A Placental Growth Factor Variant Unable to Recognize Vascular Endothelial Growth Factor (VEGF) Receptor-1 Inhibits VEGF-Dependent Tumor Angiogenesis via Heterodimerization

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Abstract

Angiogenesis is one of the crucial events for cancer development and growth. Two members of the vascular endothelial growth factor (VEGF) family, VEGF-A and placental growth factor (PIGF), which are able to heterodimerize if coexpressed in the same cell, are both required for pathologic angiogenesis. We have generated a PIGF¹ variant, named PIGF¹-DE in which the residues Asp⁷² and Glu⁷³ were substituted with Ala, which is unable to bind and activate VEGF receptor-1 but is still able to heterodimerize with VEGF. Here, we show that overexpression in tumor cells by adenoviral delivery or stable transfection of PIGF¹-DE variant significantly reduces the production of VEGF homodimer via heterodimerization, determining a strong inhibition of xenograft tumor growth and neangiogenesis, as well as significant reduction of vessel lumen and stabilization, and monocyte-macrophage infiltration. Conversely, the overexpression of PIGF¹wt, also reducing the VEGF homodimer production comparably with PIGF¹-DE variant through the generation of VEGF/PIGF heterodimer, does not inhibit tumor growth and vessel density compared with controls but induces increase of vessel lumen, vessel stabilization, and monocyte-macrophage infiltration. The property of PIGF and VEGF-A to generate heterodimer represents a successful strategy to inhibit VEGF-dependent angiogenesis. The PIGF¹-DE variant, and not PIGF¹wt as previously reported, acts as a “dominant negative” of VEGF and is a new candidate for antiangiogenic gene therapy in cancer treatment. Cancer Res; 70(5); OF1–10. ©2010 AACR.

Introduction

Angiogenesis is one of the major pathologic changes associated with several complex diseases, such as cancer, atherosclerosis, arthritis, diabetic retinopathy, and age-related macular degeneration (1, 2). Among the several molecular players involved in angiogenesis, some members of vascular endothelial growth factor (VEGF) family—VEGF-A, VEGF-B, and placental growth factor (PIGF)—and the related receptors VEGFR-1 (also known as Flt-1, recognized by all three VEGF members) and VEGF-R-2 (also known as Flk-1 in mice and KDR in human, specifically recognized by VEGF-A) have a decisive role (3). VEGFR-1 exists also as soluble form generated by alternative splicing (4), representing one of the most potent antiangiogenic molecule, as confirmed for its pivotal role in cornea avascularity (5). Recently, a soluble form of VEGFR-2 has been described that acts primarily as endogenous inhibitor of lymphatic vessel growth (6).

All members of VEGF family naturally exist as dimeric glycoproteins to interact and induce the dimerization of their receptors (7). PIGF and VEGF-A share a strict biochemical and functional relationship because, besides having VEGFR-1 as common receptor, they can form heterodimer if coexpressed in the same cell (8). The heterodimer may induce receptor heterodimerization, like VEGF, or bind to VEGF-R-1.

The role of VEGF-A is essential in both physiologic and pathologic angiogenesis, whereas that of PIGF and VEGF-B is mainly restricted to pathologic conditions. In the same manner, the VEGFR-1 signaling is not crucial in physiologic conditions but results in different contexts of pathologic angiogenesis (9, 10).

VEGF-A is the main proangiogenic factor known, and Avastin, a neutralizing monoclonal antibody (mAb) against VEGF-A, is the first antiangiogenic drug approved for cancer treatment (11). More recently, increasing attention has been...
devoted to the specific activation of VEGFR-1 for its crucial role in different pathologic conditions. Indeed, the block of VEGFR-1 or PI GF is sufficient to strongly inhibit pathologic angiogenesis associated to different kinds of pathologies, such as cancer (12, 13), atherosclerosis, arthritis, ocular neovascular diseases, and metastasis formation (14–18). Interestingly, the VEGF/PIGF heterodimer, which is able to act only in the presence of VEGFR-1, stimulates angiogenesis in a model of myocardial infarct with an extent comparable with that obtained with VEGF (19). Altogether, these data indicate how a fine-tuning of the availability of the VEGF ligands and receptors is required for a correct angiogenesis during pathologic conditions.

Among the different strategies designed to inhibit VEGF activity in pathologic angiogenesis, we have evaluated if the ability of VEGF and PIGF to generate heterodimer may be successful to inhibit pathologic angiogenesis. In this perspective, we have previously generated a PIGF1 variant, named PIGF1-DE (20), which is unable to bind and activate VEGFR-1 but keeps the ability to form heterodimer with VEGF. The basic hypothesis was that it is possible to sequester active VEGF by forcing the formation of nonfunctional PIGF1-DE/VEGF heterodimer. To verify the applicability of this strategy, in vivo growth and neoangiogenesis of xenograft tumors generated with tumor stable cell lines overexpressing PIGF1-DE, or of xenograft tumors transduced with recombinant adenovirus for PIGF1-DE, were analyzed.

