Placenta Growth Factor-1 antagonizes VEGF-induced angiogenesis and tumor growth by the formation of functionally inactive PlGF-1/VEGF heterodimers

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Summary

Tumor growth and metastasis require concomitant growth of new blood vessels, which are stimulated by angiogenic factors, including vascular endothelial growth factor (VEGF), secreted by most tumors. Whereas the angiogenic property and molecular mechanisms of VEGF have been well studied, the biological function of its related homolog, placenta growth factor (PlGF), is poorly understood. Here we demonstrate that PlGF-1, an alternatively spliced isoform of the PlGF gene, antagonizes VEGF-induced angiogenesis when both factors are coexpressed in murine fibrosarcoma cells. Overexpression of PlGF-1 in VEGF-producing tumor cells results in the formation of PlGF-1/VEGF heterodimers and depletion of the majority of mouse VEGF homodimers. The heterodimeric form of PlGF-1/VEGF lacks the ability to induce angiogenesis in vitro and in vivo. Similarly, PlGF-1/VEGF fails to activate the VEGFR-2-mediated signaling pathways. Further, PlGF-1 inhibits the growth of a murine fibrosarcoma by approximately 90% when PlGF-1-expressing tumor cells are implanted in syngeneic mice. In contrast, overexpression of human VEGF in murine tumor cells causes accelerated and exponential growth of primary fibrosarcomas and early hepatic metastases. Our data demonstrate that PlGF-1, a member of the VEGF family, acts as a natural antagonist of VEGF when both factors are synthesized in the same population of cells. The underlying mechanism is due to the formation of functionally inactive heterodimers.

Introduction

Tumor growth and metastasis are dependent on the degree of neovascularization in the tumor bed (Carmeliet and Jain, 2000; Folkman, 1995; Hanahan and Folkman, 1996). Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is a key angiogenic factor, frequently utilized by tumors and other tissues to switch on blood vessel growth (Benjamin and Keshet, 1997; Dvorak, 2000; Ferrara and Alitalo, 1999; Senger et al., 1983; Yancopoulos et al., 2000). In addition to pathological angiogenesis, VEGF is an essential factor that contributes to the development of the vascular system by stimulating vasculogenesis and angiogenesis during embryonic development (Carmeliet et al., 1996; Ferrara et al., 1996).

The VEGF family is comprised of five structurally related members, including VEGF-A, placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (Eriksson and Alitalo, 1999). The biological functions of the VEGF family are mediated by activation of three structurally homologous tyrosine kinase receptors, VEGFR-1, VEGFR-2, and VEGFR-3 (Cao et al., 1998).

SIGNIFICANCE

Although VEGF is one of the most frequently expressed angiogenic factors found in tumors, PlGF-1, as an alternatively spliced isoform of the PlGF gene and structurally related to VEGF, does not seem to induce angiogenesis. In contrast, PlGF-2, a heparin binding isoform of PlGF, is able to induce angiogenesis. Some previous studies suggested that PlGF-1 might potentiate VEGF-induced angiogenesis by competing with VEGF for the decoy VEGFR-1 receptor, thus making more VEGF molecules available for interaction with VEGFR-2, which transduces the angiogenic signals. Our present work provides evidence that PlGF-1 acts as a natural antagonist of VEGF when both factors are coexpressed in the same population of cells. The underlying antagonistic mechanism is due to the formation of functionally inactive PlGF-1/VEGF heterodimers. Our results demonstrate that other members of the VEGF family can antagonize VEGF-induced angiogenesis. Taken together with work by others, we propose a novel mechanistic principle according to which PlGF-1 plays dual roles in regulation of VEGF-induced angiogenesis in both positive and negative manners, depending upon the temporal and spatial relations of expression of these two factors. Our work also has conceptual implications for gene therapy in cancer treatment by targeting PlGF-1 to tumors.

VEGF and PlGF-2 also bind to a nontyrosine kinase receptor, neuropilin-1 (Migdal et al., 1998; Soker et al., 1998). However, the biological signals mediated by neuropilin-1 in endothelial cells are not known. According to their receptor binding patterns and angiogenic features, the members of the VEGF family can be further divided into three subgroups: (1) VEGF, which binds to VEGFR-1 and VEGFR-2, and induces vasculogenesis, angiogenesis, and vascular permeability; (2) PlGF and VEGF-B, which bind only to VEGFR-1, with unknown physiological and pathological functions; and (3) VEGF-C and VEGF-D, which interact with both VEGFR-2 and VEGFR-3, and induce both blood angiogenesis and lymphangiogenesis (Cao et al., 1998; Makinen et al., 2001; Marconcini et al., 1999; Skobe et al., 2001; Stacker et al., 2001). Accumulating evidence has suggested that VEGFR-2, in response to VEGF, mediates angiogenic signals for blood vessel growth, and VEGFR-3 transduces signals for lymphatic vessel growth (Dvorak, 2000; Ferrara and Alitalo, 1999). The function of VEGFR-1 is poorly understood. While some studies suggest a direct role in transducing angiogenic signals, others report that VEGFR-1 may act as a decoy receptor for VEGF/VEGFR-2 signaling (Hiratsuka et al., 2001; Landgren et al., 1998; Zeng et al., 2001). Similar to VEGF, PlGF can be generated as at least three alternatively spliced isoforms of the same gene: PlGF-1, PlGF-2, and PlGF-3 (Cao et al., 1997; Maglione et al., 1991; Maglione et al., 1993).

Similar to the platelet-derived growth factor (PDGF) family, all members in the VEGF family naturally exist as dimeric proteins in order to interact with their specific receptors. In addition to homodimers, PlGF and VEGF-B can form heterodimers with VEGF when these factors are produced in the same cell (Cao et al., 1996a; DiSalvo et al., 1995; Olofsson et al., 1996). We have previously reported that PlGF-1 preferentially forms heterodimers with VEGF intracellularly (Cao et al., 1996a, 1996b). PlGF-1/VEGF heterodimers are naturally present in tissues when both factors are synthesized in the same population of cells (Cao et al., 1996a, 1996b; DiSalvo et al., 1995). Our previous results and those of others showed that PlGF-1/VEGF heterodimers, unlike VEGF homodimers, exhibit only a weak stimulation of endothelial cells, suggesting that PlGF-1 might act as a negative regulator for VEGF (Cao et al., 1996a; Kurz et al., 1998). To further test this hypothesis we have studied the role of PlGF-1 in primary tumor growth and metastasis of a murine fibrosarcoma.

Results

Generation of PlGF/VEGF heterodimers

To generate heterodimeric forms of PlGF/VEGF, we expressed recombinant human PlGF-1 and $VEGF_{165}$ monomers with different molecular weights. Using protocols described in the Experimental Procedures, recombinant PlGF-1 and $VEGF_{165}$ monomers purified from bacterial lysates could be readily dimerized into a mixture of homodimers and heterodimers of PlGF-1/PlGF-1, $VEGF_{165}$ /$VEGF_{165}$, and PlGF-1/ $VEGF_{165}$ after removal of the reducing reagent, DTT. Each of these dimers was highly purified to homogeneity by affinity chromatography (Figure 1A). These dimeric proteins were converted to monomers under reducing conditions as analyzed by electrophoresis (Figure 1A).

