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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 (PlGF), which are able to heterodimerize if coexpressed in the same cell, are both required for pathologic angiogenesis. We have generated a PlGF1 variant, named PlGF1-DE in which the residues Asp^{72} and Glu^{73} 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 PlGF1-DE variant significantly reduces the production of VEGF homodimer via heterodimerization, determining a strong inhibition of xenograft tumor growth and neoangiogenesis, as well as significant reduction of vessel lumen and stabilization, and monocyte-macrophage infiltration. Conversely, the overexpression of PlGF1wt, also reducing the VEGF homodimer production comparably with PlGF1-DE variant through the generation of VEGF/PlGF 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 PlGF and VEGF-A to generate heterodimer represents a successful strategy to inhibit VEGF-dependent angiogenesis. The PlGF1-DE variant, and not PlGF1wt 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); 1804–13. ©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 (PlGF)—and the related receptors VEGFR-1 (also known as Flt-1, recognized by all three VEGF members) and VEGFR-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). PlGF 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 VEGFR-1.

The role of VEGF-A is essential in both physiologic and pathologic angiogenesis, whereas that of PlGF 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

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devoted to the specific activation of VEGFR-1 for its crucial role in different pathologic conditions. Indeed, the block of VEGFR-1 or PlGF 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/PlGF 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 PlGF to generate heterodimer may be successful to inhibit pathologic angiogenesis. In this perspective, we have previously generated a PlGF1 variant, named PlGF1-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 PlGF1-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 PlGF1-DE, or of xenograft tumors transduced with recombinant adenovirus for PlGF1-DE, were analyzed.

Materials and Methods

Plasmids. The expression vector pCDNA3 carrying the full-length human cDNA for PlGF1wt (pPlGF1wt) or the variants PlGF1-D^{72}→A-E^{73}→A (pPlGF1-DE) and PlGF1-N^{16}→A (pPlGF1-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, 1×10^7 NCI-H460 or A2780 cells were electroporated (Gene Pulser II System, 250 V/cm and 975 μF; Bio-Rad) with 50 μg of pPlGF1wt, pPlGF1-DE, pPlGF1-N, and, as a control, pCDNA3 vectors. Two days later, culture medium was supplemented with 0.8 mg/mL geneticin. After 2 wk, the G418-resistant clones were picked, amplified, and screened by ELISA to determine the PlGF concentration in the medium. For each transfection, the three clones expressing the highest amount of PlGF 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 PlGF 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 (3×10^6 per flank for A2780 or 2×10^6 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 (mm^3) = $d^2{*}D/2$, 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 mm^3. Histomorphometrical and immunohistochemical analyses and quantitative determination of VEGF and PlGF 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 PlGF1wt, PlGF1-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): 4×10^{11} pfu/mL for Ad-PlGF1wt, 2×10^{10} pfu/mL for Ad-PlGF1-DE, and 3×10^9 pfu/mL for Ad-GFP. CD1 nude mice were inoculated s.c. with 3×10^6 A2780 cells. After 10 d, tumors reached, in average, a volume of 200 mm^3. The animals were randomly divided in three groups, and intratumoral injections with 5×10^7 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 difference 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 PlGF1-DE variant. The cDNA for PlGF1-DE variant or for PlGF1wt cloned in the pCDNA3 expression vector and, as control, the empty vector was used to stably transfect two VEGF-producing PlGF-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 PlGF for each cell line were mixed. The stable cell lines generated were characterized for the concentration of secreted VEGF and PlGF homodimers and for the presence of VEGF/PlGF