Materials and Methods

Plasmids. The expression vector pCDNA3 carrying the full-length human cDNA for PIGF1wt (pPIGF1wt) or the variants PIGF1-D72→E→E→E→A (pPIGF1-DE) and PIGF1-N8→A (pPIGF1-N) were generated as previously described (20).

Cell culture and tumor stable clone generation. Human tumor cell lines NCI-H460 (from lung cancer; American Type Culture Collection) and A2780 (from ovarian carcinoma; European Collection of Animal Cell Cultures) were grown in RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L glutamine, and standard concentration of antibiotics. To generate tumor stable cell lines, 1 × 10⁶ NCI-H460 or A2780 cells were electroporated (Gene Pulser II System, 250 V/cm and 975 μF; Bio-Rad) with 50 μg of pPIGF1wt, pPIGF1-DE, pPIGF1-N, and, as a control, pCDNA3 vectors. Two days later, culture medium was supplemented with 0.8 mg/mL genetin. After 2 wk, the G418-resistant clones were picked, amplified, and screened by ELISA to determine the PIGF concentration in the medium. For each transfection, the three clones expressing the highest amount of PIGF were mixed to avoid clonal effects. Once resuscitated, cells were amplified until the fifth passage and frozen to generate a master cell bank. Therefore, for each experiments performed, we started from passage five. The same approach was followed for the generated A2780 and NCI-H460 stable clones. Cell lines, characterized by cell banks for isoenzymology and DNA profiling, were further characterized in house, evaluating morphology, the growth curve, and absence of Mycoplasma.

ELISA assays. To quantify PIGF and VEGF dimers in the cell culture medium or tumor extracts, we used the protocols described elsewhere (20–22) with some modifications (Supplementary Data).

Xenograft tumor growth and analysis. For xenograft tumor experiments, 7- to 8-wk-old CD1 male nude athymic mice (Charles River) were used. Exponentially growing tumor cells (3 × 10⁶ per flank for A2780 or 2 × 10⁵ per flank for NCI-H460) were injected s.c. and tumor growth was followed by biweekly measurements of tumor diameters with a caliper. Tumor volume (TV) was calculated according to the following formula: TV (mm³) = d²D/2, where d and D are the shortest and the longest diameters, respectively. For ethical reasons, mice were sacrificed when control tumors reached a volume of 1,500 to 2,000 mm³. Histomorphometrical and immunohistochemical analyses and quantitative determination of VEGF and PIGF dimers were performed on tumor samples (see Supplementary Data). The care and husbandry of mice and xenograft tumor experimental procedures were in accordance with European Directives no. 86/609 and with Italian D.L. 116. All the experiments were approved by the Institute of Genetics and Biophysics and the Sigma-Tau veterinarians.

Adenovirus generation and gene therapy experiments. Recombinant adenovirus C serotype 5 for PIGF1wt, PIGF1-DE, and green fluorescent protein (GFP) was generated using AdEasy Adenoviral vector system (Stratagene; ref. 23). Adenoviral preparations were purified using standard procedures and titrated by measuring the plaque-forming units (pfu): 4 × 10¹⁰ pfu/mL for Ad-PIGF1wt, 2 × 10¹⁰ pfu/mL for Ad-PIGF1-DE, and 3 × 10⁹ pfu/mL for Ad-GFP. CD1 nude mice were inoculated s.c. with 3 × 10⁶ A2780 cells. After 10 d, tumors reached, in average, a volume of 200 mm³. The animals were randomly divided in three groups, and intratumoral injections with 5 × 10⁷ pfu/30 μL PBS of virus were performed. The injection was repeated 7 d later. After 21 d from cell injection, tumors were explanted and analyzed as described above.

Statistical analysis. Data are expressed as the mean ± SE, with P < 0.05 considered statistically significant. Differences among groups were tested by one-way ANOVA; Tukey honestly different test was used as post hoc test to identify which group differences account for the significant overall ANOVA. All calculations were carried out using SPSS statistical package (version 12.1).