PlGF-1/VEGF heterodimers lack angiogenic activity

To test their in vivo angiogenic features, we analyzed all three dimeric factors in the mouse corneal angiogenesis model (Cao et al., 1998). As expected, VEGF homodimers induced a robust corneal angiogenic response with a high number of microvessels forming a primitive vascular network at the leading edge (Figure 1C). In contrast, the same amount of PlGF-1 homodimers

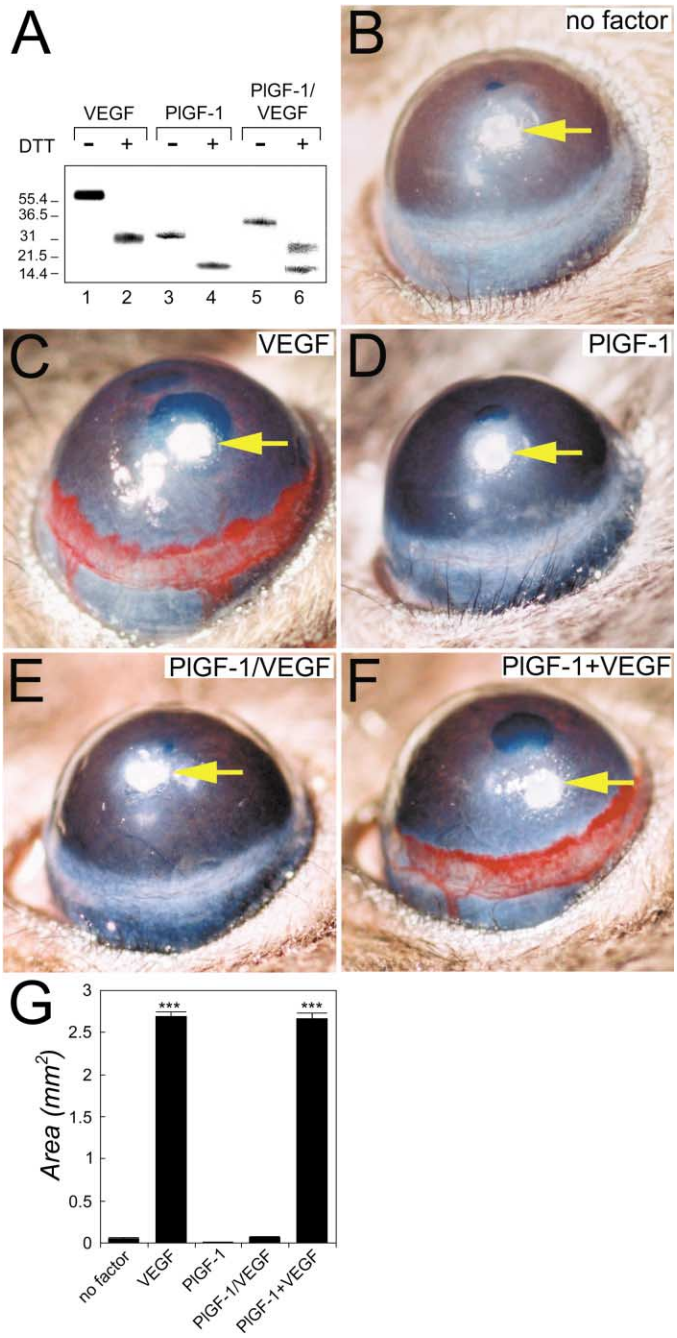

Figure 1. Corneal angiogenesis

A: VEGF, PlGF-1, and PlGF-1/VEGF were purified to homogeneity and analyzed under reducing (lanes 2, 4, and 6) and nonreducing (lanes 1, 3, and 5) conditions.

B–F: 160 ng of VEGF (**C**), PlGF-1 (**D**), PlGF-1/VEGF (**E**), or PlGF-1 (160 ng) plus VEGF (160 ng) (**F**) were implanted into corneas of C57BL/6. Polymers without growth factors were used as negative controls (**B**). Arrows point to the implanted pellets.

G: Areas of neovascularization were measured according to protocols described in the Experimental procedures.

or PlGF-1/VEGF heterodimers failed to stimulate corneal angiogenesis (Figures 1D and 1E). PlGF-1 did not seem to interfere with VEGF-induced neovascularization when both PlGF and VEGF homodimers were coimplanted in mouse corneas (Figure 1F). Quantification of corneal neovascularization showed that PlGF-1 and PlGF-1/VEGF lacked detectable angiogenic responses in this in vivo assay (Figure 1G).

Formation of PlGF-1/VEGF heterodimers in tumor cells

Because PlGF-1 forms functionally defective heterodimers with VEGF in vitro, we hypothesized that overexpression of PlGF-1 could lead VEGF molecules to form PlGF-1/VEGF heterodimers in tumor cells and thus antagonize the angiogenic activity of VEGF produced by tumors. To test this hypothesis, we expressed hPlGF-1 to a high level using a retroviral vector in a well-characterized murine fibrosarcoma with a VEGF-dependent in vivo growth (Kuo et al., 2001). The presence of hPlGF-1 and hVEGF cDNAs was confirmed by Southern blot analysis. Overexpression of hVEGF in murine fibrosarcoma cells resulted in the formation of hVEGF/hVEGF homodimers and hVEGF/mVEGF heterodimers that were coprecipitated by an antibody specific to hVEGF (Figure 2B, lane 2). The different forms of VEGF dimers were converted to monomers under reducing conditions (Figure 2B, lane 3). In addition to the $mVEGF_{164}$ isoform, $mVEGF_{120}$ also participated in the formation of heterodimers with $hVEGF_{165}$. Similarly, high levels of hPlGF-1 expression resulted in the formation of hPlGF-1/hPlGF-1 homodimers and hPlGF-1/mVEGF heterodimers as detected in complexes precipitated by a specific anti-hPlGF-1 antibody (Figure 2A). These dimers were reduced to monomers in the presence of a reducing agent (Figure 2A, lane 3).

To quantify the amounts of various dimeric molecules secreted by tumor cells, we performed a sensitive sandwich ELISA assay using specific antibodies against each factor (Table 1), either two antibodies against the same factor but raised in different species (homodimers), or two antibodies against different factors (heterodimers). As expected, a high level of mVEGF homodimers (2430 pg/ml) was detected in conditioned medium from wt T241 tumor cells. The majority of mPlGF produced by wt T241 was involved in heterodimerization with mVEGF, suggesting that mPlGF preferentially formed heterodimers with mVEGF, rather than mPlGF/mPlGF homodimers. Only low levels of mVEGF homodimers (770 pg/ml) were found in the conditioned media of hPlGF-T241-1 and hPlGF-T241-2 cells grown at the same conditions. In contrast, virtually all mVEGF molecules were present as hPlGF-1/mVEGF heterodimers in PlGF-1-overexpressing hPlGF-T241-1 (7,500 pg/ml) and hPlGF-T241-2 tumor cells (73,000 pg/ml) (Table 1). Although different levels of hPlGF were produced in hPlGF-T241-1 and hPlGF-T241-2 tumor cells, both these high levels efficiently depleted mVEGF homodimers.

