Enhances the Efficacy of Chemotherapy

Exposure of Panc02 and B16 tumor cells to the cytostatic agents gemcitabine and cyclophosphamide dose-dependently increased PIGF expression (Figure 3A; Figure S2A), providing a rationale to test whether α PIGF would enhance chemotherapy. Compared to monotherapy, combination therapy with α PIGF and gemcitabine inhibited Panc02 tumor growth more (Figure 3B). In the B16 model, α PIGF plus cyclophosphamide near completely inhibited tumor growth as compared to only the partial inhibition of tumor growth by either monotherapy alone (Figure S2B). Thus, α PIGF enhanced the tumor growth inhibitory effect of cytostatic agents.

α PIGF Enhances the Anticancer Activity of α VEGFR-2

VEGFR^R blockade increases plasma PIGF levels in cancer patients (Motzer et al., 2006; Rosen et al., 2007; Willett

et al., 2005). Plasma PIGF levels were undetectable in healthy mice, but elevated to \sim 100 pg/ml in tumor-bearing mice (Figure 3C). Treatment of tumor-bearing mice with α VEGFR-2 elevated PIGF levels in the plasma (Figure 3C) and tumors (Note S3A). Plasma VEGF levels were also increased when tumor-bearing mice were treated with α VEGFR-2 (Figure S2C), but not by α PIGF (data not shown). ELISA confirmed that tumoral VEGF levels were upregulated in tumors by α VEGFR-2, but not by α PIGF (Note S3B).

The finding that PIGF levels were upregulated by α VEGFR-2 in tumor-bearing mice prompted us to examine whether α PIGF enhanced the antitumor effect of α VEGFR-2, using the colon CT26 tumor model, as it is relatively resistant to α VEGFR-2 (see above). Compared to the partial inhibition of tumor growth by monotherapy with α VEGFR-2 or α PIGF, their combination inhibited tumor growth more completely (Figure 3D), indicating that α PIGF enhanced the antitumor activity of α VEGFR-2.

We also examined whether α PIGF might substitute for α VEGFR-2. A maximal dose of α PIGF and α VEGFR-2

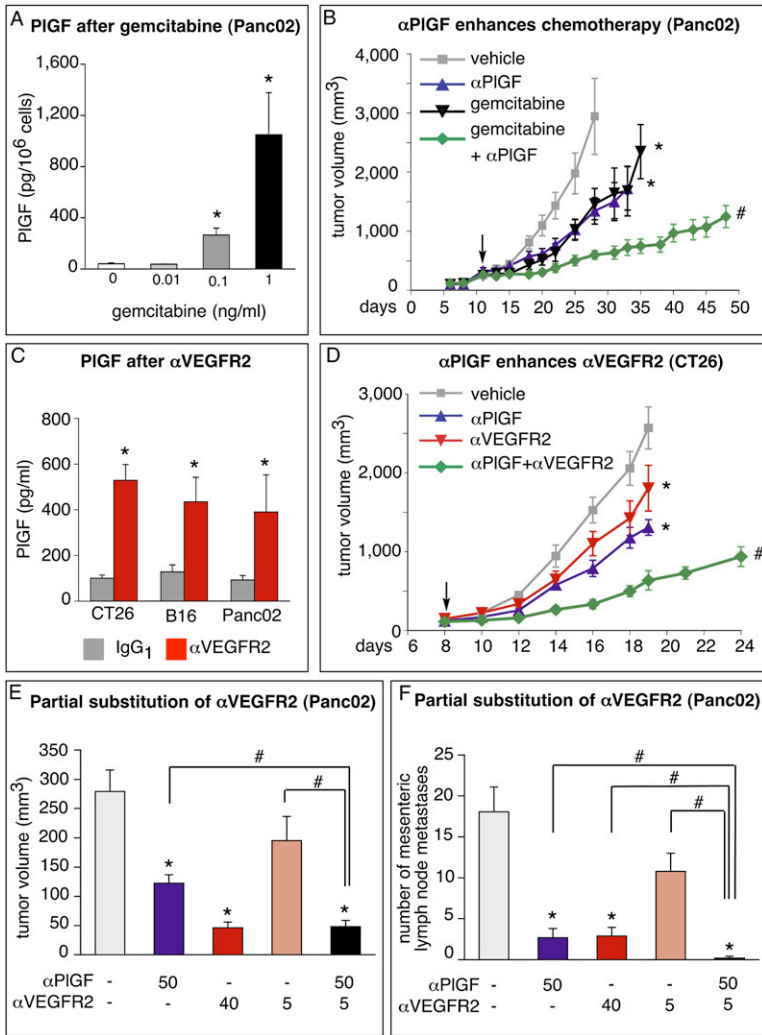


Figure 3. α PIGF Enhances Chemotherapy and α VEGFR-2 Therapy

(A) Gemcitabine upregulates PIGF release by Panc02 tumor cells (n = 5).
 (B) Panc02 tumor-bearing mice received α PIGF (50 mg/kg), gemcitabine (125 mg/kg, two times per week) or the combination. Combination therapy inhibited tumor growth more than single treatments (n = 15; #p < 0.01 versus single treatments, ANOVA). Arrow indicates start of treatment.
 (C) α VEGFR-2 increases PIGF plasma levels in CT26, B16 and Panc02 tumor bearing mice (n = 5–11).
 (D) CT26 tumor growth curves in mice receiving α PIGF (50 mg/kg), α VEGFR-2 (50 mg/kg) or the combination of both. α PIGF enhances α VEGFR-2 (n = 15; #p < 0.0001 versus single treatments, ANOVA). Arrow indicates start of treatment.
 (E) α VEGFR-2 dose dependently inhibits growth of orthotopic Panc02 tumors (n = 12). Substitution of α VEGFR-2 (5 mg/kg) by α PIGF (50 mg/kg) inhibits tumor growth to the same extent as 40 mg/kg α VEGFR-2 (n = 12; #p < 0.05 versus α VEGFR-2 (5 mg/kg) and p < 0.05 versus α PIGF).
 (F) Partial substitution of α VEGFR-2 by α PIGF reduces mesenteric lymph node metastasis as compared to single treatments (n = 12; #p < 0.05 versus α PIGF and α VEGFR-2). *p < 0.05 versus control. Error bars represent mean \pm SEM.

reduced tumor growth in Panc02 tumors by ~56% and ~83%, respectively (Figure 3E). A low dose of α VEGFR-2 (from here on defined as 5 mg/kg; three times per week) inhibited tumor growth by ~30%, but when combined with α PIGF, tumor growth was reduced by ~83%, i.e., to the same extent as monotherapy with a high dose of α VEGFR-2 (Figure 3E). Similar results were obtained when analyzing lymphatic metastasis of Panc02 tumor cells, whereby substitution of most of α VEGFR-2 by α PIGF yielded even more complete suppression than α VEGFR-2 monotherapy alone (Figure 3F). Thus, substitution of α VEGFR-2 with α PIGF resulted in a comparable or even greater antitumor/metastasis effect.