Results

Generation and characterization of NCI-H460 and A2780 stable clones overexpressing PIGF1-DE variant. The cDNA for PIGF1-DE variant or for PIGF1wt cloned in the pCDNA3 expression vector and, as control, the empty vector was used to stably transfect two VEGF-producing PIGF-nonproducing human tumor cell lines: NCI-H460 (lung carcinoma) and A2780 (ovarian carcinoma). To avoid clonal effects, the three clones expressing the highest amount of PIGF for each cell line were mixed. The stable cell lines generated were characterized for the concentration of secreted VEGF and PIGF homodimers and for the presence of VEGF/PIGF
heterodimer. As expected (Table 1), only clones transfected with PlGF1wt or PlGF1-DE were able to produce the heterodimer. For both cell lines, we observed a similar and significant reduction of secreted VEGF homodimer compared with PlGF1wt or PlGF1-DE transfected clones or detected in A2780 xenograft tumor extracts. According to this experiment, A2780-PlGF1wt cells showed a growth rate and a mean volume after 21 days, which are fully comparable with those of nontransfected or pCDNA3-transfected cells. Because A2780-pCDNA3 and A2780-PlGF1wt tumors showed a growth fully comparable with that of nontransfected cells, with a mean volume of 0.3 cm³ (Fig. 2B). In contrast, tumors generated by transfected cells showed a delayed growth. H460-pCDNA3 and H460-PlGF1wt displayed a similar growth rate without significant differences, showing at day 35 a mean volume of 1.9 and 1.7 cm³, respectively. Conversely, tumors generated by H460-PlGF1-DE cells showed a strong growth delay with a mean volume of 0.3 cm³ at day 35. This reduction was significant in comparison not only with H460 tumors (P < 0.0001) at day 27 but also with H460-PlGF1wt (P < 0.005) and H460-pCDNA3 tumors (P < 0.001) at day 27 or 35 (Fig. 2A).

Xenograft tumors generated with A2780-pCDNA3 or A2780-PlGF1wt cells showed a growth rate and a mean volume after 21 days, which are fully comparable with those of tumors generated with parental A2780 cells, with a mean volume of ~2 cm³ at day 27. In contrast, tumors generated by transfected cells showed a delayed growth. H460-pCDNA3 and H460-PlGF1wt displayed a similar growth rate without significant differences, showing at day 35 a mean volume of 1.9 and 1.7 cm³, respectively. Conversely, tumors generated by H460-PlGF1-DE cells showed a strong growth delay with a mean volume of 0.3 cm³ at day 35. This reduction was significant in comparison not only with H460 tumors (P < 0.0001) at day 27 but also with H460-PlGF1wt (P < 0.005) and H460-pCDNA3 tumors (P < 0.001) at day 27 or 35 (Fig. 2A).

Table 1. Quantification of PlGF and VEGF dimers secreted by H460 and A2780 stable clones or detected in A2780 xenograft tumor extracts

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>VEGF</th>
<th>PlGF/VEGF</th>
<th>PlGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>H460-pCDNA3</td>
<td>5.4 ± 0.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H460-PlGF1wt</td>
<td>2.5 ± 0.1*</td>
<td>6.4 ± 0.3</td>
<td>20.0 ± 0.6</td>
</tr>
<tr>
<td>H460-PlGF1-DE</td>
<td>2.2 ± 0.2*</td>
<td>5.9 ± 0.2</td>
<td>24.0 ± 0.7</td>
</tr>
<tr>
<td>A2780</td>
<td>2.1 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A2780-pCDNA3</td>
<td>1.9 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A2780-PlGF1wt</td>
<td>1.2 ± 0.1†</td>
<td>1.9 ± 0.2</td>
<td>27.4 ± 0.5</td>
</tr>
<tr>
<td>A2780-PlGF1-DE</td>
<td>1.1 ± 0.1†</td>
<td>2.0 ± 0.1</td>
<td>31.6 ± 0.8</td>
</tr>
</tbody>
</table>

NOTE: The values, expressed as ng/1 × 10⁶ cells for cell lines and as ng/mg for tumor extracts, represent the average ± SE of two independent experiments, in which each sample was analyzed in triplicate. Abbreviation: ND, not detectable.

*P < 0.0001 versus H460 and H460-pCDNA3.
†P < 0.01 versus A2780 and A2780-pCDNA3.
‡P < 0.0001 versus A2780-pCDNA3.
§P < 0.01 versus Ad-GFP.

**VEGF/PlGF heterodimer binding properties.** VEGF/PlGF heterodimer cannot induce VEGFR-2 dimerization because the PlGF moiety does not recognize VEGFR-2. Because some reports indicated this possibility (24), we first evaluated if the PlGF moiety does not recognize VEGFR-2. Because some reports indicated this possibility (24), we first evaluated if the PlGF moiety does not recognize VEGFR-2. As expected, all PlGF and VEGF dimers were able to induce VEGFR-1 phosphorylation in a stable cell line overexpressing it (293-Flt-1). Differently from VEGF, the heterodimer failed to induce VEGFR-2 phosphorylation in cells overexpressing only this receptor (PAE-KDR), whereas it was able to induce the VEGFR-2 phosphorylation on human umbilical vascular endothelial cells (HUVEC) that express both VEGFR-1 and VEGFR-2 via receptor heterodimerization (Fig. 1C). These data definitively confirmed that VEGF/PlGF binds to VEGFR-1 and may activate VEGFR-2 phosphorylation only in cells expressing both the receptors.