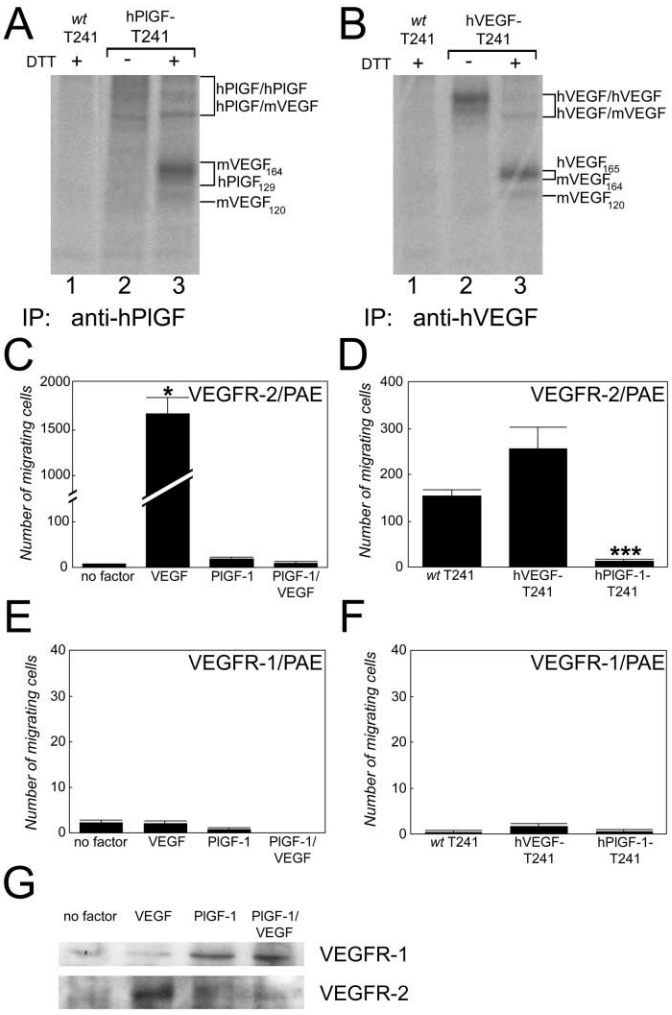

Figure 2. Dimer formation, chemotactic activity, and receptor phosphorylations

Radiolabeled complexes of hPlGF-1 homodimers and hPlGF-1/mVEGF heterodimers produced by tumor cells were immunoprecipitated by an anti-hPlGF-1 antibody (**A**). Immunocomplexes of hVEGF homodimers and hVEGF/mVEGF heterodimers were precipitated with an anti-hVEGF antibody (**B**). The dimeric (lane 2) and monomeric (lane 3) forms of growth factors were analyzed under nonreducing and reducing conditions. Conditioned medium (CM) from wt T241 cells was used as negative control (lane 1). Pure growth factors (**C** and **E**) or conditioned media (**D** and **E**) derived from wt T241, hVEGF-T241, and hPlGF-1-T241 cells were added to the lower chamber, and VEGFR-2/PAE (**C** and **D**) or VEGFR-1/PAE (**E** and **F**) cells were seeded in the upper chamber. Migrating cells were counted per optic field (32×). Data represent means (±SEM) of a triplicate of each sample. **G:** In response to growth factors, cell lysates from VEGFR-1/PAE and VEGFR-2/PAE were blotted onto a nitrocellulose membrane and detected with an anti-phospho-tyrosine antibody.

Lack of stimulatory activity on endothelial cells by PlGF-1-overexpressing cells in vitro

To study the chemotactic activity, we tested the effect of conditioned media derived from various transduced and nontransduced cells in a modified Boyden chemotaxis assay. As expected, VEGF elicited a strong chemotactic response on VEGFR-2-expressing PAE cells (Figure 2C). Similarly, conditioned medium from wt T241 cells significantly stimulated VEGFR-2/PAE cell migration (Figure 2D). High expression levels of hVEGF enhanced the chemotactic activity produced by T241 cells. In contrast, both PlGF-1 homodimers and PlGF-1/VEGF heterodimers lacked such a chemotactic effect on these endothelial cells, and expression of hPlGF-1 completely abolished the chemotactic effect produced by T241 cells (Figure 2D). None of these factors or conditioned media induced VEGFR-1/PAE cell motility (Figures 2E and 2F). Similar results were obtained with primary HUVE cells (data not shown).

Table 1. Quantification of dimeric factors secreted by tumor cells

Growth factors	wt T241	hVEGF-T241	hPlGF-T241-1	hPlGF-T241-2
	(pg/ml)	(pg/ml)	(pg/ml)	(pg/ml)
mVEGF/mVEGF	2430	2000	770	770
mPlGF-1/mPlGF-1	630	80	700	400
mPlGF-1/mVEGF	2400	140	370	230
hPlGF-1/hPlGF-1	ND	ND	7500	73000
hPlGF-1/mVEGF	ND	ND	6500	23000
hVEGF/hVEGF	ND	567000	ND	ND

ND = Not detectable

Addition of hVEGF homodimers at the concentration of 50 ng/ml to VEGFR-2/PAE cells induced a spindle-like cell shape with reorganization of actin fibers (Figure 3E), a feature that both PlGF-1 homodimers and PlGF-1/VEGF heterodimers lack (Figures 3I and 3M). Furthermore, VEGFR-1-expressing PAE cells did not respond to any of these three factor treatments (Figures 3F, 3J, and 3M). Incubation with conditioned medium from wt T241 cells resulted in an elongated spindle-like cell shape in VEGFR-2/PAE cells, similar to that induced by hVEGF (Figure 3C), confirming that T241 cells secrete high levels of mVEGF. This effect was completely abrogated after expression of hPlGF-1, indicating that a majority of the mVEGF molecules participated in formation of heterodimers with hPlGF-1 (Figure 3K). In contrast, overexpression of hVEGF in T241 cells led to remarkable cell shape changes and actin reorganization (Figure 3G). Again, conditioned media from all cell lines failed to induce a similar change in endothelial morphology of VEGFR-1/PAE cells (Figures 3D, 3H, and 3L).