α PIGF Inhibits Tumor Angiogenesis

α PIGF reduced tumor angiogenesis by ~50% in all tumors analyzed, i.e., comparably or slightly less than α VEGFR-2 (Figure 4A; Figures S3A–S3F; Table S3). Double staining for CD31 and caspase-3 revealed that α PIGF induced EC apoptosis, indicating that α PIGF induced pruning of preexisting tumor vessels (Figures 4B–4D). In Panc02 tu-

mors, a combination of α PIGF with a low dose α VEGFR-2 reduced tumor angiogenesis to the same extent as a high dose of α VEGFR-2 (Figure 4A). Also, in CT26 tumors, the combination of α PIGF plus α VEGFR-2 yielded the greatest inhibition of tumor angiogenesis (Figure S3G). Thus, α PIGF reduces tumor growth, at least in part, by inhibiting tumor angiogenesis. Similar to α VEGFR-2, α PIGF primarily reduced EC numbers, without affecting SMC counts, suggesting possible improvement of tumor vessel maturation (Figures 4E–4H).

α PIGF Inhibits Intratumoral Macrophage Recruitment

VEGFR-1⁺ macrophages promote tumor growth and angiogenesis. α PIGF reduced F4/80⁺ macrophage infiltration, maximally by 74%, in both VEGFR^{R1}-sensitive (Panc02; Figure 4I) and VEGFR^{R1}-resistant (CT26; Figures S4A, S4B, and S4E) tumors (Table S3). It also normalized circulating monocyte counts in these tumor models (Figure 4J; Table S3). In contrast, α VEGFR-2 failed to inhibit the macrophage infiltration and even slightly increased circulating

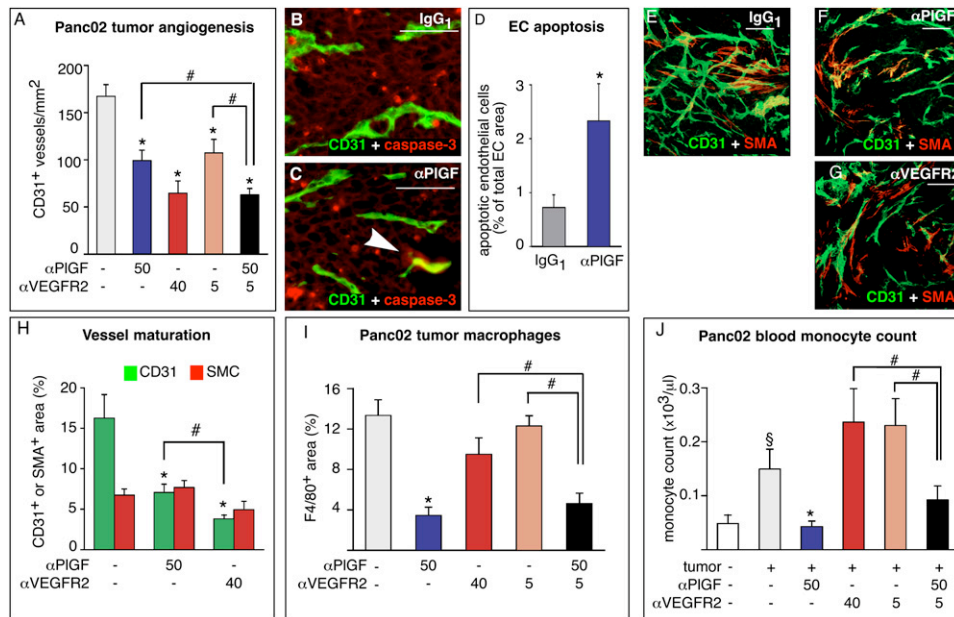


Figure 4. α PIGF Inhibits Tumor Angiogenesis and Macrophage Recruitment

(A) Quantification of CD31⁺ vessel density of orthotopic Panc02 tumors treated with control IgG₁, α PIGF, and/or α VEGFR-2. Combination of α VEGFR-2 (5 mg/kg) with α PIGF (50 mg/kg) inhibits angiogenesis comparably as 40 mg/kg α VEGFR-2 ($n = 6$; $p = \text{NS}$) and more efficiently than either α PIGF or 5 mg/kg α VEGFR-2 ($n = 6$; $p < 0.05$).

(B and C) Double staining using anti-CD31 (green) and anti-caspase-3 (red) antibodies on orthotopic Panc02 tumors treated with control IgG₁ (B) and α PIGF (C). Arrowhead indicates apoptotic EC.

(D) Bar graph represents percent of apoptotic EC area/total EC area ($n = 8$).

(E–H) Double staining for CD31 (green) and SMA (red) on Panc02 tumor sections after treatment with control IgG₁ (E), α PIGF (F) and α VEGFR-2 (G), and quantification of CD31⁺ and SMA⁺ area/tumor area (H). Both α PIGF and α VEGFR-2 decrease CD31⁺ areas without affecting SMA⁺ areas ($n = 6$). Scale bar is 100 μm .

(I and J) α PIGF, but not α VEGFR-2, inhibits intratumoral macrophage recruitment ($n = 10$) (I) and abrogates Panc02 tumor-induced blood monocytosis ($n = 6$; $^{\$}p < 0.05$ versus nontumor-bearing mice) (J). α PIGF predominates over α VEGFR-2 treatment ($n = 6$ –10). $\#p < 0.05$ as indicated. $*p < 0.05$ versus control. Error bars represent mean \pm SEM.

monocyte counts (Figures 4I and 4J; Figures S4C and S4E). Interestingly, the combination of α PIGF plus α VEGFR-2 inhibited macrophage infiltration and normalized tumor-induced monocytosis (Figures 4I and 4J; Figures S4D and S4E), indicating that α PIGF predominated over α VEGFR-2. Thus, α PIGF and α VEGFR-2 substantially differ in their mechanism of regulating tumor inflammation. Similar findings were obtained when using a VEGF inhibitor (see below).