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

	VEGF	PlGF/VEGF	PlGF
Cell lines			
H460	5.4 ± 0.4	ND	ND
H460-pCDNA3	5.5 ± 0.3	ND	ND
H460-PlGF1wt	2.5 ± 0.1*	6.4 ± 0.3	20.0 ± 0.6
H460-PlGF1-DE	2.2 ± 0.2*	5.9 ± 0.2	24.0 ± 0.7
A2780	2.1 ± 0.2	ND	ND
A2780-pCDNA3	1.9 ± 0.2	ND	ND
A2780-PlGF1wt	1.2 ± 0.1†	1.9 ± 0.2	27.4 ± 0.5
A2780-PlGF1-DE	1.1 ± 0.1†	2.0 ± 0.1	31.6 ± 0.8
Xenograft tumors			
A2780	9.9 ± 0.3	ND	ND
A2780-pCDNA3	8.8 ± 0.4	ND	ND
A2780-PlGF1wt	5.3 ± 0.3‡	9.9 ± 0.5	28.6 ± 1.5
A2780-PlGF1-DE	4.2 ± 0.2‡	11.4 ± 0.4	37.0 ± 2.7
Adenovirus-infected xenograft tumors			
Ad-GFP	8.4 ± 0.8	ND	ND
Ad-PlGF1wt	5.4 ± 0.4§	4.9 ± 0.7	7.6 ± 0.9
Ad-PlGF1-DE	5.1 ± 0.5§	5.2 ± 0.9	8.4 ± 1.1

NOTE: The values, expressed as ng/1 × 10^6 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 and A2780-pCDNA3.
§$P < 0.01$ versus Ad-GFP.

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 nontransfected or pCDNA3-transfected cells.

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 VEGF/PlGF was able to interact with VEGFR-2 in an ELISA-based assay. As expected, the heterodimer was able to bind VEGFR-1 but failed to recognize VEGFR-2, whereas VEGF was able to interact with both the receptors. Moreover, the PlGF moiety allowed the heterodimer to bind with high affinity to VEGFR-1 compared with VEGF homodimer (Fig. 1A).

Furthermore, we evaluated the ability of the heterodimer to induce receptor phosphorylation. 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 cm^3 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 cm^3, respectively. Conversely, tumors generated by H460-PlGF1-DE cells showed a strong growth delay with a mean volume of 0.3 cm^3 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 ~1.75 cm^3. In contrast, tumors generated by A2780-PlGF1-DE cells, starting from day 7, showed a significant growth delay and, at day 21, were significantly smaller than controls, with a mean volume of 0.3 cm^3 ($P < 0.005$), reaching at day 32 a mean volume of only 0.85 cm^3 (Fig. 2B). Because A2780-pCDNA3 and A2780-PlGF1wt tumors showed a growth fully comparable with that of nontransfected cells, further analyses were performed on A2780 tumors.

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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 Asn^{16} 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