Lack of activation of the VEGFR-2-mediated signaling pathway

Consistent with previous findings (Ferrara and Alitalo, 1999), VEGFR-2 became heavily phosphorylated upon VEGF stimulation (Figure 2G). In contrast, PlGF-1 and PlGF-1/VEGF were unable to induce phosphorylation of VEGFR-2 under the same conditions. However, they efficiently activated phosphorylation of VEGFR-1 (Figure 2G). To quantify the levels of intracellular inositol phosphates (InsPs), one of the mediators for VEGFR-2 signal transduction, a sensitive radiolabeling method was used. As expected, VEGF produced increases of InsP1, InsP2, and InsP3 production in VEGFR-2/PAE cells (Table 2). In contrast, neither PlGF-1, PlGF-1/VEGF, nor conditioned media of PlGF-1 overexpressing tumor cell lines stimulated significantly in-

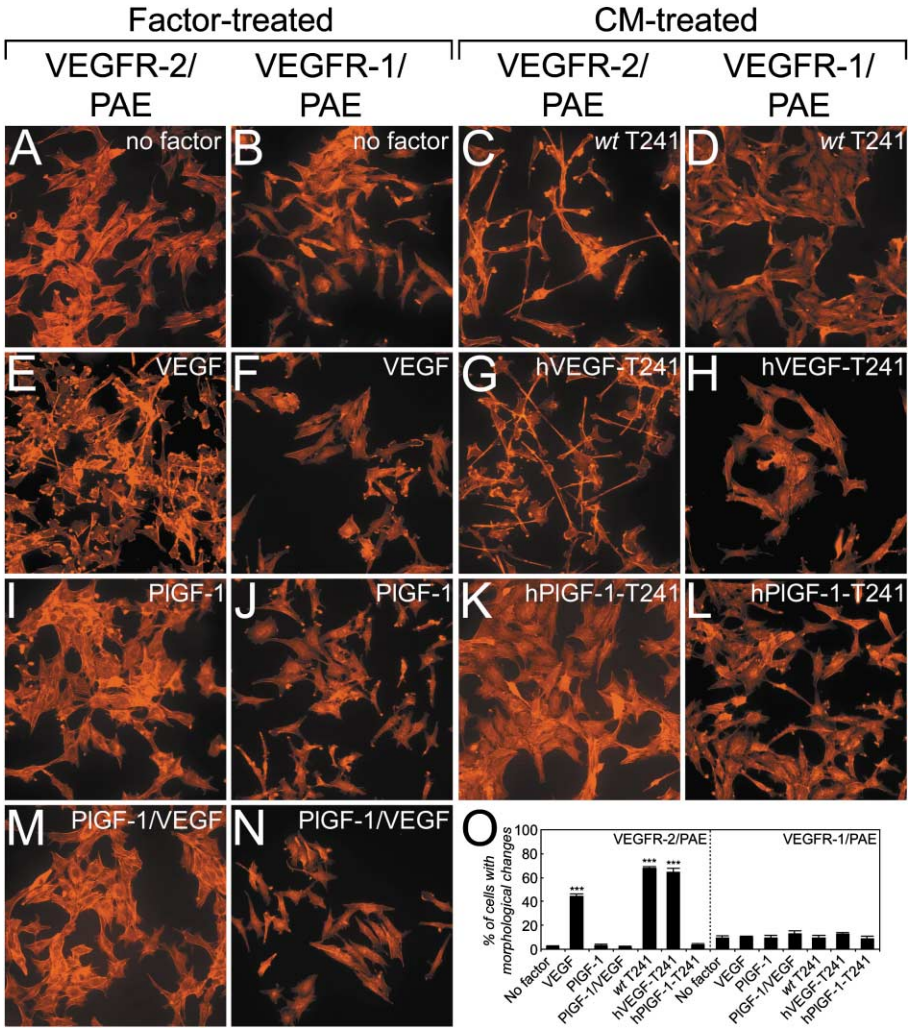

Figure 3. Endothelial cell shape changes and actin reorganization

Pure hVEGF (**E** and **F**), hPlGF-1 (**I** and **J**), PlGF-1/VEGF (**M** and **N**) or CM derived from wt T241 (**C** and **D**), hVEGF-T241 (**G** and **H**), and hPlGF-1-T421-1 (**K** and **L**) cells were added to approximately 50%–60% confluent VEGFR-2/PAE cells (**A, C, E, G, I, K,** and **M**) or VEGFR-1/PAE (**B, D, F, H, J, L,** and **N**) for 16 hr. In response to VEGF (**E**), wt T241 CM (**C**), and hVEGF-T241 CM (**G**), PAE/VEGFR-2 cells underwent dramatic spindle-like shape changes. Incubation of VEGFR-2/PAE cells without growth factors served as a negative control (**A**). VEGFR-1/PAE did not show any morphological changes in response to these stimuli (**B, D, F, H, J, L,** and **N**). **O:** Quantification of spindle-like cells.

Table 2. Analysis of inositol phosphate production

Cell line	Treatment	InsP1 (cpm)	InsP2 (cpm)	InsP3 (cpm)
VEGFR-2/PAE	No factor	1421	518	84
	VEGF	7868	1414	336
	PlGF-1	1913	331	23
	PlGF-1/VEGF	2019	692	142
	hVEGF-T241	4724	1155	402
	hPlGF-T241-1	1568	316	69
	hPlGF-T241-2	1517	335	74
VEGFR-1/PAE	No factor	261	179	77
	VEGF	371	173	56
	PlGF-1	398	147	43
	PlGF-1/VEGF	355	199	27
	hVEGF-T241	509	181	18
	hPlGF-T241-1	276	183	20
	hPlGF-T241-2	409	203	10

creased levels of InsP1, InsP2, and InsP3 in these cells. None of these growth factors or conditioned media stimulated InsP synthesis in VEGFR-1/PAE cells.

Suppression of tumor growth

Overexpression of hPlGF-1 in T241 cells did not alter the growth rates of two independent tumor clones in culture (Figure 4A). The wt T241 tumor cells grew rapidly in vivo, and visible tumors were readily detectable 6 days after implantation (Figure 4B). Expression of hVEGF in these cells significantly accelerated tumor growth (Figure 4B). Tumors expressing hVEGF grew invasively into surrounding tissues and resulted in early visible metastases in other organs such as spine and liver. In contrast, expression of hPlGF-1 remarkably delayed tumor growth, and visible tumors were only detectable by day 12 after implantation (Figure 4B). These tumors remained at a similar small average size of about 70 mm^3 by day 16 after implantation (Figure 4B). At day 16 after tumor implantation, approximately 90% inhibition of tumor growth was scored in hPlGF-1-expressing tumors as compared with the wt T241 tumors (Figures 4B and 4D). Similarly, highly significant reductions of primary tumor volumes were found in both PlGF-1-expressing tumor clones (Figure 4C). Immunohistochemical studies showed that hPlGF-1-expressing tumors had significantly reduced neovascularization as compared with wt T241 tumors (Figures 5A, 5C, and 5D). In contrast, hVEGF-expressing tumors were highly vascularized with an average of more than 300 microvessels per optical field (×10) (Figures 5B and 5D). In addition to high vessel counts, some of the microvessels were dilated with a thin endothelial layer (Figure 5B). The invasive growth feature of hVEGF-expressing tumors led to early metastasis of tumor cells into other organs such as spine and liver. Biopsy examination revealed that 100% of hVEGF-T241 tumor bearing mice had hepatic metastases (Figure 5E). Both liver volume and weight of hVEGF-T241 tumor bearing mice were significantly larger than those of control animals (Figures 5E and 5F). Overproduction of VEGF by tumor cells not only resulted in hemorrhagic lesions in hepatic metastases (Figure 5H, arrowheads), but also caused destruction of regular hepatocyte sinusoidal organization as compared to liver structures of wt T241- or hPlGF-1-T241-tumor bearing mice (Figures 5G and 5I).