Due to its binding abilities, the unique inhibitory property that wild-type heterodimer might show is to prevent VEGFR-2 heterodimerization on cells expressing exclusively VEGFR-2 via binding to receptor monomers on cell surface with its VEGF moiety. To investigate this possibility, 12-fold molecular excess of VEGF/PlGF was used to compete VEGF-induced VEGFR-2 phosphorylation on PAE-KDR cells, but no inhibition was observed (Fig. 1C).

Similar assays were performed to evaluate the binding property of PlGF1-DE/VEGF heterodimer. In ELISA-based binding assays, PlGF1-DE/VEGF as well as PlGF1-DE produced by A2780-PlGF1-DE cells lost the binding activity to VEGFR-1 (Fig. 1B). We purified PlGF1-DE/VEGF from the culture medium of H460-PlGF1-DE stable clones (Table 1) by affinity chromatography (Supplementary Data; Supplementary Fig. S1). As reported in Fig. 1D, differently from wild-type heterodimer, PlGF1-DE/VEGF failed to induce VEGFR-2 phosphorylation on HUVECs as well as VEGFR-1 phosphorylation on 293-Flt-1 cell line. These data showed that coexpression of PlGF1-DE variant in VEGF-producing cells effectively sequestered active VEGF for the generation of a nonfunctional heterodimer.

**Overexpression of PlGF1-DE strongly inhibited xenograft tumor growth.** After assessing that overexpression of PlGF1-DE or PlGF1wt did not affect the growth of stable clones in vitro (Supplementary Fig. S2), stably transfected and parental cell lines were injected s.c. in CD-1 nude mice. NCI-H460 tumors were detectable in all the animals inoculated, showing a volume of ~2 cm³ at day 27. In contrast, tumors generated by transfected cells showed a delayed growth. H460-pCDNA3 and H460-PlGF1wt displayed a similar growth rate without significant differences, showing at day 35 a mean volume of about 1.9 and 1.7 cm³, respectively. Conversely, tumors generated by H460-PlGF1-DE cells showed a strong growth delay with a mean volume of 0.3 cm³ at day 35. This reduction was significant in comparison not only with H460 tumors (P < 0.0001) at day 27 but also with H460-PlGF1wt (P < 0.005) and H460-pCDNA3 tumors (P < 0.001) at day 27 or 35 (Fig. 2A).
A2780-PlGF1-DE tumors showed reduced neoangiogenesis. Tumors were first characterized for the presence of human PlGF and VEGF dimers in tumor protein extracts using ELISA assays that did not cross-react with endogenous proteins. As reported in Table 1, A2780 cells transfected with PlGF were able to produce VEGF/PlGF heterodimer in vivo, showing similar significant reduction in VEGF homodimer production if compared with tumor generated with A2780 or A2780-pCDNA3 cells. Vessel density was determined by immunostaining with anti-CD31 antibody, and a significant reduction (P < 0.0001) was observed only in A2780-PlGF1-DE tumors (Fig. 2C and E). Furthermore, tumors were analyzed by histochemistry, and as expected for tumor with reduced vascularization, only A2780-PlGF1-DE tumors showed significant decrease in mitotic index and significant increase in percentage of necrotic area (Table 2).

The analysis just described referred to tumors with different volumes (Fig. 2B). To verify if the reduction in vessel density might be in part due to the differences of TV, we decided to generate and analyze tumors with similar volume. In addition to the three A2780 stable clones previously described, we generated a new A2780 stable cell line overexpressing the PlGF variant PlGF1-N16. The change of residue Asn16 to Ala abolished one of the glycosylation sites of PlGF, without modifying its ability in receptor binding and heterodimer generation (20). Tumors of the five experimental groups were explanted when their volume was ∼200 mm$^3$ (Table 2). The immunohistochemical analyses confirmed that only the overexpression of PlGF1-DE induced a strong significant inhibition of vessel density (P < 0.0005 versus all other groups; Fig. 2D). In addition, A2780-PlGF1-DE tumors showed a significant lower mitotic index (P < 0.001 versus all other groups) and the highest value of the percentage of necrosis (Table 2).

