Blockage of VEGF-Induced Angiogenesis by Preventing VEGF Secretion

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Abstract—Vascular endothelial growth factor (VEGF)/vascular permeability factor is one of the most frequently expressed angiogenic factors in several pathological tissues. Development of VEGF antagonists has become an important approach in the treatment of angiogenesis-dependent diseases. Here we describe a novel anti-VEGF strategy by preventing the secretion of VEGF. We utilize the fact that placenta growth factor (PlGF)-1, a member of the VEGF family lacking detectable angiogenic activity, preferentially forms intracellular heterodimers with VEGF in cells coexpressing both factors. We constructed a retroviral vector containing human PlGF-1 or VEGF with a C-terminal KDEL sequence, which is a mammalian retention signal for the endoplasmic reticulum. Transduction of murine Lewis lung carcinoma cells with the retro-hPlGF-1-KDEL construct almost completely abrogated tumor growth. Consistent with the dramatic antitumor effect, most mouse VEGF molecules remained as intracellular mVEGF/hPlGF-1 heterodimers, and only a negligible amount of mVEGF homodimers were secreted. As a result, in hPlGF-1-KDEL–expressing tumors, blood vessels remained at very low numbers and lacked branching and capillary networks. Gene transfer of a hVEGF-KDEL construct into tumor cells likewise produced a dramatic antitumor effect. Thus, our study provides a novel antiangiogenic approach by preventing the secretion of VEGF. (Circ Res. 2004;94:1443-1450.)

Key Words: PlGF-1 ■ VEGF ■ angiogenesis ■ tumor growth ■ endoplasmic reticulum

Vascular endothelial growth factor (VEGF) is a key angiogenic factor frequently used by tumors and other tissues to switch on their angiogenic phenotypes.1–4 In fact, nearly all tumors express VEGF at high levels. Recent studies show that VEGF-stimulated blood vessels are essential not only for primary tumor growth but also for metastasis.5 In addition to contributing to pathological angiogenesis, VEGF is an essential factor that contributes to formation of the circulatory system by stimulating vasculogenesis and angiogenesis during embryonic development.6,7 VEGF is the prototype of a growth factor family that contains at least 4 additional structurally related members, including placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D.8 The angiogenic signals triggered by members of the VEGF family are mainly mediated by activation of 2 structurally related homologous tyrosine kinase receptors, VEGFR-1 and VEGFR-2, both of which are expressed almost exclusively on endothelial cells.9 VEGF binds to VEGFR-1 and VEGFR-2 and induces vasculogenesis, angiogenesis, and vascular permeability. PlGF and VEGF-B only bind to VEGFR-1 with unknown physiological and pathological outcomes.9,10 However, some recent studies suggest that PlGF-2 may contribute to the differentiation of endothelial precursor cells.11 In addition to VEGFR-1 and VEGFR-2, a lymphatic endothelial cell–specific tyrosine kinase receptor, VEGFR-3, has been identified.12 VEGF-C and VEGF-D interact with VEGFR-2 and VEGFR-3 and induce blood angiogenesis and lymphangiogenesis.9,14–17 The function of VEGFR-1 is poorly understood. Some studies suggest a direct role for the receptor in transducing angiogenic signals, whereas others report that VEGFR-1 may act as a decoy receptor for VEGF/VEGFR-2 signaling.18–20 Recently, VEGFR-1 was found to play a critical role in recruiting stem cell–differentiated endothelial cells into newly formed blood vessels.5,12 Similar to VEGF, alternative splicing of human PlGF transcripts generates at least 3 isoforms of the mature PlGF protein, PlGF-1, PlGF-2, and PlGF-3.21–23 All members of the VEGF family exist naturally as dimeric proteins in order to interact with their specific receptors. Based on their expression patterns, homodimers and heterodimers with distinct biological activities are formed. We have previously reported that PlGF-1 preferentially forms heterodimers intracellularly with VEGF,24,25 PlGF-1/VEGF heterodimers are present by nature in tissues when both factors are produced in the same cell.24,25 Various approaches have been developed as therapeutic strategies to block VEGF function. Consequently, anti-VEGF reagents, including VEGF neutralizing antibodies, VEGF antisense oligonucleotides, soluble VEGF receptors, anti-VEGF receptor antibodies, and intracellular signaling inhibitors, have produced promising antitumor effects in animal models.26–29 However, early clinical evaluation of these...
anti-VEGF compounds has presented varying results. Very recently, a humanized anti-VEGF antibody generated positive results in patients in randomized, double-blinded, and placebo trials. This early clinical study indicates that anti-VEGF agents are important for the treatment of angiogenesis-dependent diseases. The approaches used today are mainly based on the development and administration of functional recombinant protein antagonists that either neutralize the extracellular VEGF function or block VEGF signaling in target cells. However, none of these strategies is intended to block secretion of VEGF in tumor cells. The disadvantages of current therapeutic strategies are many, including difficulties in manufacturing active recombinant protein, high-dose requirements, high costs for manufactures and consumers, and the probable need for lifetime treatment of the patient. Because of their relatively short half-lives, recombinant proteins must be administrated repeatedly by injection once to several times daily. Gene therapy as an alternative approach can bypass several of the disadvantages of protein therapy. In this report, we provide a novel anti-VEGF strategy by blocking its secretion in tumor cells.