Discussion

The members of the VEGF family are considered to be key angiogenic mediators contributing to both physiological and pathological angiogenesis (Schratzberger et al., 2000). Our present study demonstrates that PlGF-1, a member of this family, could act as an antagonist of its prominent homolog, VEGF. The antagonistic effect of PlGF-1 in opposing VEGF requires coexpression of both factors in the same cells. Our results show that the molecular mechanism of the anti-VEGF effect by PlGF-1 is due to the formation of functionally inactive PlGF-1/VEGF heterodimers. These heterodimers fail to activate the key angio-

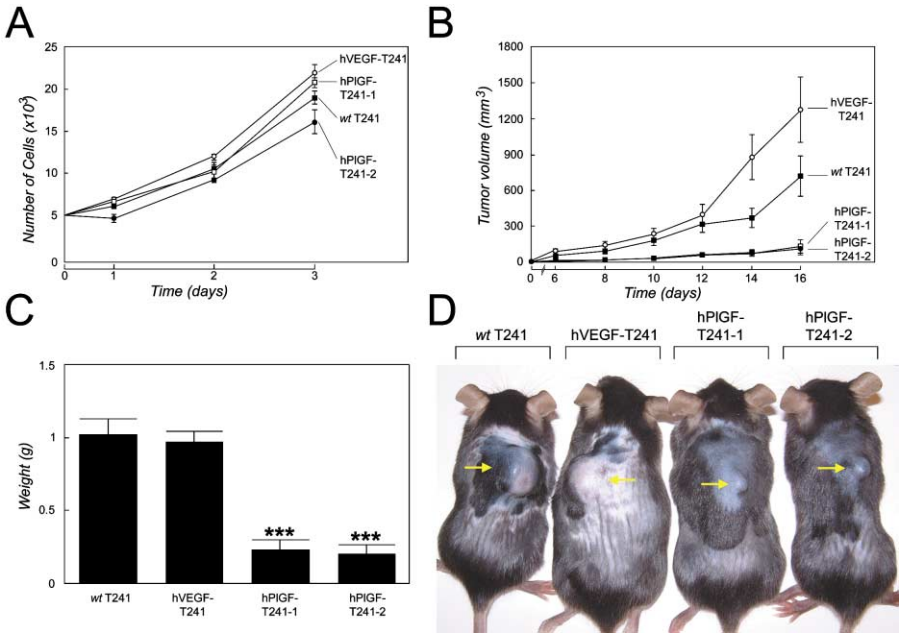

Figure 4. Suppression of tumor growth

A: Tumor cell growth rates. Monolayers of wt T241, hPlGF-T241-1, hPlGF-T241-2, and VEGF-T241 tumor cells growing in 24-well plates at the density of 5,000 cells/well were incubated for indicated time points and cell numbers were counted. **B:** Approximately 1 × 10^6 cells of wt T241, hVEGF-T241, hPlGF-T241-1, or hPlGF-T241-2 cells were subcutaneously injected into 6-week-old C57BL/6 male mice. Tumor sizes were measured every other day. At day 14 after tumor implantation, typical tumor appearance was photographed (**D**) and tumor burdens were weighed after necropsy at day 18 (**C**). Yellow arrows indicate the implanted tumors (**D**).