PIGF-Dependent Macrophage Recruitment Confers Resistance to α VEGFR-2

CT26 tumors produced comparable amounts of VEGF and PIGF (data not shown), yet α VEGFR-2 inhibited their growth much less than α PIGF (see above). Since both antibodies comparably inhibited angiogenesis, the resistance of CT26 tumors to α VEGFR-2 could not be explained by its antiangiogenic activity alone. As α PIGF, but not α VEGFR-2, inhibited macrophage infiltration, we assessed whether the tumor responsiveness to α VEGFR-2 was dependent on tumor inflammation. We therefore used clodronate liposomes (referred to as clodrolip) to deplete macrophages (Zeisberger et al., 2006). Clodrolip slowed down

tumor growth in control mice but did not enhance the effect of α PIGF (Figure 5A). α PIGF alone was equipotent in inhibiting intratumoral macrophage infiltration, and clodrolip only slightly amplified the anti-inflammatory effect of α PIGF (Figure 5B). Tumor angiogenesis was reduced by clodrolip, indicating that macrophages were proangiogenic (Figure 5C). However, clodrolip did not further amplify the antiangiogenic effect of α PIGF, suggesting that α PIGF alone already efficiently depleted growing tumors from proangiogenic macrophages (Figure 5C). The finding that α PIGF inhibited tumor growth and angiogenesis more than clodrolip, while comparably inhibiting intratumoral macrophage infiltration, suggests that α PIGF inhibits tumor growth not only via an anti-inflammatory activity, but also via its antiangiogenic activity. Similar results were observed in orthotopic Panc02 tumors (Figures S4F–S4H).

In contrast, the combination of α VEGFR-2 and clodrolip was more effective in inhibiting tumor growth, indicating that depletion of mononuclear cells by clodrolip sensitized CT26 tumors to the inhibitory effect of α VEGFR-2 (Figure 5D). Since α VEGFR-2 inhibited tumor angiogenesis without affecting tumor macrophage infiltration and clodrolip amplified the antiangiogenic activity of α VEGFR-2

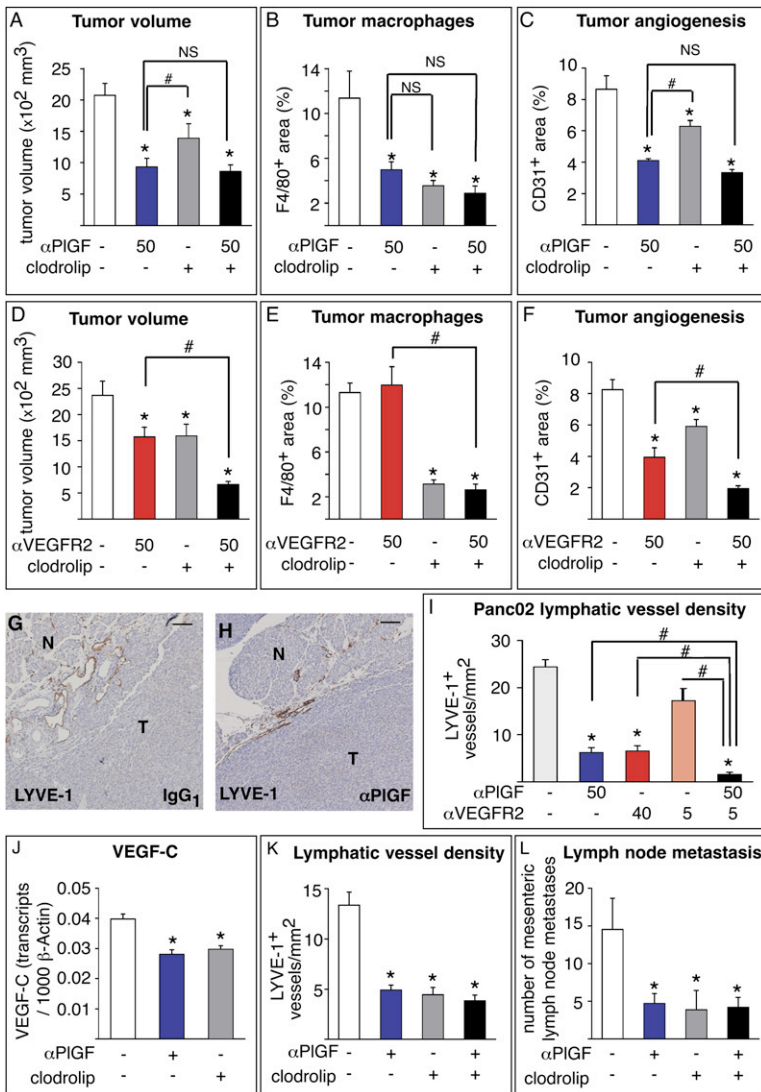


Figure 5. Macrophage Recruitment Confers Resistance to α VEGFR-2

(A–C) Macrophage depletion by clodrolip inhibits tumor growth but does not further enhance the antitumor effect of α PIGF (n = 15) (A) because the latter depletes tumors already from macrophages (n = 15) (B). Macrophage depletion inhibits angiogenesis but does not further enhance the antiangiogenic effect of α PIGF (n = 5) (C). Note that α PIGF inhibits tumor growth (A) and angiogenesis (C) more than clodrolip. #p < 0.05.

(D–F) Clodrolip enhances the antitumor effect of α VEGFR-2 (n = 15) (D) by depleting tumor macrophages (N = 6) (E), and by inhibiting tumor angiogenesis (n = 5) #p < 0.05 versus α VEGFR-2 alone (F).

(G–I) LYVE-1⁺ immunostaining of peritumoral lymphatic vessels in Panc02 tumors treated with control IgG₁ (G) or α PIGF (H). Scale bar is 100 μ m. α PIGF and α VEGFR-2 (40 mg/kg) inhibit peritumoral lymphatic vessel density (I). Combination of α VEGFR-2 (5 mg/kg) and α PIGF reduces lymphatic vessel density more than single treatments (n = 7; #p < 0.05).

(J–L) Clodrolip and α PIGF reduce intratumoral VEGF-C transcript levels (J), lymphatic vessel density (K) and lymph node metastasis (L) comparably (n = 15). *p < 0.05 versus control. Error bars represent mean \pm SEM.

(Figures 5E and 5F), the infiltration of proangiogenic macrophages contributed to the resistance of CT26 tumors to α VEGFR-2. Thus, α PIGF and α VEGFR-2 act via distinct mechanisms: by blocking both tumor angiogenesis and inflammation, α PIGF inhibits growth of a VEGFR^{R1}-resistant tumor; in contrast, the antiangiogenic activity of α VEGFR-2 is unable to suppress growth of this VEGFR^{R1}-resistant tumor, since it fails to inhibit infiltration of proangiogenic macrophages.