Materials and Methods

Animals
Female 6- to 7-week-old C57Bl/6 mice were acclimated and caged in groups of 3-6. Animals were anesthetized by injection of a mixture of dormicum and hypnorm (1:1) before all procedures and euthanized by a lethal dose of CO2. All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Board.

Generation and Purification
PIGF-1/VEGF165 Heterodimers
Recombinant human PIGF-1 and VEGF165 monomers were expressed in Escherichia coli and purified as previously described (Details are available in an online supplement available at http://circres.ahajournals.org).

Retroviral Vector Design and Tumor Cell Transduction
Cloning and transfection of human PIGF-1, PIGF-1-KDEL, VEGF165, and VEGF165-KDEL into 293 T cells were performed according to previously published procedures (see the online supplement for details).

Tumor Cell Proliferation Assay
Vector-transduced, hPIGF-1-transduced, hPIGF-1-KDEL-transduced, and hVEGF-KDEL-transduced and wt Lewis lung carcinoma (LLC) cells were seeded at a density of 1×10⁶ cells/well in 24-well plates in DMEM medium (5% FCS, 37°C). Trypsinized cells were resuspended in Isoton II solution ( Beckman Coulter, Sweden) and counted in a Coulter Counter at various time points. Triplicates were used for each sample.

Cell Shape Assay and Actin Staining
Porcine aortic endothelial (PAE) cells expressing either VEGFR-1 or VEGFR-2 were grown on coverslips in 12-well plates in Ham F12 medium (10% FCS) as previously described. Fixation and actin staining were performed according our previously published procedures (see the online supplement for details).

Chemotaxis Assay
The motility responses of VEGFR-1/PAE and VEGFR-2/PAE cells to various recombinant growth factors and LLC-conditioned media were assayed using a modified Boyden chamber technique previously described (see the online supplement for details).

Enzyme-Linked Immunosorbent Assay
All sandwich enzyme-linked immunosorbent assays (ELISAs) were performed using the Quantikine ELISA system (R&D Systems) according to the manufacturer’s instructions and our previously published methods (see the online supplement for details).

Tumor Studies in Mice
Tumor studies were performed as previously described (see the online supplement for details).

Confocal Microscopy Analysis
To directly visualize tumor vascularization, whole mount staining and confocal microscopy analysis were performed. Tumors were dissected into thin tissue slices and fixed in 3% PFA overnight. Antibody epitopes were exposed by proteinase K (20 μg/mL) digestion and methanol permeabilization. Endogenous biotin and avidin activity was blocked before staining with a biotinylated rat antimouse monoclonal antibody against CD31 (Pharmingen). Blood vessels were detected with SA-Cy3 (Jackson ImmunoResearch Laboratories Inc) and visualized by confocal microscopy (Zeiss Confocal LSM510 Microscope). By scanning 16 thin sections (5- to 6-μm distance) of each sample, 3-dimensional images of the tissue sample were assembled. Areas of CD31-positive signals were quantified using the color range and histogram tool in a Photoshop 7.0 program.

Terminal Deoxynucleotidyl-Transferase–Mediated dUTP Nick-End Labeling Staining
Apoptotic analysis of tumor tissues was performed according to manufacturer’s instructions (see the online supplement for details).

Statistical Analysis
Statistical analysis was performed using standard Student t-test in Microsoft Excel. P<0.05 and P<0.001 were deemed as significant and highly significant, respectively.

Results
Generation of Retroviral Vectors Containing PIGF-KDEL or VEGF-KDEL
Both PIGF-1 and VEGF are released via the classical secretory pathway and their functional dimers are formed in the endoplasmic reticulum (ER). For construction of ER-retained PIGF-1 or VEGF, the C-terminus of human PIGF-1 or VEGF was fused with KDEL, a mammalian ER-retention signal. The fusion gene products were cloned separately into a retroviral vector containing green fluorescent protein (GFP) as a marker, and recombinant retroviruses were used to transduce a well-characterized murine LLC cell line. The in vivo growth of these cells is VEGF dependent. The presence of hPIGF-1 and hVEGF cDNAs was confirmed by Southern blot analysis and GFP positive cells were sorted by fluorescence-activated cell sorter (FACS) analysis.