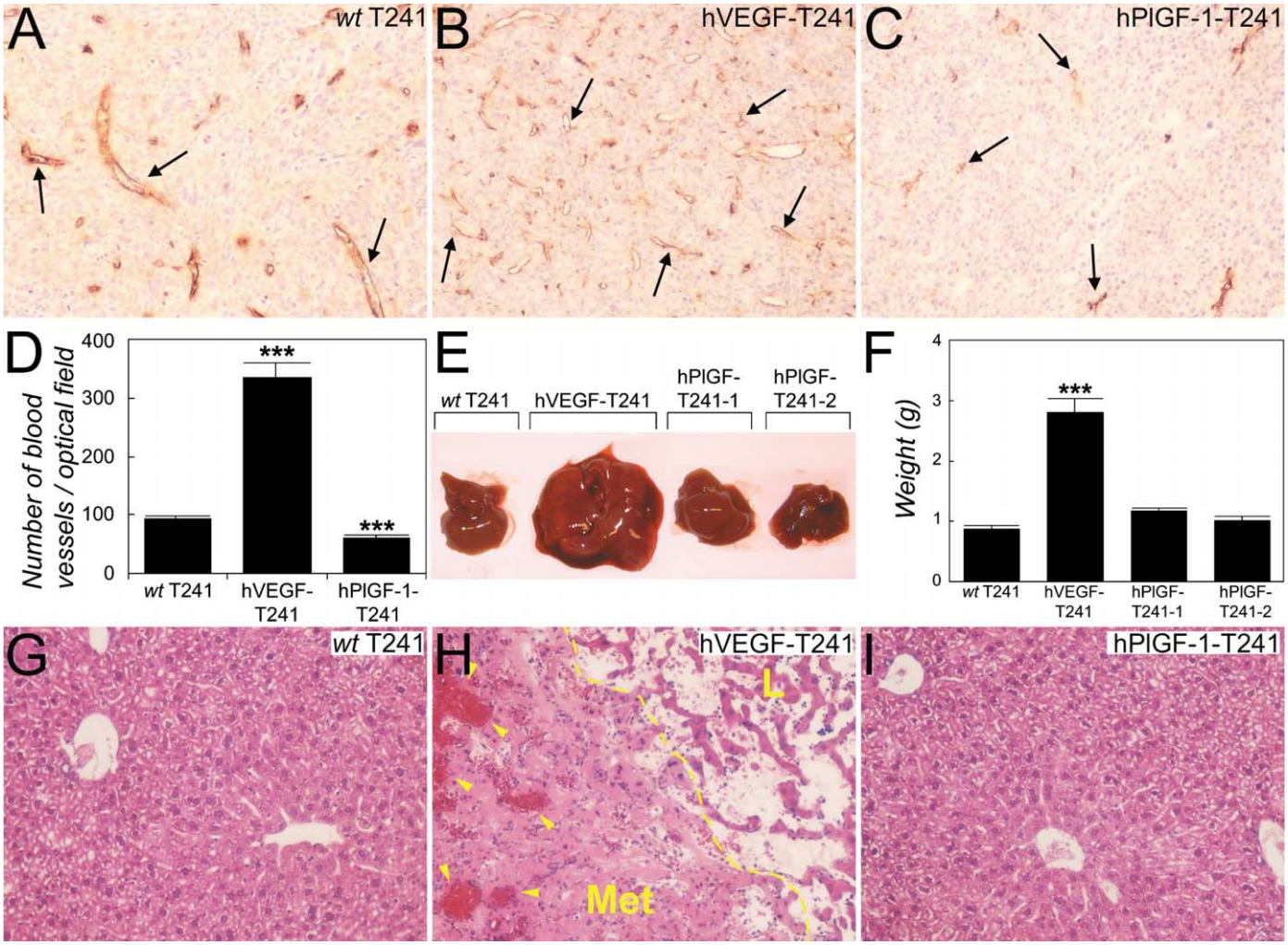

Figure 5. Immunohistochemical analysis of primary tumors and hepatic metastases

At day 18 after tumor implantation, primary tumors (**A**–**C**) were resected and stained with an anti-CD31 antibody. Arrows point to tumor microvessels (**A**–**C**). Microvessel density was counted under a light microscope in at least 5 random fields (**G**). ***, $P < 0.001$. Livers of each group were optically analyzed for their appearance (**E**). Large numbers of hepatic metastases were visible on the surface of all livers of hVEGF-T241-bearing mice (**E**). Liver burdens were weighed and mean values of each group (6 mice) were presented as ±SEM (**F**). ***, $P < 0.001$. Histological analysis of hepatic metastases was performed after staining with hemotoxylin-eosin at 10× magnification (**G–I**). Hepatic metastases in hVEGF-T241-implanted mice (**H**) are marked with a dashed line. Arrowheads (**H**) point to hemorrhagic microvessels in metastases. Met, metastasis; L, liver.

genic receptor of VEGF, VEGFR-2, and VEGFR-2-mediated signaling pathways. We further provide evidence showing that gene delivery of PlGF-1 to VEGF-producing tumors suppresses tumor growth and may have conceptual implications in the treatment of cancer and metastasis.

PlGF-1 was discovered more than 10 years ago as the second member of the VEGF family based on its structural homology with VEGF (Maglione et al., 1991, 1993; Persico et al., 1999; Schratzberger et al., 2000). Since the identification of this molecule, it has been speculated that PlGF-1 might act as an angiogenic factor (Maglione et al., 1991). Whereas a couple of studies suggest that it stimulates endothelial cell proliferation or migration, most other work demonstrates that PlGF-1 alone is insufficient to induce neovascularization (Cao et al., 1996a, 1996b; Carmeliet and Jain, 2000; Clauss et al., 1996; Park et al., 1994). VEGFR-1 is the only known signaling receptor for PlGF-1 (Kendall and Thomas, 1993; Park et al., 1994). Deletion of VEGFR-1 results in early embryonic lethality due to disorganization and inappropriate remodeling of the vascular tube structures (Fong et al., 1995). However, the defective phenotype of VEGFR-1 null mice shows that endothelial cell number is not affected, suggesting that VEGFR-1-mediated signals are not critical for endothelial cell proliferation and migration. Interestingly, removal of the tyrosine kinase domain of VEGFR-1 did not alter developmental angiogenesis (Hiratsuka et al., 1998). Therefore, VEGFR-1 may function as a decoy receptor by limiting the bioavailability of VEGF to bind to VEGFR-2. In support of this hypothesis, some reports show that administration of PlGF-1 protein in cell culture and animal models leads to potentiation of VEGF-induced angiogenesis in vitro and in vivo, possibly by competing with VEGF for the binding site of VEGFR-1 (Carmeliet et al., 2001; Park et al., 1994). However, the VEGF recep-

tor-mediated signals might be more complex because VEGFR-1 and VEGFR-2 form both homodimers and heterodimers (Huang et al., 2001). It is not known if VEGFR-1/VEGFR-2 heterodimers mediate angiogenic responses, and if so, which ligands trigger angiogenesis.

Within the PlGF family, at least three PlGF isoforms (i.e., PlGF-1, PlGF-2, and PlGF-3) have been identified, all generated by alternative splicing of the same gene (Cao et al., 1997; Maglione et al., 1991, 1993). It appears that PlGF-2, a heparin binding isoform, possesses additional biochemical and biological properties, such as binding to the neuropilin-1 receptor (Migdal et al., 1998). PlGF-1 is less active than PlGF-2 in stimulation of endothelial cell growth and angiogenesis (Sawano et al., 1996; Ziche et al., 1997). Thus, it is possible that additional biological activities of PlGF-2, but not PlGF-1, including stimulation of angiogenesis, could be mediated by the neuropilin-1 receptor. The fact that activation of neuropilin-1 potentiates angiogenic responses mediated by VEGF tyrosine kinase receptors supports this finding (Soker et al., 1998). Thus, it is not surprising that transfection of the PlGF-2 gene in tumor cells does not significantly alter tumor growth (Hiratsuka et al., 2001). It is possible that PlGF-1, but not PlGF-2, is a natural antagonist for VEGF when produced as a heterodimer with VEGF. Thus, regulation of alternative splicing of PlGF isoforms in various tissues could possibly act as an operator in the switch of VEGF-induced angiogenesis.

A recent study suggests that PlGF-1 produced by tumor and host cells may positively contribute to angiogenesis and tumor growth (Carmeliet et al., 2001). However, these data do not seem to conflict with our current observation. In that report, the level of PlGF-1 expression in the tumors was several times lower than that of VEGF. It is not known whether all VEGF molecules expressed in that tumor participate in heterodimerization with PlGF-1. In addition, PlGF-1 expressed in host cells cannot form heterodimers with VEGF produced by the tumor, and may thus instead potentiate VEGF-induced tumor angiogenesis. Our present work demonstrates that high levels of PlGF-1 expression in VEGF-producing tumors force VEGF to form functionally inactive heterodimers and thus neutralize VEGF activity. We should emphasize that PlGF-1 antagonizes VEGF-induced angiogenesis only if both factors are simultaneously synthesized in the same population of cells. Therefore, PlGF-1, as shown in Figure 6, may play dual roles in regulation of VEGF-induced angiogenesis and tumor growth. When these two factors are produced in different cell populations, PlGF-1 competes with VEGF for VEGFR-1, increasing the bioavailability of VEGF, thus allowing excess of VEGF molecules to bind VEGFR-2, which triggers angiogenic responses. On the other hand, PlGF-1 acts as an antagonist for VEGF by formation of biologically inactive PlGF-1/VEGF heterodimers and depletion of VEGF homodimers when both factors are coexpressed in the same cell populations.