α PIGF Inhibits Tumor Lymphangiogenesis

To explain how α PIGF inhibited lymphatic metastasis of orthotopic Panc02 tumors, we analyzed lymphangiogenesis. α PIGF reduced LYVE-1⁺ peritumoral lymphatic vessel density and area by 75% and 87%, respectively (Figures 5G–5I; Figure S3H). Consistent with previous findings that VEGF stimulates lymphangiogenesis, α VEGFR-2 also inhibited lymphangiogenesis in Panc02 tumors (Figure 5I). Notably, combination of a low dose of α VEGFR-2

plus α PIGF resulted in a significantly greater inhibition of lymphangiogenesis than single treatments (Figure 5I). Thus, both α PIGF and α VEGFR-2 suppressed peritumoral lymphangiogenesis and amplified each other’s antilymphangiogenic activity. Since lymphatic endothelial cells do not express VEGFR-1, we studied if α PIGF inhibited lymphangiogenesis indirectly. Indeed, α PIGF decreased *Vegf-C* levels, lymphangiogenesis, and lymph node metastasis comparably as clodrolip, suggesting that α PIGF inhibited the recruitment of prolymphangiogenic macrophages (Figures 5J–5L).

Negligible Induction of a Rescue Angiogenic Program by α PIGF

Resistance to antiangiogenic agents is, at least in part, determined by the induction of a compensatory angiogenic program. We therefore analyzed the tumor expression of several angiogenic genes. Treatment of VEGFR^{R1}-sensitive and -resistant tumor-bearing mice with α VEGFR-2, but

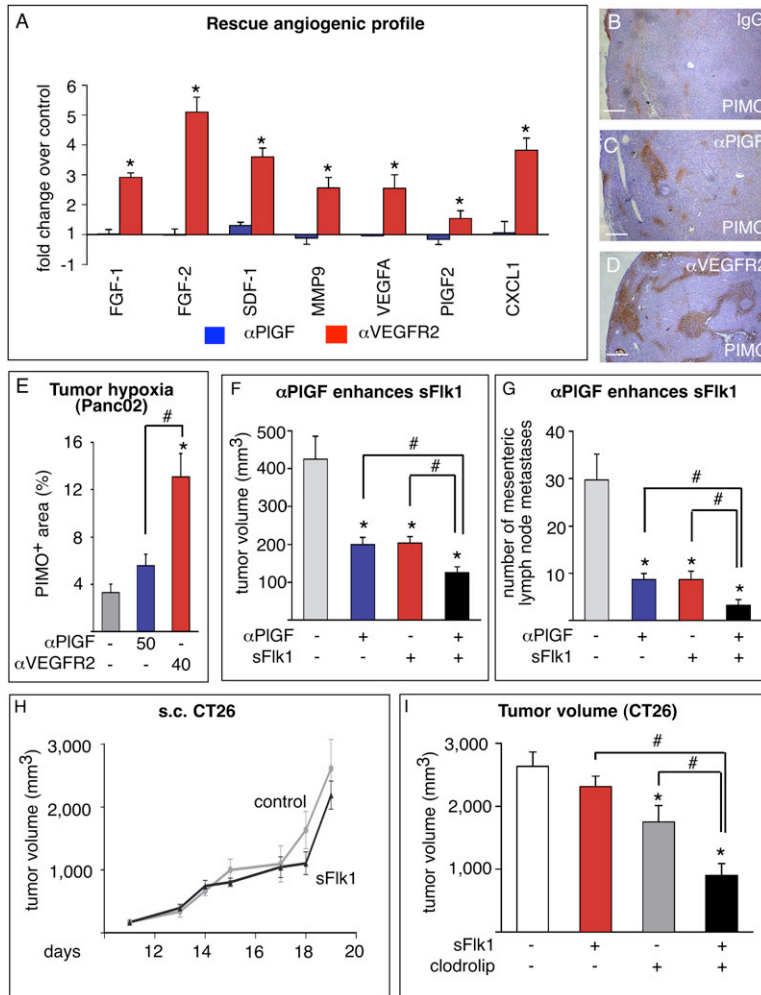


Figure 6. α PIGF Does Not Rescue Angiogenesis and Enhances VEGF Trap Therapy

(A) α PIGF does not induce angiogenic gene expression in contrast to α VEGFR-2. Data are fold change over control ($n = 3-5$; see Note S4 for absolute values).

(B-E) Immunostaining for hypoxyprobe-1 on orthotopic Panc02 tumors treated with control IgG, (B), α PIGF (C), or α VEGFR-2 (40 mg/kg) (D). Scale bar is 200 μ m. α VEGFR-2, but not α PIGF, induces tumor hypoxia ($n = 10$; # $p < 0.05$ versus α PIGF) (E).

(F and G) α PIGF enhances sFlk1 therapy on tumor growth (F) and lymphatic metastasis (G) in orthotopic Panc02 tumors ($n = 15$; # $p < 0.05$ versus α PIGF and sFlk1).

(H and I) Macrophages confer resistance to sFlk1 in CT26 tumors. CT26 are resistant to sFlk1 (H), but depletion of macrophages by clodrolip enhances the antitumor effect of sFlk1 ($n = 15$) (I). * $p < 0.05$ versus control. Error bars represent mean \pm SEM.

not α PIGF, upregulated proangiogenic genes such as *Sdf-1*, *Fgf-1*, *Fgf-2*, *Vegf*, *Plgf*, *Mmp9*, and *Cxcl1* (Figure 6A). This upregulation was specific, as other genes involved in angiogenesis, such as *Csf*, *Ccl-2*, *Egf*, and *Vegfr-3*, were not upregulated (data not shown). Tumor hypoxia, resulting from the antiangiogenic treatment, may underlie the induction of such an angiogenic rescue program. We therefore assessed tumor hypoxia by staining for the hypoxia-marker pimonidazole (PIMO; Figures 6B–6D). In orthotopic Panc02 tumors, α VEGFR-2 (but not α PIGF) significantly increased the PIMO⁺ fraction of the tumor (Figure 6E). Further, α VEGFR-2 reduced the vessel area more than α PIGF (lumen/tumor area: $4.6 \pm 0.4\%$ in controls versus $3.3 \pm 0.4\%$ after α PIGF and $1.2 \pm 0.2\%$ after α VEGFR-2; $n = 5$; $p = 0.04$ versus α PIGF; $p < 0.0001$ versus α VEGFR-2). In addition, α VEGFR-2, but not α PIGF, reduced in vivo tumor perfusion (tumor/kidney perfusion: $48 \pm 10\%$ in controls versus $17 \pm 3\%$ after α VEGFR-2, $n = 14$; $p < 0.01$; and $45 \pm 9\%$ after α PIGF, $n = 14$; $p = \text{NS}$). Similarly, in CT26 tumors, α VEGFR-2 caused more tumor cell hypoxia than α PIGF, resulting in larger necrotic tumor areas (Figures S4I and S4J). Thus, compared to

α VEGFR-2, α PIGF caused less severe intratumoral hypoxia, which may explain the minimal rescue angiogenic program.