Blockage of VEGF Secretion in Tumor Cells
To quantify the amounts of intracellular and extracellular dimeric molecules, a sensitive sandwich ELISA assay was used to analyze cell lysates and conditioned media from
transduced and non-transduced LLC cell lines. As expected, a high level of mVEGF homodimers was detected in conditioned media from the controls, wt LLC, and vector-transduced LLC cells (Table). The majority of mPlGF-1 produced by wt and vector-transduced LLC cells was involved in heterodimerization with mVEGF, suggesting that mPlGF-1 preferentially formed heterodimers with mVEGF, rather than forming mPlGF-1/mPlGF-1 homodimers. Overexpression of hVEGF in these cells resulted in sufficient secretion of heterodimers of hVEGF/mVEGF molecules (3779 pg/mL) in addition to hVEGF/hVEGF homodimers (41880 pg/mL). In contrast, transduction of LLC cells with hVEGF-KDEL prevented VEGF secretion. Only a minor part of the hVEGF/mVEGF (268 pg/mL) and hVEGF/hVEGF (628 pg/mL) was present in conditioned medium compared with the large portion retained intracellularly (1578 pg/mL and 2624 pg/mL, respectively). This demonstrates the consequence of KDEL being retained in the ER. Consistent with our previous report, virtually all mVEGF molecules were present as hPlGF-1/mVEGF heterodimers in the conditioned medium of hPlGF-1-overexpressing LLC cells (5581 pg/mL) (Table). The preferential formation of hPlGF-1/mVEGF heterodimers in these tumor cells resulted in a remarkable depletion of secreted mVEGF homodimers (Table). Remarkably, gene delivery of hPlGF-1-KDEL in LLC cells not only forced nearly all mVEGF molecules to form hPlGF-1/mVEGF heterodimers but also prevented the secretion of hPlGF-1/mVEGF heterodimers and hPlGF-1/hPlGF-1 homodimers. The majority of each kind of heterodimer and homodimer was present intracellularly, and only minor portions were present in the conditioned medium.

Depletion of Endothelial Stimulatory Activity Released by Tumor Cells

To monitor the VEGF-mediated endothelial activity, we determined the endothelial chemotactic activity of conditioned media from various transduced tumor cells using a modified Boyden chemotaxis assay. The VEGFR-1- and VEGFR-2- expressing PAE cells have previously been used to detect VEGF activity.6 When purified recombinant dimeric growth factors were analyzed in this assay, only VEGF homodimers could significantly induce the motility of VEGFR-2/PAE cells (Figure 1A). Neither PIGF-1 homodimers nor PIGF-1/VEGF heterodimers induced a cell motility exceeding the background level. As expected, conditioned media from non-transduced or vector-transduced LLC cells significantly stimulated VEGFR-2/PAE cell migration (Figure 1B and 1C). However, overexpression of PIGF-1 or PIGF-1-KDEL dramatically blocked LLC cell-produced VEGF activity (P<0.001) (Figure 1B). High expression levels of hVEGF enhanced the chemotactic activity of the LLC cells (Figure 1C). In contrast, overexpression of VEGF-KDEL in these tumor cells drastically abolished VEGF-2/PAE cell migration in comparison to the controls (P<0.001) (Figure 1C). None of the recombinant factors or conditioned media induced VEGFR-1/PAE cell motility.