Unlike VEGF, the expression pattern of PlGF-1 under physiological conditions is limited to only a few tissues in adult mammals. Placenta is perhaps the only tissue that expresses PlGF-1 at extremely high levels. VEGF has been detected at high levels in the placenta as well. However, a recent study indicates that mRNAs for VEGF and PlGF-1 are not colocalized, and it is thus unlikely that there is any significant production of PlGF-1/VEGF heterodimers in the placenta (Clark et al., 1998). Accordingly,

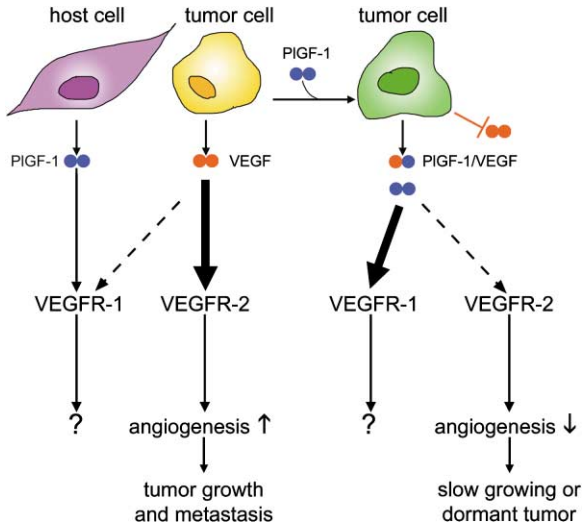

Figure 6. Schematic representation of dual roles of PlGF-1 in regulation of VEGF-induced angiogenesis

When PlGF-1 and VEGF are produced in different cells, PlGF-1 enhances the VEGF-stimulated angiogenesis. In contrast, PlGF-1 antagonizes the angiogenic activity of VEGF when both factors are coexpressed in the same population of cells.

PlGF-1 could potentiate, rather than inhibit, VEGF-induced angiogenesis in the placenta, as shown in Figure 6.

Our present findings are similar to the functional properties of the angiopoietin family, in which Ang-2 acts as a natural antagonist of Ang-1 (Maisonpierre et al., 1997). However, the antagonistic mechanism of Ang-2 is different from that of PlGF-1 in such a way that Ang-2 competes with Ang-1 for binding to the Tie-2 receptor, whereas PlGF-1 forms functionally inactive heterodimers with VEGF. Our findings provide an example of a growth factor acting as an antagonist of its structurally related analog. Our present work not only increases the understanding of the complex functions of the VEGF family, but also provides a novel conceptual implication of PlGF-1 as an antagonist in the treatment of VEGF-dependent diseases.

Experimental procedures

Animals

Male 6- to 7-week-old C57BL/6 mice were acclimated and caged in groups of six or less. Animals were anesthetized by an injection of a mixture of dormicum and hypnorm (1:1) before all procedures and killed with a lethal dose of CO_2. All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Board.

Generation and purification PlGF-1/$VEGF_{165}$ heterodimers

Recombinant human PlGF-1 and $VEGF_{165}$ monomers were expressed in *E. Coli* as previously described (Maisonpierre et al., 1997). An equimolar of mixture of PlGF-1 and $VEGF_{165}$ at a total protein concentration of 0.5 mg/ml was incubated in 20 mM Tris-HCl (pH 8.0), 6 M guanidine-HCl, and 10 mM DTT. After overnight incubation at 4°C, the protein solution was dialyzed overnight at 4°C against 10 volumes of 2 M urea, 2 mM GSH (glutathione-SH), and 0.5 mM GSSG (glutathione-S-S-glutathione) in 20 mM Tris-HCl (pH 8.0). Using this refolding protocol, a mixture of homodimeric PlGF-1 and $VEGF_{165}$ as well as heterodimeric PlGF-1/VEGF was generated.

The homodimeric and the heterodimeric proteins were separated from one another by affinity chromatography using a goat polyclonal anti-VEGF antibody affinity column and a polyclonal goat anti-PlGF antibody affinity

column. The protein solution was applied at a flow rate of 2 ml/min initially on the anti-VEGF antibody-affinity column equilibrated with PBS. The column was then washed at the same flow rate with PBS until the absorbance reading at 280 nm reached baseline level. VEGF homodimers and PlGF-1/VEGF heterodimers, but not PlGF homodimers, were retained by the column and eluted with 0.1 M sodium citrate, 0.3 M NaCl (elution buffer). The VEGF homodimers and PlGF-1/VEGF heterodimers eluted from the anti-VEGF column were dialyzed against 20 volumes of PBS at 4°C for 4 hr and applied to the anti-PlGF-affinity column under the same conditions. The heterodimer was then eluted by the same elution buffer. The purified PlGF-1, VEGF, and PlGF-1/VEGF proteins were finally dialyzed against PBS and analyzed by SDS-PAGE, followed by measurement of protein concentrations.

Retroviral vector design and tumor cell transduction

Complementary cDNAs coding for human PlGF-1_{129} and $VEGF_{165}$ were cloned into the Murine Stem Cell Virus (MSCV) vector (kindly provided by Dr. R. Hawley at the Holland Laboratory, Rockville, MD) containing EGFP. Retroviral supernatants were generated by transfecting retroviral constructs into 293T cells along with expression plasmids encoding ecotropic gag/pol and the Vesicular Stomatitis Virus-Glycoprotein (VSV-G) envelope using a classical $CaPO_4$ transfection method (Pawliuk et al., 1994). Murine T241 fibrosarcoma cells grown in log phase were exposed to filtered viral supernatants in the presence of 8 μg/ml of protamine sulfate on RetronectinTM (Biowhittaker, East Rutherford, NJ) coated culture dishes for 6 hr on two consecutive days. EGFP positive cells were sorted using a FACStar+ (Becton Dickinson, San Jose, CA) equipped with a 5 W argon and 30 mW neon laser. PCR and Southern blot analyses were performed using standard methods (Pawliuk et al., 1994).

Tumor cell proliferation assay

wt T241, hVEGF-T241, and hPlGF-1-T241 cells were seeded at a density of 1×10^4 cells/well in 24-well plates in DME medium supplemented with 10% FCS and incubated at 37°C. Cells were trypsinized, resuspended in Isoton II solution (Beckman Coulter, Sweden), and counted in a Coulter Counter at various time points. All experiments were performed in triplicates.

Cell shape assay and actin staining

VEGFR-1/PAE and VEGFR-2/PAE cells were grown on coverslips in 12-well plates to about 40%–60% confluency in Ham's F12 medium supplemented with 10% FCS as previously described (Cao et al., 1998). The medium was removed and replaced with fresh Ham's F12 medium containing 2% FCS with or without 50 ng/ml of VEGF, PlGF-1, PlGF-1/VEGF, or 25% (v/v) of conditioned media. After 16 hr, cells were fixed with 3% paraformaldehyde (PFA) in PBS (pH 7.5) for 30 min, rinsed three times with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 15 min. The cells were then washed three times with PBS and stained for 30 min with 1 μg/ml of TRITC-phalloidin (Sigma) in PBS. After washing three times with PBS, the coverslips were mounted in a mixture of glycerol and PBS (9:1). Cells were examined in a combined light and fluorescence microscope, and spindle-like cells were counted in 3–4 optical fields (20×). Data represents mean % (±SEM)

Chemotaxis assay

The motility response of VEGFR-expressing PAE cells to various growth factors and conditioned media was assayed by a modified Boyden chamber technique as previously described using micropore nitrocellulose filters (8 μm thick, 8 μm pores) (Cao et al., 1998). Cells were trypsinized and resuspended at 0.8×10^6 cells/ml in serum-free medium containing 0.2% BSA. The cells (40,000 cells per well) were applicated to the upper chamber in medium with or without 50 ng/ml of VEGF, PlGF-1, PlGF-1/VEGF heterodimers, or 25% (v/v) conditioned media from different retrovirally transduced cells in the lower chamber. After 4 hr at 37°C, the Boyden chamber was disassembled and cells attached to the filter were fixed in methanol and stained with Giemsa solution. All experiments were performed in triplicate. The cells that had migrated through the filter were counted and plotted as number of migrating cells per optic field (×32).