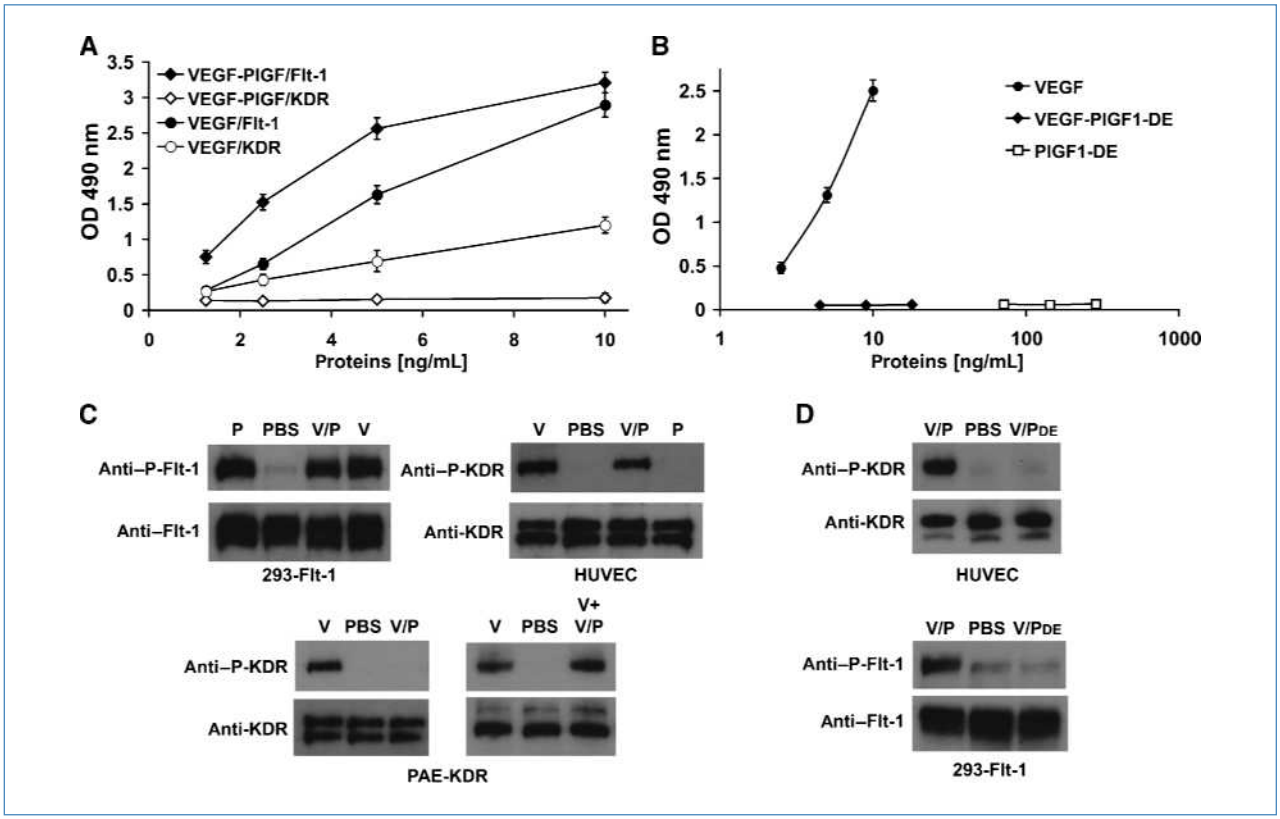

Figure 1. Receptor binding and activation exerted by PlGF and VEGF dimers. A, binding of human VEGF homodimer and VEGF/PlGF heterodimer (1.25–10 ng/mL) to coated human VEGFR-1 and VEGFR-2 (0.5 μg/mL) in ELISA-based assay. B, binding of VEGF homodimer (2.5–10 pg), VEGF/PlGF1-DE heterodimer (4.5–18 pg), and PlGF1-DE homodimer (71.5–286 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/P_{DE}) 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.

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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 ($P \leq 0.05$), 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 ($P \leq 0.0002$ 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-