In addition to chemotaxis, we assayed morphological changes of PAE cells expressing VEGFR-1 or VEGFR-2 as an independent criteria for evaluation of tumor cell-released VEGF activity. Addition of recombinant 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 1E), a feature that both PIGF-1 homodimers and PIGF-1/VEGF heterodimers fail to do (data not shown). Incubation with conditioned media from non-transfected LLC cells resulted in the VEGFR-2/PAE cells attaining an elongated spindle-like shape (Figure 1G), similar to that induced by rhVEGF. In contrast, the VEGF-induced cell shape changes were completely blocked by expression of either PIGF-1-KDEL or VEGF-KDEL in these tumor cells (Figure 1G, 1H, and 1I). Again, conditioned media from both transduced and non-transduced LLC cell lines. As expected, a high level of mVEGF homodimers was detected in conditioned media from the controls, wt LLC, and vector-transduced LLC cells (Table). The majority of mPlGF-1 produced by wt and vector-transduced LLC cells was involved in heterodimerization with mVEGF, suggesting that mPlGF-1 preferentially formed heterodimers with mVEGF, rather than forming mPlGF-1/mPlGF-1 homodimers. Overexpression of hVEGF in these cells resulted in sufficient secretion of heterodimers of hVEGF/mVEGF molecules (3779 pg/mL) in addition to hVEGF/hVEGF homodimers (41880 pg/mL). In contrast, transduction of LLC cells with hVEGF-KDEL prevented VEGF secretion. Only a minor part of the hVEGF/mVEGF (268 pg/mL) and hVEGF/hVEGF (628 pg/mL) was present in conditioned medium compared with the large portion retained intracellularly (1578 pg/mL and 2624 pg/mL, respectively). This demonstrates the consequence of KDEL being retained in the ER. Consistent with our previous report, virtually all mVEGF molecules were present as hPlGF-1/mVEGF heterodimers in the conditioned medium of hPlGF-1-overexpressing LLC cells (5581 pg/mL) (Table). The preferential formation of hPlGF-1/mVEGF heterodimers in these tumor cells resulted in a remarkable depletion of secreted mVEGF homodimers (Table). Remarkably, gene delivery of hPlGF-1-KDEL in LLC cells not only forced nearly all mVEGF molecules to form hPlGF-1/mVEGF heterodimers but also prevented the secretion of hPlGF-1/mVEGF heterodimers and hPlGF-1/hPlGF-1 homodimers. The majority of each kind of heterodimer and homodimer was present intracellularly, and only minor portions were present in the conditioned medium.

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ND indicates not detectable.
cell lines failed to induce a similar change in morphology of VEGFR-1/PAE cells (data not shown).

**Suppression of Tumor Growth**

Although PlGF-1 and VEGF are considered to be growth factors acting specifically on blood vessel endothelial cells, overexpression and retention of these factors in the ER might affect tumor cell growth. To exclude this possibility, the growth rate of PlGF-1-KDEL and VEGF-KDEL LLC cells were compared with those of control cells. Transduction of PlGF-1-KDEL or PlGF-1 into LLC cells did not alter the growth rates in culture as compared with wt-transduced and vector-transduced LLC cells, indicating that accumulation of PlGF-1 in the ER compartment did not affect tumor cell growth in vitro (Figure 2A). Similarly, VEGF-KDEL–transduced LLC cells did not demonstrate an altered growth rate in vitro when compared with both VEGF overexpressing cells and the control cell lines (Figure 3A). In the controls, visible tumors were readily detectable 5 days after implantation and grew to the size of the Swedish ethical limit (1500 mm$^3$) within 2 weeks after implantation (Figures 2B and 3B). Consistent with our recent findings in a murine T241 fibrosarcoma model, expression of hPlGF-1 in LLC remarkably delayed tumor growth and visible tumors were only detectable by day 10 after implantation. At day 14 after tumor implantation, $\approx 90\%$ inhibition of tumor growth was scored in hPlGF-1–expressing tumors as compared with wt-transduced and vector-transduced tumors (Figure 2B). The tumors remained small, at a similar average size of $<200$ mm$^3$, by day 16 after implantation (Figure 2C).

At day 14 after implantation, hPlGF-1-KDEL-LLC cells only gave rise to barely detectable tumors (40 mm$^3$) (Figure 2B and 2C). These tumors all remained small over the next 3 weeks during prolongation of the experiments (Figure 2C). Although hPlGF-1-LLC tumors continued to grow exponentially to an average size of $>600$ mm$^3$ 3 weeks after implantation, hPlGF-1-KDEL-LLC tumors only reached an average size of $<100$ mm$^3$ (Figure 2C). Thus, the measured tumor volumes of hPlGF-1-KDEL-LLC and hPlGF-1-LLC were significantly different ($P<0.001$). The hPlGF-1-KDEL-LLC tumors demonstrated a marked delay in growth rate in vivo when compared with the hPlGF-1-LLC tumors.

To further study if fusion of the KDEL sequence to VEGF could inhibit tumor growth, hVEGF-KDEL-LLC cells were implanted into C57Bl/6 mice. Although wt and vector-transfected LLC cells produced VEGF at high levels, overexpression of hVEGF in these cells further accelerated tumor growth. After only 10 days, the hVEGF-LLC tumors had reached an average size of 1400 mm$^3$ (close to the Swedish ethical limit) (Figure 3B and 3C), whereas both wt-LLC and vector-LLC cells needed 14 days to produce tumors of similar size (Figure 3C). Mice carrying hVEGF-LLC tumors were euthanized at day 10 after implantation. At that time, $90\%$ inhibition was detected in hVEGF-KDEL-LLC tumors in comparison to hVEGF-LLC tumors (Figure 3B and 3C). In contrast to hVEGF-LLC, implantation of hVEGF-KDEL-LLC cells produced $\approx 50\%$ inhibition of tumor growth at day 14 when compared with wt and vector tumors (Figure 3B). These differences in in vivo tumor growth were not caused by altered tumor cell growth rates because all transduced and nontransduced tumor cells grew at a similar rate in vitro (Figures 2A and 3A).
Suppression of VEGF-Induced Tumor Neovascularization