α PIGF Enhances VEGF-Trap Therapy

To assess whether α PIGF mechanistically also differs from a specific VEGF inhibitor, we used a soluble form of VEGFR-2 (sFlk1) that “traps” VEGF selectively (i.e., thus, not PIGF or VEGF-B). Upon hydroporation, circulating sFlk1 was elevated during the entire experiment to levels ($2,900 \pm 790$ ng/ml), previously reported to inhibit tumor growth (Davidoff et al., 2002). sFlk1 inhibited orthotopic Panc02 tumor growth, angiogenesis, and lymph node metastasis (Figures 6F and 6G; Table S4) with plasma sFlk1 levels correlating with tumor growth inhibition (data not shown). The combination of α PIGF with sFlk1 was more efficient in inhibiting tumor growth and lymphatic metastasis than each treatment alone (Figures 6F and 6G). When using VEGFR^{R1}-resistant CT26 tumors, sFlk1 inhibited tumor growth only insignificantly by 20% (Figure 6H) without any correlation between sFlk1 levels and tumor inhibition, confirming that this tumor is resistant to VEGFR^{R1}

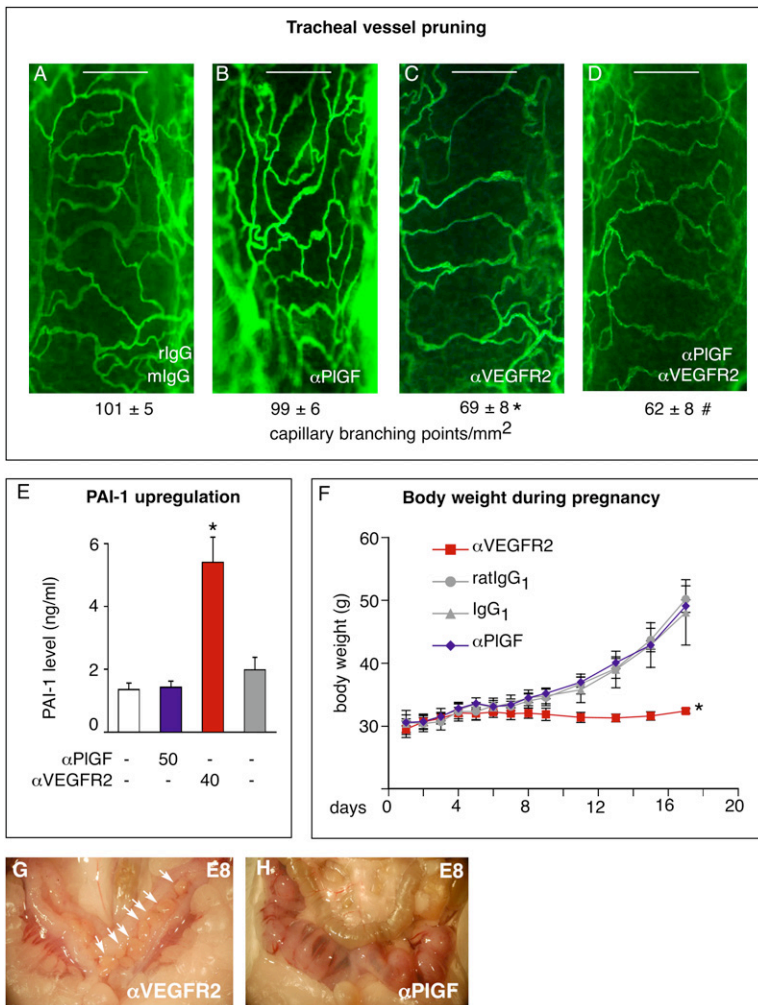


Figure 7. αPIGF Does Not Affect Healthy Vessels

(A–D) Immunofluorescent pictures of isolectin perfused tracheas of mice treated with control IgG₁ (A), αPIGF (B), or αVEGFR-2 (C) reveal that αVEGFR-2, but not αPIGF, induces tracheal vessel regression (n = 14). Combination of αPIGF and αVEGFR-2 does not further reduce capillary branching points/mm² compared to αVEGFR-2 alone (n = 14; #p = NS versus αVEGFR-2) (D). Scale bar is 100 μm.

(E) αPIGF does not upregulate plasma PAI-1 levels in healthy mice (n = 5). White and gray bars represent murine and rat IgG₁, respectively.

(F) Mice were treated with αPIGF, αVEGFR-2, or control IgG₁ throughout pregnancy, and body weight was monitored. αVEGFR-2, but not αPIGF, arrests pregnancy and embryonic development from day E7 onward (n = 10).

(G and H) Upon resection at day E8, the uteri of mice treated with αVEGFR-2 contained dead early stage embryos (n = 10) (G), whereas αPIGF did not impair embryonic development (n = 10) (H). *p < 0.05 versus control. Error bars represent mean ± SEM.

therapy. Similar to αVEGFR-2, sFlk1 inhibited tumor angiogenesis but failed to inhibit intratumoral macrophage recruitment (Table S4), while clodrolip rendered this tumor again sensitive to sFlk1 (Figure 6I). Thus, even despite its antiangiogenic activity, sFlk1, like αVEGFR-2, is unable to inhibit macrophage recruitment and, hence, CT26 tumor growth. αPIGF, by contrast, differs from these VEGFR^{R1}s by inhibiting angiogenesis and macrophage infiltration and, thereby, growth of this VEGFR^{R1}-resistant tumor. Unlike αPIGF, sFlk1 also switched on an angiogenic rescue program, including *Vegf*, *Plgf*, and *Fgf-2* (data not shown).