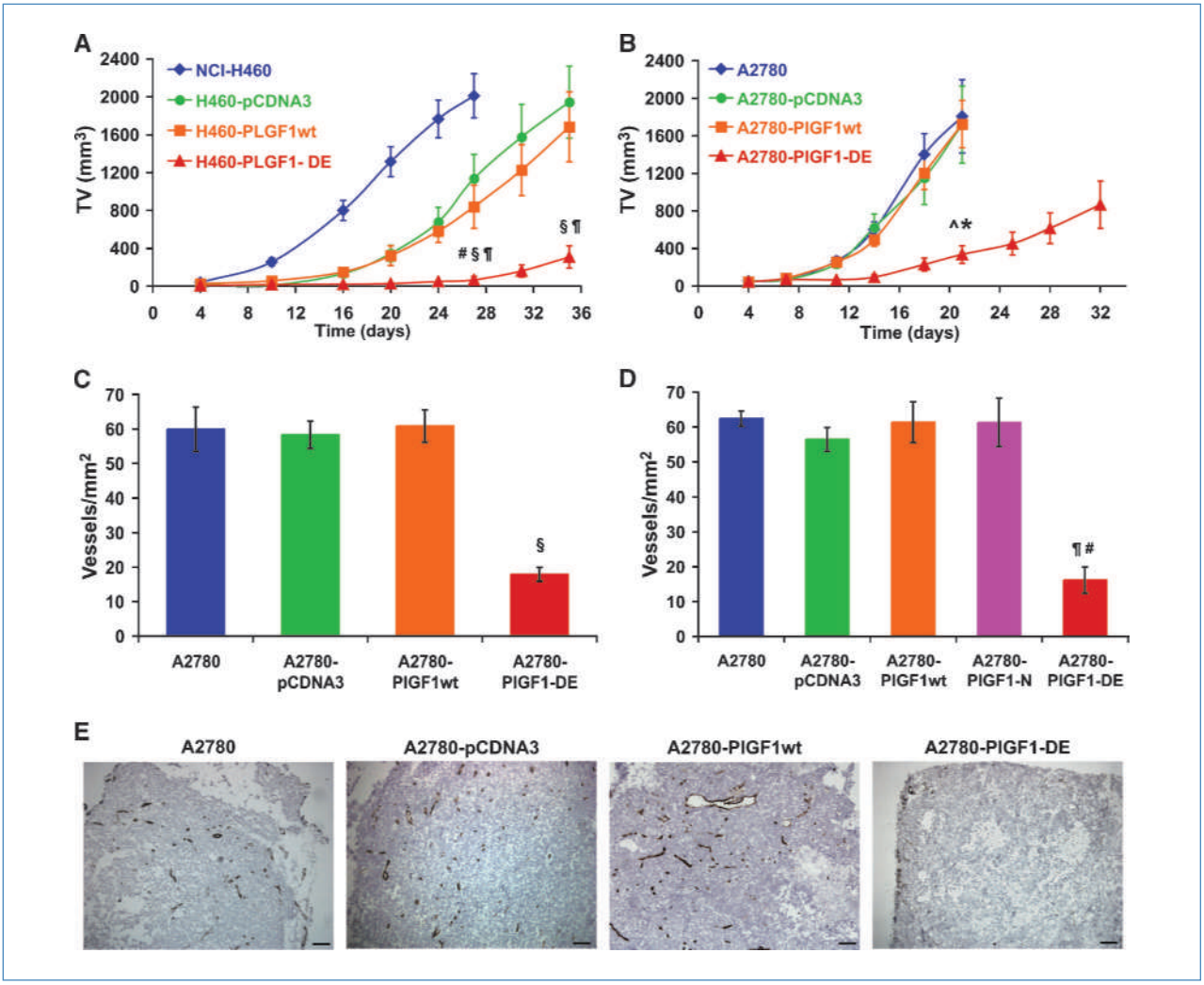

Figure 2. PlGF1-DE variant inhibits tumor growth and neoangiogenesis. Exponentially growing NCI-H460 (2×10^6 cells; A) and A2780 (3×10^6 cells; B) stable clones, and the respective wild-type cells, were s.c. injected into 8-wk-old CD1 nude athymic mice ($n = 10$ for NCI-H460 groups; $n = 7$ for A2780 groups). TV was measured twice a week and data are represented as the mean ± SE. #, $P < 0.0001$ versus NCI-H460 tumors; §, $P < 0.005$ versus H460-PlGF1wt tumors; ¶, $P < 0.001$ versus H460-pCDNA3 tumors; ^, $P < 0.005$ versus A2780 and A2780-pCDNA3 tumors; *, $P < 0.0005$ versus A2780-PlGF1wt. Vessel density was calculated analyzing five optical fields for each tumor, counting CD31-positive vessels. Data are represented as the mean ± SE. Vessel density of tumors with different volume (C; §, $P < 0.0001$ versus all other groups) or with similar volume (D; see Table 2). ¶, $P < 0.0001$ versus A2780 and A2780-pCDNA3; #, $P < 0.0005$ versus A2780-PlGF1wt and A2780-PlGF1-N. E, representative pictures of CD31 staining of A2780 tumors. Scale bar, 100 μm.