To study tumor neovascularization, we performed immuno-histochemical analysis using an anti-CD31 antibody. Human PI GF-1-LLC and PI GF-1-KDEL-LLC tumors had significantly reduced neovascularization as compared with wt-or vector-transduced LLC tumors (Figure 4A, 4B, 4E, 4F, and 4G). However, hPI GF-1-KDEL was significantly more potent than PI GF-1 in blocking tumor neovascularization (Figure 4F and 4G). Transduction of LLC with hVEGF-KDEL also dramatically blocked tumor neovascularization. In contrast to hPI GF-1, hPI GF-1-KDEL, and hVEGF-KDEL, transduction of LLC with hVEGF alone remarkably increased tumor neovascularization (Figure 4C and 4G), with an average of more than 350 microvessels per optical field (×10).

Confocal analysis of tumor vasculatures revealed that wt and vector-transduced tumors contained high numbers of vessels with a high density of capillary sprouts (Figure 4H and 4I). Interestingly, extremely high numbers of capillaries or microvessels, which were likely to fuse into primitive vascular plexuses, were found in hVEGF-LLC tumors (Figure 4J and 4N). This type of vascular structure appeared to be leaky and hemorrhagic because autopsy examination of the hVEGF-LLC tumor tissues revealed large internal volumes of hemorrhagic fluids. In contrast, transduction of LLC tumors with hVEGF-KDEL blocked capillary sprout formation and resulted in formation of vascular architectures lacking the usual vascular branches (Figure 4K). Remarkably, overexpression of hPI GF-1-KDEL in LLC tumors led not only to a drastic reduction in vessel numbers but also to a nearly complete depletion of microcapillaries (Figure 4M and 4N). Similarly, PI GF-1-LLC tumors lacked vascular sprouts (Figure 4L). These data demonstrate that overexpression of ER-retained hPI GF-1-KDEL or hVEGF-KDEL proteins in mouse tumors sufficiently blocks mouse VEGF secretion and tumor neovascularization.

Induction of Tumor Cell Apoptosis

Blood vessels growing into tumors not only supply the cells with nutrients and O2 but also provide them with survival factors. Therefore, suppression of tumor angiogenesis might influence the rate of tumor cell apoptosis. To assess tumor...
cell apoptosis, we performed a TUNEL staining. Overexpression of hVEGF significantly reduced the number of apoptotic tumor cells \((P<0.05)\) when compared with controls (Figure 5A–5C and 5G), which suggests that hVEGF-induced vessels were able to supply additional survival factors and thereby prevent apoptosis of tumor cells. However, transduction of hVEGF-KDEL in LLC tumor cells resulted in a significant increase in apoptosis \((P<0.001)\) (Figure 5D and 5G). According to our previous results, even a small increase in tumor cell apoptosis could have a great impact on tumor volume because the turnover rate of tumor cells is relatively fast. These data indicate that in hVEGF-KDEL–transduced and hPlGF-1-KDEL–transduced tumors a massive number of tumor cells undergo apoptosis because of insufficient blood supply.

**Discussion**

VEGF is believed to be one of the key factors that switches on an angiogenic phenotype in most, if not all, tumors. The role of VEGF in regulation of disease is not only limited to cancer. Other angiogenesis-dependent diseases, including diabetic retinopathy, age-related macular degeneration, atherosclerosis-related ischemic heart disease, and stroke, are all related to VEGF activity. Thus, the development of VEGF antagonists has become one of the central focuses in current anti-angiogenic therapy for the treatment of cancer and other common diseases. These antagonists target VEGF ligands, receptors, and intracellular signaling components.

In animal models, successful delivery of several VEGF antagonists has produced remarkable effects in blocking pathological progression and in improving disease conditions. For example, VEGF neutralizing antibodies potently block tumor growth in mice. Promising results from animal studies have encouraged researchers to test these compounds for use in the treatment of human diseases. In fact, >10 different VEGF antagonists have entered into clinical trials for the treatment of human cancers. Very recently, an anti-VEGF neutralizing antibody, avastin, was found to significantly extend the lifespan of patients with colon–rectal cancers.