Differences in vessels and cell infiltration in tumors overexpressing PlGF1-DE or PlGF1wt. To assess which differences are generated in tumors overexpressing PlGF1wt or PlGF1-DE, we first evaluated the distribution of vessels based on their lumen (Supplementary Data; ref. 14). In both the series of tumor analyzed, A2780-PlGF1-DE tumors showed significant increase in small vessels and decrease of medium and large vessels. Interestingly, A2780-PlGF1wt and

Figure 1. Receptor binding and activation exerted by PlGF and VEGF dimers. A, binding of human VEGF homodimer and VEGF/PlGF heterodimer (1.25–10 ng/mL) to coated human VEGFR-1 and VEGFR-2 (0.5 μg/mL) in ELISA-based assay. B, binding of VEGF homodimer (2.5–10 pg), VEGF/PlGF1-DE heterodimer (4.5–18 pg), and PlGF1-DE homodimer (7.5–26 pg) present in the supernatant of A2780-PlGF1-DE stable clones to coated VEGFR-1 (0.5 μg/mL). C, Western blot analysis of VEGFR-1 and VEGFR-2 phosphorylation (anti-P-Flt-1 and anti-P-KDR) induced by 50 ng/mL of VEGF (V) or VEGF/PlGF (V/P) and 20 ng/mL of PlGF (P) on cells expressing only VEGFR-1 (293-Flt-1), VEGFR-2 (PAE-KDR), or both receptors (HUVEC). VEGF/PlGF (500 ng/mL) was unable to inhibit VEGF-induced VEGFR-2 phosphorylation on PAE-KDR cells. D, Western blot analysis showing that purified VEGF/PlGF1-DE (V/PDE) used at 50 ng/mL was unable to induce VEGFR-1 and VEGFR-2 phosphorylation on cells expressing only VEGFR-1 or both receptors. Anti-Flt-1 or anti-KDR antibodies were used for normalization.
A2780-PlGF1-N16 tumors showed the opposite vessel distribution, with a significant decrease of small vessels and a significant increase of large vessels (Supplementary Fig. S3).

Furthermore, we assessed the extent of vessel stabilization evaluating the density of vessels surrounded by smooth muscle cells (SMC) by immunostaining with antibody against smooth muscle α-actin (SMA). A2780-PlGF1-DE tumors showed reduced vessel stabilization compared with other groups (P ≤ 0.05), whereas both A2780-PlGF1wt and A2780-PlGF1-N tumors presented significant increases in stabilized vessel density (P < 0.01) compared with control tumors (Fig. 3A and B).

Finally, we evaluated the monocyte-macrophage infiltration by immunostaining with anti-F4/80 antibody. A2780-PlGF1-DE tumors showed a strong and significant reduction of F4/80-positive cell area compared with other tumor groups (P < 0.0001), whereas tumors generated with cells overexpressing active PlGF1 showed a remarkable and significant increase of F4/80-positive cell area (P ≤ 0.0002 versus A2780 and A2780-pCDNA3; Fig. 3C and D).

**Ad-PlGF1-DE inhibited A2780 xenograft tumor growth and neoangiogenesis.** To validate the use of PlGF1-DE variant as inhibitor of VEGF-dependent tumor angiogenesis, gene therapy experiments using adenoviral vectors were per-
formed (25, 26). Adenovirus carrying cDNAs for PlGF1wt (Ad-PlGF1wt), PlGF1-DE (Ad-PlGF1-DE) or, as control, GFP (Ad-GFP) was generated. First, we showed that recombinant adenoviruses were able to transduce in vitro the A2780 cells (Supplementary Data; Supplementary Fig. S4) and that, after infection with Ad-PlGF1wt and Ad-PlGF1-DE, the reduction of VEGF homodimer and the production of VEGF/PlGF heterodimer were detectable (Supplementary Table S1).

To evaluate in vivo the antitumoral activity of adenovirus-mediated PlGF1-DE gene transfer, A2780 exponentially growing xenograft tumors were infected by intratumoral injection (27–29) of 5 × 10⁷ pfu, starting at day 10 from tumor cell injection (mean TV, ∼ 200 mm³). Only tumors transduced with Ad-PlGF1-DE showed a strong and significant growth inhibition (P < 0.01; Fig. 4A). The immunohistochemical analyses showed that injection of Ad-PlGF1-DE strongly inhibited the vessel density (P ≤ 0.005), the vessel stabilization (P ≤ 0.005), and the monocyte-macrophage infiltration (P < 0.0001) compared with Ad-GFP and Ad-PlGF1wt (Fig. 4B–D). In the same manner, the infection with Ad-PlGF1wt did not alter the tumor growth and vessel density but significantly stimulated vessel stabilization (P = 0.0009 versus Ad-GFP) and monocyte-macrophage infiltration (P = 0.0001 versus Ad-GFP; Fig. 4). Moreover, histomorphometrical analysis confirmed that adenovirus-mediated PlGF1-DE delivery determined significant reduction of mitotic index (P ≤ 0.01) and increase of percentage of necrosis (P ≤ 0.001) compared with controls (Table 2).