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Table 2. Histomorphometrical analysis of A2780 tumors

	Volume (mm^3)	Mitotic index	% of necrosis
Xenograft tumors (different volume)			
A2780	1,807.1 ± 390.9	56.8 ± 3.2	25.0 ± 2.7
A2780-pCDNA3	1,719.2 ± 410.3	55.7 ± 3.4	23.2 ± 3.6
A2780-PlGF1wt	1,724.5 ± 251.9	49.6 ± 3.1	18.5 ± 2.5
A2780-PlGF1-DE	865.7 ± 251.8	31.9 ± 3.5*,†	36.1 ± 4.2‡,†
Xenograft tumors (similar volume)			
A2780	204.0 ± 35.1	37.7 ± 3.3	24.4 ± 7.5
A2780-pCDNA3	174.4 ± 10.5	33.8 ± 2.5	24.5 ± 8.3
A2780-PlGF1wt	200.7 ± 22.2	40.9 ± 2.7	20.5 ± 7.5
A2780-PlGF1-N	218.0 ± 16.1	37.4 ± 3.2	17.9 ± 3.1
A2780-PlGF1-DE	184.9 ± 19.0	19.7 ± 1.2§,‖	33.8 ± 8.8
Adenovirus-infected xenograft tumors			
Ad-GFP	2,075.3 ± 383.2	75.5 ± 6.4	28.2 ± 3.0
Ad-PlGF1wt	1,970.7 ± 433.4	79.4 ± 2.6	23.9 ± 3.3
Ad-PlGF1-DE	520.4 ± 131.5	53.8 ± 4.2¶,**	38.4 ± 5.6††,‡‡

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.

†$P < 0.005$ versus A2780-PlGF1wt.

‡$P < 0.05$ versus A2780 and A2780-pCDNA3.

§$P < 0.001$ versus A2780, A2780-pCDNA3, and A2780-PlGF1-N.

‖$P = 0.0001$ versus A2780-PlGF1wt.

¶$P = 0.015$ versus Ad-GFP.

**$P = 0.0002$ versus Ad-PlGF1wt.

††$P = 0.0011$ versus Ad-GFP.

‡‡$P = 0.0001$ versus Ad-PlGF1wt.

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 5×10^7 pfu, starting at day 10 from tumor cell injection (mean TV, ~200 mm^3). 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 ($P \leq 0.005$), the vessel stabilization ($P \leq 0.005$), 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 ($P \leq 0.01$) and increase of percentage of necrosis ($P \leq 0.001$) 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.

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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:

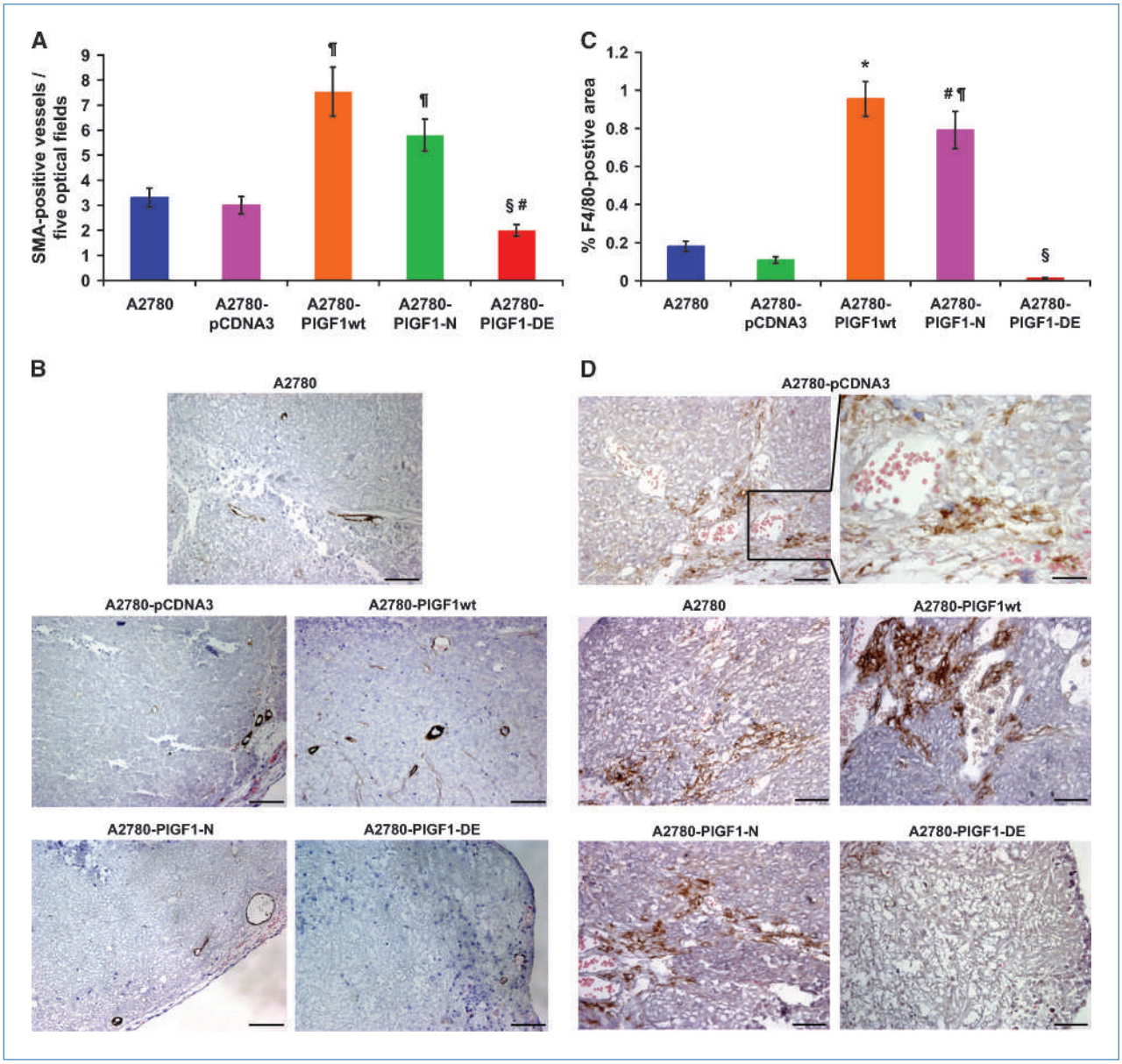

Figure 3. Vessel stabilization and monocyte-macrophage infiltration in tumors overexpressing PlGF1-DE or PlGF1wt. The analyses were performed on five optical fields for each tumor and data are represented as the mean ± SE. A, newly formed vessels covered by SMCs were evaluated counting SMA-positive vessels. A2780-PlGF1-DE tumors showed reduced vessel stabilization compared with all other groups, whereas both A2780-PlGF1wt and A2780-PlGF1-N tumors showed increases in stabilized vessel density. §, $P < 0.05$ versus A2780 and A2780-pCDNA3; #, $P < 0.001$ versus A2780-PlGF1wt and A2780-PlGF1-N; ¶, $P < 0.01$ versus A2780 and A2780-pCDNA3. B, representative pictures of SMA staining of A2780 tumors. Scale bar, 100 μm. C, the area of monocyte-macrophage infiltration in the xenograft tumors was evaluated by immunostaining with anti-F4/80 antibody. A2780-PlGF1-DE tumors showed a reduced F4/80-positive area compared with all other tumor groups. §, $P < 0.0001$. Conversely, A2780-PlGF1wt or A2780-PlGF1-N tumors showed an increase of F4/80-positive cell area. *, $P < 0.0001$ versus A2780 and A2780-pCDNA3; #, $P = 0.0002$ versus A2780; ¶, $P = 0.0001$ versus A2780-pCDNA3. D, representative pictures of F4/80 staining of A2780 tumors. Scale bar, 50 μm. A high magnification picture of A2780-pCDNA3 tumors is shown (scale bar, 20 μm).