Safety Profile of the αPIGF Antibody

Treatment with VEGFR^{R1}s causes thrombosis, hypertension, microvascular pruning in healthy organs, interruption of pregnancy, and other side effects (Hurwitz et al., 2004; Kamba et al., 2006; Kramer and Lipp, 2007). However, treatment of healthy mice with αPIGF did not affect body weight, did not cause any obvious organ pathology upon inspection and histological analysis, did not alter the clinical chemistry or hematological blood profile, and did not increase proteinuria (data not shown). When analyzing

the morphology of quiescent vessels in different healthy organs, treatment with αVEGFR-2, but not αPIGF, for 3 weeks reduced the number of capillary branching points/mm² in the trachea by 34 ± 2% (Figures 7A–7C) and capillary profiles/mm of follicle perimeter in the thyroid gland by 21 ± 1% (n = 14; p < 0.05; Table S5). Moreover, the combination of αPIGF and αVEGFR-2 did not aggravate the adverse effect of αVEGFR-2 on vessel pruning (Figure 7D). Thus, αPIGF enhanced the antitumor growth efficacy of αVEGFR-2 without, however, aggravating its toxicity.

We also measured the circulating levels of plasminogen activator inhibitor-1 (PAI-1), a fibrinolytic inhibitor of clot lysis, released by stressed ECs and known risk factor of thrombosis. Treatment with αVEGFR-2, but not αPIGF, caused a 2.7-fold increase of PAI-1 levels (n = 5; p < 0.05; Figure 7E). Furthermore, after 3 weeks of treatment, the mean blood pressure was elevated in mice given αVEGFR-2 (73 ± 3.2 mmHg after IgG₁ versus 95 ± 3.9 mmHg after αVEGFR-2; n = 9; p < 0.002), but not αPIGF (74 ± 5.3 mmHg after IgG₁ versus 74 ± 3.9 mmHg after αPIGF; n = 9; p = NS). The relative teratogenicity of αPIGF and αVEGFR-2 was analyzed in pregnant mice from the

day of plug-check throughout pregnancy. Mice treated with α VEGFR-2 failed to gain weight beyond day 6, suggesting that embryonic development was arrested (Figure 7F). At day 8 of pregnancy, their uteri contained dead, partially resorbed early-stage embryos and a pale placenta, indicating that α VEGFR-2 inhibited placental vascular development (Figures 7G and 7H). In contrast, α PIGF did not interrupt the pregnancy-associated weight gain, nor did it abort embryonic development, and resulted in normal litters and healthy pups, that grew up normally (Table S5). In contrast to α VEGFR-2, α PIGF did also not aggravate the cardiotoxicity induced by doxorubicin (data not shown). sFlk1 and VEGFR tyrosine kinase inhibitors had a comparable toxicity profile to α VEGFR-2 (data not shown).

DISCUSSION

The primary findings of this study are (1) α PIGF monotherapy inhibits the growth and metastasis of >12 different tumor models; (2) cytotoxic drugs upregulate PIGF expression by tumor cells, and α PIGF amplifies the antitumor effect of chemotherapy; (3) α VEGFR-2 and sFlk1 therapy upregulate PIGF levels, and α PIGF enhances and partially substitutes α VEGFR-2 and sFlk1 monotherapy; (4) α PIGF mimics α VEGFR-2 and sFlk1 in inhibiting tumor angiogenesis but differs by inhibiting intratumoral macrophage infiltration and acts, thus, via complementary mechanisms to VEGFR^{R1}s; (5) unlike α VEGFR-2, α PIGF does not induce an angiogenic rescue/antiangiogenic escape program (which might lead to resistance against antiangiogenic therapy) because it inhibits macrophage infiltration and does not cause severe hypoxia; and (6) α PIGF is not associated with typical VEGFR^{R1} side effects but exhibits a superior safety profile. This study is the first to document the pharmacological properties of a specific PIGF-inhibitor (Note S5).

Mechanisms of α PIGF

The antitumor activity of α PIGF depends, in part, on its pleiotropic effects. First, PIGF stimulates EC growth and migration (Autiero et al., 2003; Carmeliet et al., 2001), mobilizes angiocompetent bone marrow progenitors (Hattori et al., 2002; Kaplan et al., 2005), and acts as a survival factor for existing vessels. Thus, α PIGF not only inhibits new vessel growth, but also causes regression of existing tumor vessels. Second, α PIGF impaired tumor lymphangiogenesis. Our finding that α PIGF augmented the antilymphangiogenic activity of α VEGFR-2 suggests that it acts via complementary VEGF-unrelated mechanisms. Indeed, consistent with the role of macrophages in pathological lymphangiogenesis (Cursiefen et al., 2004), clodrolip inhibited lymphatic metastasis, lymphangiogenesis, and tumoral *Vegf-C* levels as effectively as α PIGF, indicating that the effect of α PIGF on lymphatic vessels is mainly mediated via macrophage inhibition. An inflammation-dependent role of PIGF in lymphangiogenesis might explain why PIGF^{-/-} mice do not exhibit spontaneous lymphatic defects (Carmeliet et al., 2001).

Third, α PIGF is anti-inflammatory, consistent with the fact that PIGF chemoattracts VEGFR-1⁺ macrophages (Luttun et al., 2002a; Pipp et al., 2003). Tumor-associated macrophages (TAMs) participate in angiogenesis by secreting angiogenic factors or indirectly by producing proteases that release sequestered angiogenic factors. An increase in TAMs is associated with tumor progression, metastasis, and poor prognosis, both in humans as well as in various implanted or spontaneously arising tumor models in mice (Pollard, 2004). Thus, by inhibiting their recruitment, α PIGF also inhibits tumor growth. Whether α PIGF also blocks the role of macrophages in establishing the premetastatic niche, as suggested by genetic data using VEGFR-1/TK^{-/-} mice (Hiratsuka et al., 2002), remains to be established. Another mechanism of α PIGF may relate to its cytostatic activity for malignant cells (data not shown). The relative role of the antiangiogenic, antilymphangiogenic, anti-inflammatory, and cytostatic activity of α PIGF in spontaneously arising tumor models in transgenic mice is currently under investigation. Initial findings that loss of PIGF inhibits carcinogen-induced skin cancer growth promise to use such models to evaluate the activity of α PIGF.