In addition, the concentration of VEGF and PlGF dimers was evaluated in protein extracts of adenovirus-transduced tumors. Once again, a significant reduction of VEGF and the presence of VEGF/PlGF heterodimer were measurable only in Ad-PlGF1wt–infected and Ad-PlGF1-DE–infected tumors (Table 1).

Finally, because adenovirus is able to transduce also mouse cells that take part in tumor formation (30), many of which, such as myeloid and endothelial cells, are able to produce PlGF, we decided to perform ELISA on tumor protein extracts to evaluate if the dimer mPlGF/hPlGF was detectable. This dimer was detected only in the Ad-PlGF1wt and Ad-PlGF1-DE tumor extracts (0.65 ± 0.21 and 0.38 ± 0.17 pg/mg, respectively), indicating that the infection with Ad-PlGF1-DE determined also inhibition of endogenous mPlGF via formation of the inactive mPlGF/hPlGF1-DE dimer.

### Table 2. Histomorphometrical analysis of A2780 tumors

<table>
<thead>
<tr>
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<th>Volume (mm³)</th>
<th>Mitotic index</th>
<th>% of necrosis</th>
</tr>
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<tbody>
<tr>
<td><strong>Xenograft tumors (different volume)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2780</td>
<td>1,807.1 ± 390.9</td>
<td>56.8 ± 3.2</td>
<td>25.0 ± 2.7</td>
</tr>
<tr>
<td>A2780-pCDNA3</td>
<td>1,719.2 ± 410.3</td>
<td>55.7 ± 3.4</td>
<td>23.2 ± 3.6</td>
</tr>
<tr>
<td>A2780-PlGF1wt</td>
<td>1,724.5 ± 251.9</td>
<td>49.6 ± 3.1</td>
<td>18.5 ± 2.5</td>
</tr>
<tr>
<td>A2780-PlGF1-DE</td>
<td>865.7 ± 251.8</td>
<td>31.9 ± 3.5 †</td>
<td>36.1 ± 4.2 ††</td>
</tr>
<tr>
<td><strong>Xenograft tumors (similar volume)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2780</td>
<td>204.0 ± 35.1</td>
<td>37.7 ± 3.3</td>
<td>24.4 ± 7.5</td>
</tr>
<tr>
<td>A2780-pCDNA3</td>
<td>174.4 ± 10.5</td>
<td>33.8 ± 2.5</td>
<td>24.5 ± 8.3</td>
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<tr>
<td>A2780-PlGF1wt</td>
<td>200.7 ± 22.2</td>
<td>40.9 ± 2.7</td>
<td>20.5 ± 7.5</td>
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<tr>
<td>A2780-PlGF1-N</td>
<td>218.0 ± 16.1</td>
<td>37.4 ± 3.2</td>
<td>17.9 ± 3.1</td>
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<td>A2780-PlGF1-DE</td>
<td>184.9 ± 19.0</td>
<td>19.7 ± 1.2 ‡</td>
<td>33.8 ± 8.8</td>
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<tr>
<td><strong>Adenovirus-infected xenograft tumors</strong></td>
<td></td>
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<tr>
<td>Ad-GFP</td>
<td>2,075.3 ± 383.2</td>
<td>75.5 ± 6.4</td>
<td>28.2 ± 3.0</td>
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<td>Ad-PlGF1wt</td>
<td>1,970.7 ± 433.4</td>
<td>79.4 ± 2.6</td>
<td>23.9 ± 3.3</td>
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<tr>
<td>Ad-PlGF1-DE</td>
<td>520.4 ± 131.5</td>
<td>53.8 ± 4.2 ‡‡</td>
<td>38.4 ± 5.6 ‡‡</td>
</tr>
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</table>

**NOTE:** Data are represented as the average ± SE. For mitotic index, values represent the number of mitotically active cells. For necrosis, values represent the % of necrotic area of tumors.