In this work, we describe a novel therapeutic approach to prevent VEGF secretion from tumor cells. Because tumor cells lack high-affinity VEGF receptors, sequestration of VEGF as an intracellular protein would not result in activation of “intracrine” signaling pathways. Consistent with this principle, we have found that overexpression of VEGF did not alter tumor cell growth rates in vitro. To prevent VEGF secretion, we fused a 4-amino-acid peptide, which retains secretory proteins within the ER of mammalian cells, with the C-terminus of PlGF-1. The critical principle applied to our approach is to use PlGF-1, which forms biologically inactive heterodimers with VEGF, as bait. Overexpression of hPlGF-1-KDEL in tumor cells enforces the majority, if not all, of endogenous VEGF monomers to form heterodimers with PlGF-1. Thus, this strategy almost completely inhibits the extracellular release of VEGF produced by tumor cells. In addition to heterodimers, most PlGF-1 homodimers were retained in the ER compartment without further secretion. Prevention of PlGF-1 homodimer secretion is an important step in further suppressing VEGF function. VEGF has a higher binding affinity for VEGFR-1 compared with VEGFR-2. Excessive amounts of extracellular PlGF-1 could compete with VEGF for binding to the VEGFR-1 receptor. Thus, prevention of PlGF-1 homodimer secretion will reduce the availability of VEGF to interact with VEGFR-2, the
tyrosine kinase receptor that transduces both the angiogenic and vascular leakage signals of VEGF. Prevention of PlGF-1/VEGF heterodimer secretion may further inhibit angiogenic activity as the heterodimers may have some unknown angiogenic properties. Because many tumors overexpress PlGF-1 and PlGF-2,24 blockage of endogenous PlGF secretion by hPlGF-1-KDEL could further reduce VEGF-induced angiogenesis and tumor growth. Thus, our approach blocks VEGF at 2 levels, both intracellularly and extracellularly.

As expected, overexpression of hPlGF-1-KDEL in tumor cells exhibits a more potent antitumor activity than native PlGF-1. Our tumor model clearly demonstrates a block of the angiogenic switch in tumors transfected with PlGF-1-KDEL. In contrast, PlGF-1-producing tumors only delayed this angiogenic switch. Although transduction of hVEGF into tumor cells further potentiates tumor angiogenesis and tumor growth, overexpression of hVEGF-KDEL potently suppresses tumor growth as compared with control tumors. Our present work is aimed to prove the concept of retaining a VEGF-binding protein within the secretory compartments to prevent the secretion and function of VEGF. In addition to the ER-retaining sequence, coupling of other intracellular retention signals, such as the Golgi retention signal, to a VEGF-binding protein can in principle also be used to prevent VEGF secretion. In the present study, PlGF-1 is used as bait for VEGF. However, other VEGF-binding proteins, such as VEGF receptors, neuropilin, and VEGF-B, may also block secretion of VEGF. Such intracellular molecules can be used as targets for development of anti-VEGF gene therapies. Studies using genetic mouse models that give rise to spontaneous tumors show that presence of VEGF is critical to induce an angiogenic switch during tumor growth.40 Low levels of VEGF in tumors are insufficient to switch on angiogenesis and tumor growth.41 These findings suggest that suppression of the VEGF function below a threshold level is enough to prevent tumor growth. Thus, gene delivery of PlGF-1-KDEL into tumor cells may not necessarily require transfection of every single tumor cell.

PlGF-1 may regulate VEGF-induced angiogenesis in both positive and negative manners. Systemic delivery of PlGF-1 homodimers may potentiate VEGF-induced angiogenesis as PlGF-1 competes with VEGF for binding to the VEGFR-1 receptor, a potential decoy receptor. As a result of this competition, more VEGF becomes available and can interact with the VEGFR-2 receptor, which is the active receptor in transducing angiogenic responses. Thus, systemic delivery of PlGF-1 homodimers may in this case potentate the angiogenic activity of VEGF. In contrast, when PlGF-1 and VEGF are coexpressed in the same cell, biologically inactive PlGF-1/VEGF heterodimers are formed.5 Thus, PlGF-1 negatively regulates the function of VEGF in cells that coexpress both factors. PlGF homodimers and PlGF/VEGF heterodimers have been reported by other researchers to induce angiogenesis. However, most of these studies were performed using PlGF-2 or mouse PlGF, which corresponds to the human PlGF-2 isoform, rather than PlGF-1.12 We have been using PlGF-1 in all our studies. It is possible that various spliced isoforms of PlGF may have different angiogenic activities. In our current approach, PlGF-1 is retained as an intracellular protein. Thus, gene therapy based on this concept will most likely not induce angiogenesis as PlGF-1 homodimers, as well as PlGF-1/VEGF heterodimers, will not be secreted from the cell.