Our data also indicate that α PIGF, by inhibiting macrophage recruitment, enhances the responsiveness of tumors to VEGFR^{R1}s. Indeed, CT26 tumors are relatively resistant to α VEGFR-2 and sFlk1, but, following macrophage depletion, they became sensitive to these VEGFR^{R1}s. Thus, macrophages counteract VEGFR^{R1}s, presumably by releasing angiogenic factors other than VEGF. Since macrophages do not express VEGFR-2 (Clauss et al., 1996), α VEGFR-2 failed to inhibit macrophage tumor infiltration. sFlk1 was also ineffective—this is perhaps more surprising, as VEGF also chemoattracts macrophages. However, α VEGFR-2 and sFlk1 also upregulated the expression of the macrophage chemoattractants PIGF, VEGF, FGF-2, and G-CSF, which might have counteracted VEGFR^{R1} treatment. Thus, by blocking macrophage infiltration, α PIGF not only reduced tumor growth, but also rendered tumors more responsive to VEGFR^{R1}s.

Enhancement of Chemotherapy and VEGFR^{R1} Inhibitor Therapy by α PIGF

Like bevacizumab in patients (Hurwitz et al., 2004), α PIGF amplified the effect of chemotherapy in preclinical mouse tumor models. This may, at least in part, be attributable to the fact that cytotoxic agents upregulate PIGF in tumor cells. Obviously, additional mechanisms might contribute. For instance, it remains to be determined whether the effects of α PIGF on tumor vessel perfusion will improve the delivery of cytotoxic drugs, or whether α PIGF might also inhibit EPC mobilization in response to chemotherapy.

Treatment with a single antiangiogenic agent may lead to acquired drug resistance based on an escape mechanism via induction of an angiogenic rescue program; for instance, FGF is upregulated after α VEGFR-2 treatment (Casanovas et al., 2005). Plasma PIGF levels are upregulated 3- to >10-fold in colorectal cancer patients treated with bevacizumab (Willett et al., 2005), as well as in renal cell

cancer patients treated with Sunitinib (Motzer et al., 2006). Induction of PIGF expression has also been proposed to contribute to human xenografted tumor recurrence after immunoradiation therapy (Taylor et al., 2003). Furthermore, PIGF expression correlates with the tumor stage, vascularity, metastasis, recurrence, and survival in different malignancies. In this study, α VEGFR-2 and sFlk1 upregulated plasma PIGF levels in tumor-bearing mice, mimicking the effect of VEGF^{R1}s in clinical trials. Combination treatment of α PIGF with α VEGFR-2 and sFlk1 resulted in more sustained tumor growth inhibition than monotherapy with each agent alone. Thus, not only the upregulation of PIGF by VEGF^{R1}s, but also the pleiotropic and complementary mechanisms of α PIGF relative to VEGF or VEGFR-2 selective inhibitors, suggest that α PIGF may be useful for monotherapy, as well as an adjunct to VEGF^{R1}s.

Resistance to α PIGF?

An important question is how to avoid/minimize resistance to antiangiogenic agents. Antiangiogenesis is expected to prune the tumor vasculature, thereby depriving tumor cells from oxygen and causing tumor necrosis. The resultant hypoxia is, however, a strong stimulus for the expression of angiogenic cytokines, which induce an angiogenic rescue program that could evoke resistance to VEGF^{R1}s (Sweeney et al., 2003). Our studies show that hypoxia upregulated PIGF release from tumor cells and that α VEGFR-2 not only inhibited tumor angiogenesis but also increased tumor hypoxia and upregulated the production of PIGF and other angiogenic molecules such as FGF-1, FGF-2, SDF-1, CXCL1, and MMP-9. Each of these cytokines can be produced by tumor cells, macrophages, and other stromal cells and are hypoxia-inducible (Casanovas et al., 2005; Semenza, 2003). sFlk1 induced a similar angiogenic rescue program (data not shown).

In contrast, α PIGF did not switch on such an angiogenic rescue program. Indeed, though α PIGF reduced tumor vessel density, it did not cause as severe hypoxia and necrosis as α VEGFR-2, suggesting that the residual vasculature permitted a critical threshold of oxygenation. The larger vascular perfusion area and preserved perfusion in the residual tumors after α PIGF as compared to α VEGFR-2 are consistent with such model. Besides the lower hypoxia, the inhibition of intratumoral macrophage infiltration may also explain the lack of upregulation of these angiogenic factors in α PIGF-treated tumors. Also, α PIGF overruled the effect of α VEGFR-2 on tumor macrophage infiltration and, coincidentally, on the upregulation of VEGF by α VEGFR-2 (although α VEGFR-2 upregulated tumor VEGF levels, α PIGF/ α VEGFR-2 combination normalized VEGF levels again; data not shown). Thus, α PIGF enhances the antitumor efficacy of α VEGFR-2, yet it does not increase, but might even decrease, the resistance to α VEGFR-2.

Safety and Therapeutic Profile of α PIGF

The safety of antiangiogenic agents will be a determining factor for their wider spread use in particular oncological

indications (children, pregnant women, and patients at risk for developing VEGF^{R1}-related side effects) and beyond oncology. Side effects of VEGF^{R1}s include teratogenicity, pruning of healthy vessels, thrombosis, hypertension, and other effects. Fetal development depends on the formation of a vascularized placenta—VEGF is essential for this process (Carmeliet et al., 1996; Ferrara et al., 1996). Not surprisingly, therefore, VEGF^{R1}s interrupted pregnancy by inhibiting angiogenesis in the placenta. In contrast, α PIGF did not affect fetal development consistent with genetic data that embryos develop normally in the absence of PIGF (Carmeliet et al., 2001). VEGF^{R1}s do, however, also cause pruning of quiescent vessels in healthy tissues because they require VEGF survival signals for their maintenance. α PIGF did not prune such quiescent vessels, presumably not only because these fenestrated ECs lack VEGFR-1 (Kamba et al., 2006), but also because expression of PIGF (unlike VEGF) is negligible in quiescent tissues in baseline conditions (data not shown). In addition, α PIGF did not aggravate vessel pruning induced by α VEGFR-2, while the combination more effectively inhibited tumor angiogenesis and growth. Data that a VEGF-trap (capturing VEGF, VEGF-B, and PIGF) did not aggravate the neonatal vascular defects induced by an anti-VEGF antibody are consistent with our findings (Malik et al., 2006).

Some of the toxicity of the VEGF^{R1}s has been ascribed to the deprivation of quiescent ECs from critical VEGF-dependent survival and maintenance signals, thereby inducing EC dysfunction, leading to a hypertensive and prothrombotic state. Indeed, VEGF upregulates the vasodilating nitric oxide and maintains the endothelium in an antithrombotic state by upregulating tPA and downregulating PAI-1 (Carmeliet et al., 1997). In contrast, PIGF is only a survival signal for growing ECs, but not for quiescent vessels (as suggested by the PIGF knockout phenotype), and is not detectable in healthy vessels. Whatever the mechanisms, the safety of α PIGF is striking and medically relevant.