*P < 0.0005 versus A2780 and A2780-pCDNA3.
†P < 0.05 versus Ad-PlGF1wt.
‡P < 0.01 versus Ad-GFP.
§P < 0.001 versus A2780 and Ad-PlGF1-N.
¶P < 0.0001 versus Ad-GFP.
**P = 0.0002 versus Ad-GFP.
***P = 0.0011 versus Ad-GFP.
††P = 0.0001 versus Ad-PlGF1wt.
Discussion

In this report, we have shown that the property of VEGF and PlGF to form heterodimer when coexpressed in the same cell (8) may represent a successful strategy to reduce the production of active VEGF, inhibiting VEGF-dependent angiogenesis. This inhibition was attained using a mutant of human PlGF1 that lost the ability to bind and activate VEGFR-1 but was still able to heterodimerize with VEGF. The overexpression of PlGF1-DE variant in tumor VEGF-producing cells, besides determining the reduction of active VEGF by heterodimerization process, produced two inactive dimers:
PIGF1-DE homodimer and PIGF1-DE/VEGF heterodimer. As result, two different human tumor cell lines derived from lung and ovarian carcinomas, stably transfected with PIGF1-DE, showed severe growth impairment when grafted in vivo compared with parental cells and with cells transfected with empty vector or PIGF1wt. Most importantly, in the xenograft model of ovarian carcinoma, similar results were obtained when the overexpression of PIGF1-DE started 10 days after tumor cell injection, following the intratumoral delivery of Ad-PIGF1-DE. Conversely, the overexpression of PIGF1wt in both experimental approaches did not inhibit tumor growth, indicating that the effects produced by the reduction of VEGF homodimer via heterodimerization were abolished by the overexpression of active PIGF1wt homodimer and the generation of VEGF/PIGF heterodimer. Indeed, we have shown that wild-type heterodimer is able to induce VEGFR-1 phosphorylation and VEGFR-1/VEGFR-2 heterodimerization and phosphorylation and that it may not act as inhibitor preventing VEGFR-2 dimerization.

Previously, it has been reported that overexpression by stable transfection of human PIGF1wt in a mouse tumor cell line (31) or human PIGF2wt in human tumor cell lines (32) per se was sufficient to inhibit xenograft tumor growth and neoangiogenesis, suggesting that wild-type PIGF homodimer and VEGF/PIGF heterodimer overexpressed by grafted tumor cells have no role in tumor development. Conversely, other reports indicated that overexpression of murine PIGF in glioma cells weakly stimulated and not inhibited the tumor growth and survival (33, 34). Recently, it has been reported that PIGF produced by tumor cells is crucial for the generation of large-diameter microvessels and for vessel stabilization (35), in agreement with results here presented.

In the last years, controversial data have been reported on the activity of PIGF homodimer and VEGF/PIGF heterodimer, but many reports, as our data, suggest how these two molecules and VEGFR-1 essential for their activity are deeply involved in pathologic angiogenesis (9, 10).

Most of the published data indicated that the heterodimer was active, with an efficacy about comparable with that of VEGF homodimer, as mitogen on endothelial cells (8, 24), in chemotactic activity on endothelial cells (24), stimulation of neovascularization in corneal pocket assay (36), survival of Plgf−/− primary endothelial cells, and increase of tube formation of PAE cells transfected with both VEGFR-1 and VEGFR-2 (19). In vivo, recombinant heterodimer was able to stimulate angiogenesis in a model of myocardial infarct with an extent comparable with that obtained with VEGF (19). Conversely, in the report describing the inhibitory activity of PIGF1wt in tumor growth (31), it was reported that the heterodimer was inactive in corneal pocket assay and that it

**Figure 4.** Ad-PIGF1-DE inhibits tumor growth and neovascularization. A, A2780 cells were s.c. injected into 8-wk-old CD1 nude athymic mice (n = 7 for group). At days 10 and 17, 5 × 10⁷ pfu of each adenovirus was injected intratumorally. TV was measured every 3 d and data are represented as the mean ± SE. *, P = 0.0024 versus Ad-GFP; **, P = 0.0076 versus Ad-PIGF1wt. Immunohistochemical analyses were performed on five optical fields for each tumor and data are represented as the mean ± SE. B, vessel density was calculated counting CD31-positive vessels. ¶, P = 0.0037 versus Ad-GFP; §, P < 0.0001 versus Ad-PIGF1wt. C, newly formed vessels covered by SMCs were evaluated counting SMA-positive vessels. ¶, P = 0.0053 versus Ad-GFP; §, P < 0.0001 versus Ad-PIGF1wt; ○, P = 0.0009 versus Ad-GFP. D, area of monocyte-macrophage infiltration in the xenograft tumors was evaluated by immunostaining with anti-F4/80 antibody. ¶, P = 0.0001 versus Ad-GFP and Ad-PIGF1wt; **, P = 0.0001 versus Ad-GFP.