Our data indicate that coupling of the KDEL sequence to hVEGF sufficiently blocks endogenous mouse VEGF secretion and antagonizes its activity. In summary, our study presents a novel and effective therapeutic anti-VEGF approach. We hope that this novel concept eventually will be used, either alone or in combination with other anti-VEGF methods, in the treatment of human cancer and other angiogenesis-dependent diseases.

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In an article by Björndahl et al (Circ Res. 2004;94:1443–1450), Figures 4D, 4K, 5C, and 5E contained inaccurate information because of technical errors that occurred during the preparation of the figures. The images shown in Figure 4D and 4K were incorrect, and Figure 5C and 5E required additional layers of blue. The corrected Figures 4 and 5 are shown here. The text, figure legends, arrangement of all figures, all quantification graphs, and the conclusions of this article are not affected by these corrections. The authors apologize for these errors.

Figure 4.
Figure 5.
Supplementary information

**Experimental Procedures**

**Generation and purification PIGF-1/VEGF\textsubscript{165} heterodimers**

Recombinant human PIGF-1 and VEGF\textsubscript{165} monomers were expressed in *E. Coli* and purified as previously described\textsuperscript{1}. Briefly, an equimolar mixture of PIGF-1- and VEGF\textsubscript{165} homodimers, at a total protein concentration of 0.5 mg/ml, was incubated in reducing buffer at 4°C over night. The following day the protein solution was dialyzed against 10 volumes of refolding buffer. Using this refolding protocol, a mixture of homodimeric PIGF-1 and VEGF\textsubscript{165} as well as heterodimeric PIGF-1/VEGF was generated.

The homodimeric and heterodimeric proteins were separated by affinity chromatography using a goat polyclonal anti-hVEGF affinity column and a polyclonal goat anti-hPIGF affinity column. The protein solution, was applied at a flow rate of 2 ml/min onto the anti-hVEGF-affinity column pre-equilibrated with PBS. The column was then washed at the same flow rate with PBS until the absorbance reading at 280 nm reached base-line level. VEGF homodimers and PIGF-1/VEGF heterodimers, but not PIGF homodimers, were retained in the column and eluted with 0.1 M sodium citrate, pH 2.5, 0.3 M NaCl. The dimers eluted from the anti-VEGF column were instantly neutralized with 2 M Tris buffer, pH 8, and subsequently dialyzed against 20 volumes of PBS at 4°C for 4 h. After dialysis the protein sample was applied to a PBS pre-equilibrated anti-PIGF affinity column using the same conditions. Only heterodimers were retained and eluted from the column. Purified hPIGF-1, hVEGF, and hPIGF-1/hVEGF proteins were finally dialyzed against PBS and analyzed by SDS-PAGE under both reducing and non-reducing conditions, followed by measurement of protein concentrations.
Retroviral vector design and tumor cell transduction

Complementary DNAs coding for human PlGF-1,129, PlGF-1,129-KDEL, VEGF165, and VEGF165-KDEL were cloned into the Murine Stem Cell Virus (MSCV) vector containing GFP. Transfection of retroviral constructs into 293T cells along with expression plasmids encoding ecotropic gag/pol and the Vesicular Stomatitis Virus-Glycoprotein (VSV-G) envelope protein using a classical CaPO₄ transfection method generated retroviral supernatants. Murine LLC 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 hours on two consecutive days. GFP 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.

Cell Shape Assay and Actin Staining

PAE cells expressing either VEGFR-1 or VEGFR-2 were grown on coverslips in 12-well plates, in Ham’s F12 medium supplemented with 10% FCS as previously described¹. At a confluency of about 40-60%, the medium was replaced with fresh Ham’s F12 medium containing only 2% FCS and 50 ng/ml of either recombinant factors (hVEGF, hPlGF-1, or hPlGF-1/hVEGF) or 25% (v/v) of conditioned media from LLC cell lines, including wt, vector, hPlGF-1, hPlGF-1-KDEL, hVEGF, or hVEGF-1-KDEL. Non-treated cells served as a negative control. After incubation for 16 h, 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 stained for 30 min with 1 µg/ml of TRITC-phalloidin (Sigma) diluted in PBS. Cells were examined in a combined light and fluorescence
microscope and spindle-like cells were counted in 5 optical fields (20x). Data represents mean % (±SEM).