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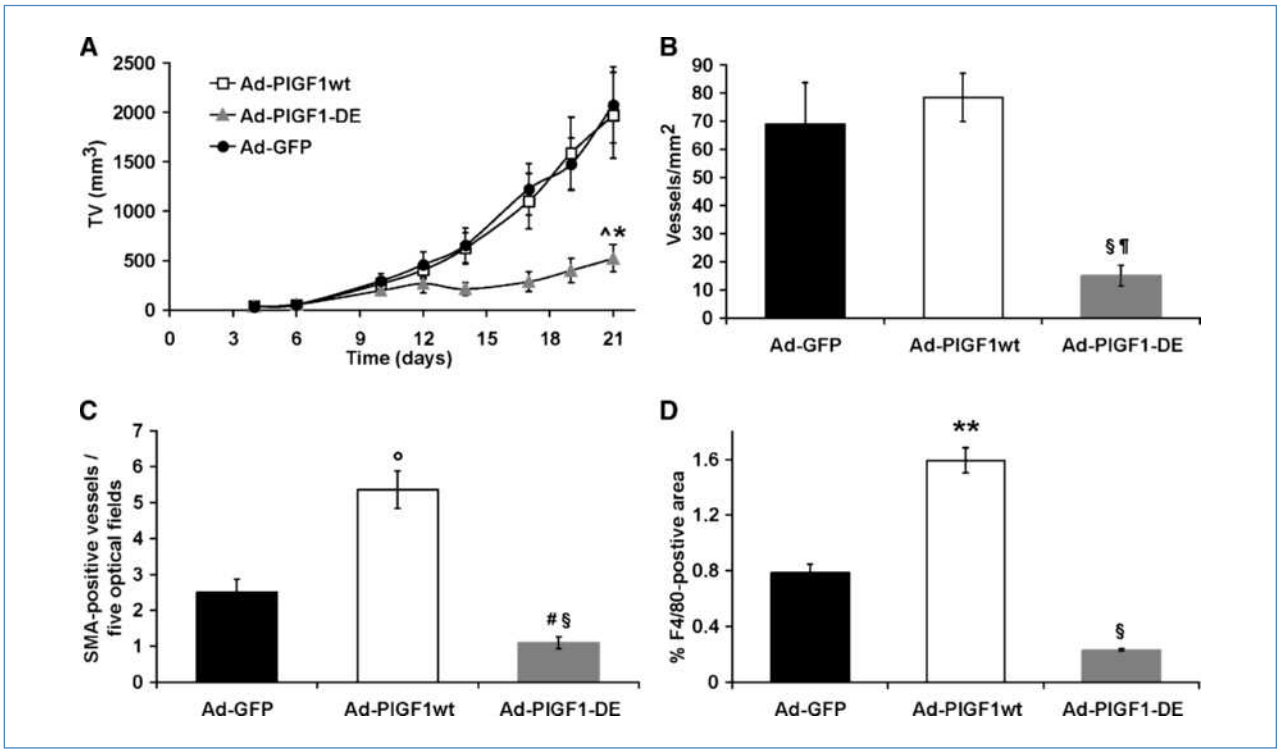

Figure 4. Ad-PlGF1-DE inhibits tumor growth and neovascularization. A, A2780 cells were s.c. injected into 8-wk-old CD1 nude athymic mice (n = 7 for group). At days 10 and 17, 5 × 10^7 pfu of each adenovirus was injected intratumorally. TV was measured every 3 d and data are represented as the mean ± SE. ^, P = 0.0024 versus Ad-GFP; *, P = 0.0076 versus Ad-PlGF1wt. 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-PlGF1wt. C, newly formed vessels covered by SMCs were evaluated counting SMA-positive vessels. #, P = 0.0053 versus Ad-GFP; §, P < 0.0001 versus Ad-PlGF1wt; ○, 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-PlGF1wt; **, P = 0.0001 versus Ad-GFP.