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PlGF−/−esis (37 with the analysis of monocyte-macrophage. Once again, these data agree
a crucial role of both VEGF and PlGF factors in the recruit-
creased in PlGF1-overexpressing tumors, confirming the
overexpressing PlGF1wt showed a significant increase in terms
of PlGF/VEGFR-1 in the recruitment of bone marrow
derived and Ad-PlGF1-DE A2780
the opposite situation, in agreement with the reported role
P l G F 1a n dV E G F /P l G F i nA 2 7 8 0 - P l G F 1t u m o r s d e t e r m i n e d
with the control tumors, whereas overexpression of active
PlGF1-DE or PlGF1wt produced similar amounts of VEGF, they
showed important and significant differences.

The volume of tumors generated with two different trans-
fected tumor cell lines overexpressing PlGF1wt was significantly
higher compared with the tumors overexpressing PlGF1-DE var-
ient, and the same was observed comparing Ad-PlGF1wt–trans-
duced and Ad-PlGF1-DE A2780–transduced tumors. Tumors
overexpressing PlGF1wt showed a significant increase in terms
of vessel density compared with PlGF1–overexpressing tu-
mors, and as expected for less vascularized tumors, PlGF1-DE
tumors had a reduced mitotic index and an increase of necrotic
area compared with PlGF1wt tumors.

The analysis of vessel dimension indicates that the reduc-
tion of VEGF in A2780-PlGF1-DE tumors determined a signif-
icant decrease of medium and large vessels in comparison
with the control tumors, whereas overexpression of active
PlGF1 and VEGF/PlGF in A2780-PlGF1 tumors determined the
opposite situation, in agreement with the reported role
of PlGF/VEGFR-1 in the recruitment of bone marrow–derived
endothelial and hematopoietic precursors in tumor angiogen-
(37–39) and with data on tumor angiogenesis obtained in
Plg f−/− mice (14).

The expression of VEGFR-1 and its activation is decisive
for the recruitment of SMCs to stabilize the neovessels (40,
41). The SMA immunohistochemical analysis showed that the
decrease of VEGF in PlGF1-DE–overexpressing A2780
tumors determined a reduction of vessel stabilization if
compared with control tumors, whereas the overexpression
in tumors of active PlGF1 and VEGF/PlGF induced a strong
recruitment of SMA-positive cells with a significant increase
of density of stabilized vessels.

Finally, monocyte-macrophage recruitment is a crucial
step for a correct neoangiogenesis process, and many reports
have shown how this mechanism is mainly mediated by
VEGFR-1 (42–44). The recruitment of F4/80-positive cells
we observed is significantly reduced in PlGF1-DE–overex-
pressing tumors, whereas it is strongly and significantly in-
creased in PlGF1-overexpressing tumors, confirming the
crucial role of both VEGF and PlGF factors in the recruit-
ment of monocyte-macrophage. Once again, these data agree
with the analysis of Plg f−/− mice (14).

Moreover, we have transfected or transduced human PlGF
cDNAs in human tumor cells to quantify in vivo only PlGF
and VEGF dimers produced by tumor cells. The quantitative
data we obtained seem to be coherent because in the culture
medium of both stable clones or transduced cells and in the
tumor extracts, we found a quantity of VEGF/PlGF heterodi-
mer corresponding to about twice the VEGF reduction ob-
served, as expected, because, for each molecule of VEGF
depleted, two molecules of heterodimer may be formed. This
was not the case in the previous reports that described
PlGF1wt–inhibitory activity in tumor angiogenesis because
much more heterodimer, in the case of PlGF1 (31), or less
heterodimer, in the case of PlGF2 (32), was described
compared with the VEGF decrease.

Interestingly, the gene therapy approach with PlGF1-DE
variant may be effective not only for the ability to decrease
the concentration of active VEGF via heterodimerization but
also for the inhibition of activity of the endogenous PlGF by
the generation of nonfunctional mPlGF/hPlGF1-DE dimer.

In conclusion, our results show that the PlGF1-DE variant
strongly inhibits VEGF- and PlGF-dependent angiogenesis via
heterodimerization and represent a new tool for tumor anti-
angiogenic gene therapy approach. At the same time, these re-
results confirm the active role of wild-type PlGF and VEGF/PlGF
in pathologic angiogenesis, particularly in the recruitment
of cells of hematopoietic origins, SMCs, and monocyte-
macrophage required in angiogenic process. Furthermore,
these data confirm the feasibility and the efficacy of the use
of adenoviral vectors in cancer therapy via intratumoral deliv-
ery, which is fast becoming one component of a multimodality
treatment approach to advanced refractory cancer, along with
surgery, radiotherapy, and chemotherapy (25, 26).

Cancer and all pathologies, in which VEGF- or PlGF-driven
angiogenesis is involved, represent a possible target for gene
therapy with PlGF1-DE variant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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