Chemotaxis Assay

The motility responses of VEGFR-1/PAE and VEGFR-2/PAE cells to various recombinant growth factors and LLC conditioned media were assayed using a modified Boyden chamber technique previously described1. Briefly, the ability of VEGFR-expressing PAE cells to migrate through a micropore nitrocellulose filter (8 μm thick, 8 μm pores) was measured as a criterion for chemotactic stimuli. Serum-free medium supplemented with 0.2% BSA and 50 ng/ml of either recombinant factors (hVEGF, hPlGF-1 or hPlGF-1/hVEGF) or 25% (v/v) of conditioned media from different retro-virally transduced cells was added to the lower chambers. Non-treated cells served as a negative control. Approximately 40,000 cells were seeded in each upper chamber. After 4 h incubation at 37°C, cells attached to the filter were fixed in methanol and stained with Giemsa solution. Quadruplicates of each sample were used, and all experiments were performed three times. The cells that had migrated through the filter were counted using a light microscope and plotted as numbers of migrating cells per optic field (32x).

ELISA assay

All sandwich ELISAs were performed using the Quantikine ELISA system (R&D Systems) according to the manufacturer’s instructions. Briefly, standard mouse (m)VEGF and samples were added to a 96-well microplate pre-coated with an affinity purified polyclonal antibody specific for mVEGF. Homodimers containing mVEGF were detected by an enzyme-linked polyclonal antibody specific for mVEGF. Similarly, homodimers of mPlGF-1, hVEGF,
and hPIGF-1 were measured using the Quantikine M mPIGF-1 ELISA kit, Quantikine hVEGF ELISA kit, and Quantikine hPIGF ELISA kit, respectively.

The amount of heterodimers was measured with cross-matching capture and detection antibodies using the same ELISA kits mentioned above. For mVEGF/mPIGF-1 heterodimers, samples were added to microplates pre-coated with anti-mVEGF. Enzyme-linked anti-mPIGF-1 was then used to detect the mVEGF/mPIGF-1 heterodimer. To calibrate the assay, a recombinant mPIGF-1 standard was analyzed simultaneously on the PlGF-1 plate. No cross reactivity was observed from the homodimers. Likewise, the amount of mVEGF/hVEGF heterodimers were measured using microplates pre-coated with anti-hVEGF antibodies, and anti-mVEGF conjugates for detection of heterodimers. Recombinant mVEGF standards analyzed on the mVEGF plates were used for calibration. Approximately 1-2% of cross reactivity was observed from each homodimer, and the results were corrected accordingly. The amount of mVEGF/hPIGF-1 heterodimers was measured using microplates pre-coated with anti-mVEGF antibodies, and an anti-hPIGF-1 conjugate was used for detection. Recombinant hPIGF-1 standards analyzed on the hPIGF-1 plates were used for calibration. Approximately 3% cross reactivity was observed from the hPIGF-1 homodimer, and the results were corrected accordingly.

**Tumor studies in mice**

Wild type-LLC, vector transfected-LLC, and LLC cells expressing hPIGF-1, hPIGF-1-KDEL, hVEGF, or hVEGF-KDEL were used for tumor implantation studies in 6-7-wk-old syngeneic C57Bl/6 mice. Approximately $1 \times 10^6$ tumor cells were implanted subcutaneously on the back of each mouse. Six mice were used in both treated and control groups. Primary tumors were measured using digital calipers on the days indicated. Tumor volumes were
calculated according to the formula: width\(^2\) x length x 0.52, and when the Swedish ethical limit (1500 mm\(^3\)) was reached the tumors were removed.

**Histology**

Tumors were surgically removed when they reached the Swedish ethical limit. Immunohistochemistry was carried out as previously described for quantification of tumor vessels. Briefly, tumor sections (6 µm) were immunostained using a biotinylated monoclonal antibody against CD31 (Pharmingen, San Diego, CA), followed by horseradish peroxidase (HRP) conjugated streptavidin (SA). Peroxidase activity was developed using diaminobenzidine (DAB, Vector Laboratories Inc., Burlingame, CA). The sections were photographed and blood vessels were counted under a light microscope in 6 optical fields (20x). Data represents mean % (±SEM).

**TUNEL staining**

For detection of apoptotic cells in the tumors a TUNEL staining was performed. Tumor tissues were fixed with 3% paraformaldehyde, dehydrated and embedded in paraffin. Dewaxed and rehydrated tissue sections (5 µm thickness) were TUNEL stained according to a standard, but modified, fluorescein in situ Death Detection protocol (Amersham). Briefly, the tissues were treated with 20 µg/ml\(^{-1}\) proteinase K (LifeTechnologies). The sections were treated with a TUNEL reaction mixture and incubated in a humid atmosphere at 37 °C for 1 h, followed by counter staining with Hoescht 33258 (500 ng/ml\(^{-1}\)). The sections were photographed and apoptotic cells were counted under a fluorescence microscope in 10 optical fields (40x). Data represents mean determinants (±SEM).
References