Because of the excellent safety profile, α PIGF could be combined with VEGF^{R1}s to increase efficacy without increased toxicity or resistance. Besides, single α PIGF therapy may also offer novel opportunities for the treatment of pathological conditions, for which the adverse effects of VEGF^{R1}s may be excessive and prohibitive, such as cancer in children and young (pregnant) women, or perhaps in patients at risk for thrombotic, cardiac, or other complications. Our data also warrant further analysis of systemic α PIGF treatment for ocular neovascularization as an alternative to intraocular administration of antiangiogenic agents.

EXPERIMENTAL PROCEDURES

Generation and Characterization of α PIGF

α PIGF was selected and produced using in vitro hybridoma cell culture technologies developed at ThromboGenics NV. α PIGF was purified from cell culture supernatant by affinity chromatography on ProSep

vA Ultra (Millipore). Affinities of α PIGF were determined by using Biacore assays. Competitive inhibition studies were performed by ELISA on immobilized soluble receptors using biotinylated growth-factor-specific antibodies (all R&D Systems) and on hVEGFR-1 overexpressing cells using ^{125}I -radiolabeled ligand. α PIGF was used at submolar to 1000-fold molar excess. For FRET analysis, porcine aortic endothelial (PAE) cells transfected with two plasmids expressing Npn1 fused to the cyan (ECFP) and the yellow (EYFP) variants of GFP were used. For VEGFR-1 tyrosine phosphorylation studies, 293T cells overexpressing VEGFR-1 were used (Errico et al., 2004).

Pharmacokinetics

Mice were i.p. injected with cold or ^{125}I -radiolabeled α PIGF or α VEGFR-2. Antibody plasma levels up to 21 days were determined by ELISA or by counting total and TCA-precipitable radioactivity using a gamma counter.

In Vitro Migration Assay

Subconfluent cell monolayers were grown in serum-free medium containing microchemotaxis chambers, of which the bottom wells were filled with diluted chemoattractants. After incubation at 37°C for 10 hr, filters were Giemsa stained and cells were counted.

Syngeneic Tumor Models

10^6 B16.F10, CT26, EL-4 and Panc02 cells were injected subcutaneously (s.c.) When tumors had grown to $\sim 100\text{ mm}^3$, mice were treated with α PIGF, α VEGFR-2 (DC101; ATCC) and isotype control IgG₁s, sFlk1 (via hydroproportion) or chemotherapy (used at ED₅₀). For orthotopic tumors, 10^6 Panc02 cells were injected into the head of the pancreas via abdominal midline incision, and treatment started when tumors reached $\sim 30\text{ mm}^3$.

sFlk1 Hydroproportion

40 $\mu\text{g}/\text{mouse}$ of a vector encoding sFlk1 or a control vector (Davidoff et al., 2002) were suspended in 2.5 ml of Ringer's solution and injected into the tail vein. sFlk1 plasma protein levels were determined using a sVEGFR-2 immunoassay (R&D Systems).

Macrophage Depletion

Clodronate encapsulated liposomes (clodrolip) were administered i.p. 24 hr after tumor cell implantation (100 mg/kg); repeated injections (50 mg/kg) every fourth day prevented macrophage repopulation (Zeisberger et al., 2006).

ELISA

Concentrations of PIGF, VEGF, and VEGF/PIGF heterodimers were quantified using PIGF and VEGF immunoassays (R&D Systems). Values from cell culture media were normalized to end-stage cell count ($\text{pg}/10^6$ cells). Similar data were obtained when correcting the data for protein content.

Histology and Morphometric Analyses

All methods for histology and immunostaining on paraffin and cryosections have been described (Carmeliet et al., 1996; Lutun et al., 2002b). The following primary antibodies were used: rat anti-CD31, rat anti-F4/80/Mac1, rat anti-CD45 (all Becton Dickinson), rabbit anti-LYVE-1 (Cell Signaling), mouse anti-SMC α actin (Sigma), and rat anti-caspase-3 (Abcam). Signals were detected using fluorescently conjugated secondary antibodies (Alexa 488 or 546, Molecular Probes) or peroxidase-labeled IgGs (Dako) followed by tyramide signal amplification (Perkin Elmer, Life Sciences). (Lymph)-angiogenesis and tumor inflammation were assessed by quantification of CD31⁺ and LYVE-1⁺ microvascular density, or CD31⁺, LYVE-1⁺, and F4/80⁺ area/total tumor area using a Zeiss Axioplan microscope with KS300 image analysis software.

qRT-PCR

Quantitative RT-PCR was performed as described (Carmeliet et al., 2001; Lutun et al., 2002b) using the probes and primers listed in Table S6.

Hypoxia Assessment and Tumor Perfusion

Tumor hypoxia was detected 2 hr after injection of 60 mg/kg pimonidazole hydrochloride into tumor-bearing mice. To detect the formation of pimonidazole adducts, tumor sections were immunostained with hypoxyprobe-1-Mab1 (Chemicon) following the manufacturer's instructions. Tumor perfusion was analyzed using fluorescent microspheres (Lutun et al., 2002b).

Assessment of the Safety Profile of α PIGF

Female Swiss mice were treated with α PIGF, α VEGFR-2, control IgG₁, or sFlk1 for 3 weeks. Tracheal vessel patency was assessed by in vivo injection of FITC-labeled *Lycopersicon esculentum* lectin. Tracheas were removed, immersed in fixative for 1 hr and processed as whole mounts for immunohistochemistry (Kamba et al., 2006). Capillary branching points per unit area overlying the cartilage rings were counted. Vascular density in paraffin sections of thyroid was expressed as a number of capillary profiles per millimeter of follicle perimeter. PAI-1 plasma levels were determined by ELISA (Declercq et al., 1995), and blood pressure was assessed by intraluminal hemodynamic measurements.

Statistics

Data represent mean \pm SEM of representative experiments unless otherwise stated. Statistical significance was calculated by Student's t test or ANOVA where indicated (Prism v4.0b), considering $p < 0.05$ as statistically significant. IC₅₀ was calculated using WinNonlin v5.1.

Supplemental Data

The Supplemental Data include five supplemental notes, four supplemental figures, six supplemental tables, and Supplemental Experimental Procedures, and can be found with this article online at <http://www.cell.com/cgi/content/full/131/3/463/DC1/>.

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