PlGF1-DE homodimer and PlGF1-DE/VEGF heterodimer. As result, two different human tumor cell lines derived from lung and ovarian carcinomas, stably transfected with PlGF1-DE, showed severe growth impairment when grafted *in vivo* compared with parental cells and with cells transfected with empty vector or PlGF1wt. Most importantly, in the xenograft model of ovarian carcinoma, similar results were obtained when the overexpression of PlGF1-DE started 10 days after tumor cell injection, following the intratumoral delivery of Ad-PlGF1-DE.

Conversely, the overexpression of PlGF1wt 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 PlGF1wt homodimer and the generation of VEGF/PlGF 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 PlGF1wt in a mouse tumor cell line (31) or human PlGF2wt in human tumor cell lines (32) per se was sufficient to inhibit xenograft tumor growth and neoangiogenesis, suggesting that wild-type PlGF homodimer and VEGF/PlGF heterodimer overexpressed by grafted tumor cells have no role in tumor development. Conversely, other reports indicated that overexpression of murine PlGF in glioma cells weakly stimulated and not inhibited the tumor growth and survival (33, 34). Recently, it has been reported that PlGF 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 PlGF homodimer and VEGF/PlGF 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 PlGF1wt in tumor growth (31), it was reported that the heterodimer was inactive in corneal pocket assay and that it

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did not affect cultured PAE cells in different assays, but this effect was only evaluated after transfection of VEGFR-2 without VEGFR-1, and here, we have definitively confirmed that the presence of VEGFR-1 is absolutely required for heterodimer activity.

Our data strongly support the view that PlGF homodimer and VEGF/PlGF heterodimer are functional active molecules in angiogenesis process. In fact, despite that stable transfected or adenovirus-infected tumors overexpressing PlGF1-DE or PlGF1wt produced similar amounts of VEGF, they showed important and significant differences.

The volume of tumors generated with two different transfected tumor cell lines overexpressing PlGF1wt was significantly higher compared with the tumors overexpressing PlGF1-DE variant, and the same was observed comparing Ad-PlGF1wt–transduced and Ad-PlGF1-DE A2780–transduced tumors. Tumors overexpressing PlGF1wt showed a significant increase in terms of vessel density compared with PlGF1-DE–overexpressing tumors, 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 reduction of VEGF in A2780-PlGF1-DE tumors determined a significant 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 angiogenesis (37–39) and with data on tumor angiogenesis obtained in *Plgf*$^{-/-}$ 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–overexpressing tumors, whereas it is strongly and significantly increased in PlGF1-overexpressing tumors, confirming the crucial role of both VEGF and PlGF factors in the recruitment of monocyte-macrophage. Once again, these data agree with the analysis of *Plgf*$^{-/-}$ 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 heterodimer corresponding to about twice the VEGF reduction observed, 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 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 delivery, 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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Cancer Research

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

In this article (Cancer Res 2010;70:1804–13), which was published in the March 1, 2010 issue of *Cancer Research* (1), the affiliation of Dr. Lucio Pastore is incorrect; the correct affiliation is CEINGE-Biotecnologie Avanzate, Naples, Italy.

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1. Tarallo V, Vesci L, Capasso O, Esposito MT, Riccioni T, Pastore L, Orlandi A, Pisano C, De Falco S. A placental growth factor variant unable to recognize vascular endothelial growth factor (VEGF) receptor-1 inhibits VEGF-dependent tumor angiogenesis via heterodimerization. Cancer Res 2010;70:1804–13.

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