Detection of phosphorylated VEGF receptors

VEGFR-1/PAE and VEGFR-2/PAE cells were grown to 70%–80% confluency in 60 mm dishes, starved for 16 hr in serum-free F12/Ham, and stimulated with 50 ng/ml VEGF, PlGF-1, or PlGF-1/VEGF for 8 min at 37°C. The cells were washed with ice cold PBS/0.1 mM NaV_3O_4 and lysed on ice in 200 μl lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% Na-deoxycholate, 0.5% Triton X-100, 1 mM PMSF, 1 ng/ml aprotinin/leupeptin, 0.1 mM NaV_3O_4). The proteins were blotted onto nitrocellulose membranes and blocked overnight in 5% BSA in TBS-T, and probed with a primary monoclonal mouse antibody against pTyr (PY20, Pharmingen, San Diego, CA), a secondary HRP-conjugated antibody against mIgG, and detected using the ECL+ system.

HPLC analysis of inositol phosphate production

For inositol phosphate measurements, cells were grown to confluency in culture dishes (6 cm^2) and incubated for 24 hr with 10 μCi/ml *myo*-[2-^{3}H]inositol in Krebs-Ringer bicarbonate medium containing 0.5 mM adenosine, 10 mM inosine, and 5 mM β-hydroxybutyrate. After incubation, cells were washed twice with KRB medium and cellular activity was stopped by adding 250 μl ice-cold 400 mM PCA. After centrifugation for 2 min at 1200 g, 250 μl of the supernatant from each tube was neutralized by addition of 47 μl ice-cold 2.5 M KOH + 0.5 M HEPES. Inositol phosphates were separated on a HPLC system using an anion-exchange column (mono Q HR 5/5, Pharmacia Biotech, Sweden) equilibrated with 10 mM NH_4COOH. The inositol phosphates were eluted by a 0%–100% gradient of 1.7 M NH_4COOH (pH 3.7).

Metabolic labeling

Cells growing to subconfluency in 25 cm^2 flasks were incubated with methionine- and serum-free DMEM for 45 min, followed by pulse-labeling with 50 μCi/ml of ^{35}S-methionine (Amersham-Pharmacia Biotech, Uppsala, Sweden) for 16 hr. The labeled medium was removed and subjected to immunoprecipitation with goat anti-human PlGF-1, goat anti-mouse VEGF, and mouse anti-human VEGF antibodies (R&D Systems Inc., Minneapolis, MN) for 1 hr at 4°C. The immunocomplexes were precipitated with Protein A-Sepharose or Protein G-Sepharose for 1 hr at 4°C. The immunoprecipitated materials were released by a SDS buffer and separated by a Nu-PAGE gel under reducing or nonreducing conditions.

ELISA assay

The sandwich ELISAs were performed using the Quantikine ELISA (R&D Systems) according to the manufacturer's instructions. Briefly, the standard mouse (m) VEGF and samples were added onto a 96-well microplate precoated with an affinity purified polyclonal antibody specific for mVEGF, which were detected by an enzyme-linked polyclonal antibody specific for mVEGF. Similarly, homodimers of mPlGF, hVEGF, and hPlGF were measured using the Quantikine M mPlGF ELISA kit, Quantikine hVEGF ELISA kit, and Quantikine hPlGF ELISA kit, respectively. These three kits contain specific monoclonal antibodies as captures.

The heterodimers were measured by crossmatching capture and detection antibodies using the same ELISA kits mentioned above. For mVEGF/mPlGF heterodimers, samples were added onto microplates precoated with anti-mVEGF. Enzyme-linked anti-mPlGF was then used to detect the mVEGF/mPlGF heterodimer. To calibrate the assay, recombinant mPlGF standard was analyzed simultaneously on the PlGF plate. No crossreactivity was observed from the homodimers. Likewise, mouse/human VEGF heterodimer was measured using microplates precoated with anti-hVEGF and anti-mVEGF conjugates. Recombinant mVEGF standards analyzed on the mVEGF plates were used for calibration. Approximately 1%–2% of crossreactivity was observed from each homodimer, and the results were corrected accordingly. mVEGF/hPlGF heterodimer was measured using microplates precoated with anti-mVEGF and the anti-hPlGF conjugate. Recombinant hPlGF standards analyzed on the hPlGF plates were used for calibration. Approximately 3% of crossreactivity were observed from the hPlGF homodimer, and the results were corrected accordingly.

Mouse corneal micropocket assay

The mouse corneal assay was performed according to procedures previously described (Cao et al., 1998, 1996b). Corneal micropockets were created with a modified von Graefe cataract knife in both eyes of each male 5- to 6-week-old C57BL/6 mouse. A micropellet (0.35 × 0.35 mm) of sucrose aluminum sulfate (Bukh Meditec, Copenhagen, Denmark) coated with hydron polymer type NCC (IFN Sciences, New Brunswick, NJ) containing 160 ng of PlGF-1, VEGF, or PlGF-1/VEGF was implanted into each corneal pocket.

The pellet was positioned 1.0–1.2 mm from the corneal limbus. After implantation, erythromycin/ophthalmic ointment was applied to each eye. Eyes were examined by a slit-lamp biomicroscope on day 5 after pellet implantation. Vessel length and clock hours of circumferential neovascularization were measured.

Tumor studies in mice

Wild-type, hPlGF-1-expressing (hPlGF-T241-1 and hPlGF-T241-2) and hVEGF-expressing (hVEGF-T241) murine T241 fibrosarcoma cells were used for tumor implantation studies in 6- to 7-week-old syngeneic C57BL/6 mice. Approximately 1×10^6 tumor cells were implanted subcutaneously in each mouse. Six to seven mice were used in the treated and control groups. Primary tumors were measured using digital calipers on the days indicated. Tumor volumes were calculated according to the formula: width^2 $\times$ length $\times$ 0.52 as previously reported (Cao et al., 1999).

Histology

At day 18 after implantation, mice were sacrificed, and tumors and livers were fixed in 3% PFA, dehydrated, and embedded in paraffin. Embedded samples were sectioned and 4 μm sections were immunostained using biotinylated monoclonal antibodies against CD31 (Pharmingen, San Diego, CA). Peroxidase activity was developed with diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA) and sections were counterstained using Mayer's hematoxylin and Surgipath eosin. The tyramide signal amplification (TSA) kit (NEN Life Science, Boston, MA) was used to enhance staining.

Statistical analysis

Statistical analysis was carried out using Student's two-tailed t test in Microsoft Excel. P-values below 0.05 (*) and 0.001 (***) were deemed significant and extremely significant, respectively.

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