Cancer Therapy: Preclinical

Placental Growth Factor Upregulation Is a Host Response to Antiangiogenic Therapy

Rebecca G. Bagley, Yi Ren, William Weber, Min Yao, Leslie Kurtzberg, Jason Pinckney, Dinesh Bangari, Cokey Nguyen, William Brondyk, Johanne Kaplan, and Beverly A. Teicher

Abstract

Purpose: Placental growth factor (PIGF) is an angiogenic protein. Upregulation of PIGF has been observed in the clinic following antiangiogenic regimens targeting the VEGF pathway. PIGF has been proposed as a therapeutic target for oncology. sFLT01 is a novel fusion protein that neutralizes mouse and human PIGF (mPIGF, hPIGF) and mouse and human VEGF-A (mVEGF-A, hVEGF-A). It was tested in syngeneic and xenograft tumor models to evaluate the effects of simultaneously neutralizing PIGF and VEGF-A and to investigate changes observed in the clinic in preclinical models.

Experimental Design: Production of PIGF and VEGF-A by B16F10 and A673 cancer cells in vitro was assessed. Mice with subcutaneous B16F10 melanoma or A673 sarcoma tumors were treated with sFLT01. Tumor volumes and microvessel density (MVD) were measured to assess efficacy. Serum levels of hVEGF-A, hPIGF, and mPIGF at early and late time points were determined by ELISA.

Results: Exposure of cancer cell lines to sFLT01 caused a decrease in VEGF secretion. sFLT01 inhibited tumor growth, prolonged survival, and decreased MVD. Analysis of serum collected from treated mice showed that sFLT01 administration caused a marked increase in circulating mPIGF but not hPIGF or hVEGF. sFLT01 treatment also increased circulating mPIGF levels in non–tumor-bearing mice.

Conclusion: With the tumor cell lines and mouse models we used, antiangiogenic therapies that target both PIGF and VEGF may elicit a host response rather than, or in addition to, a malignant cell response that contribute to therapeutic resistance and tumor escape as suggested by others. Clin Cancer Res; 17(5); 976–88. ©2011 AACR.

Introduction

The realization that tumors require vasculature to grow and metastasize gave rise to the development of antiangiogenic therapies to treat cancer. Food and Drug Administration–approved antiangiogenic agents include the humanized monoclonal antibody against VEGF-A bevacizumab and the multitargeted small molecule tyrosine kinase receptor inhibitors sunitinib, pazopanib, and sorafenib, which potententially inhibit the VEGF and PDGF (platelet derived growth factor) pathways. Although therapies that target vasculature are often included in clinical standard-of-care regimens, the benefit can be modest with little improvement in overall survival (1). There is a need for the identification of factors that enable tumor escape and confer antiangiogenic agent resistance and the identification of new targets for the next generation of antiangiogenic agents.

Placental growth factor (PIGF) can form heterodimers with VEGF and several splice variants of PIGF exist that bind to VEGFR-1/Flt-1 and/or neuropilin-1 (2–5). The contribution of PIGF to angiogenesis was shown in transgenic mice in which the overexpression of PIGF resulted in a substantial increase in vasculature, including the number of vessels, branching points, size of the vessels, and increased vascular permeability (6). PIGF is expressed by the placenta, endothelial cells, and osteogenic cells and promotes angiogenesis during wound healing, ischemia, and inflammation (7, 8). It is induced under hypoxic conditions that occur in many solid tumors (9), thereby promoting tumor neovascularization and enhancing the survival of tumor endothelial cells and macrophages (7, 10, 11).

PIGF is upregulated in many malignant diseases and thus may be a useful therapeutic target. It is more highly expressed in small and non–small cell lung cancers and in renal cell carcinomas than in the corresponding normal tissues (12–14). PIGF transcripts were present in human cervical squamous cell carcinomas and were upregulated in human prostate cancer cells (15, 16). Human melanoma cells and melanocytes also secrete and respond to PIGF (17, 18). In addition, PIGF is of interest as a target for breast and gastric cancers in which the expression is higher than in other cancers (19, 20). PIGF enhanced the...
Therapies that target the VEGF pathway offer some benefit but often do not significantly increase overall survival. The factors that contribute to drug resistance and tumor escape are not clearly defined. Placental growth factor (PlGF) has been implicated as a contributor in resistance to anti-VEGF regimens. It has been proposed as a therapeutic target for oncology. We show that simultaneous neutralization of VEGF-A and PlGF in both tumor-bearing and -naive mice elicits an acute host response resulting in elevated serum PI GF levels. The results presented here raise questions about the specific involvement of the tumor microenvironment in the increases of serum PI GF levels that are observed in the clinic and the utility of targeting PlGF as a second-line antiangiogenic therapy. Furthermore, increased circulating PI GF may be associated with pharmacodynamics and decreased response to VEGF-A therapy but may not necessarily indicate efficacy or disease progression.

We have investigated whether a therapy that targets both VEGF and PI GF can be efficacious with a novel fusion protein, sFLT01 (33), which has a molecular weight of approximately 80 kDa and consists of the VEGF/PI GF-binding domain ofVEGFR-1/Flt-1 fused to the Fc portion of human IgG1 through a polyglycine linker (9Gly; ref. 34). Intravitreal delivery of an AAV2 vector encoding sFLT01 (AAV2.sFLT01) was efficacious as a gene therapy in murine and nonhuman primate models of retinal neovascularization (34, 35). sFLT01 functions as a soluble VEGFR-1 decoy receptor and neutralizes mouse VEGF-A and PI GF (mVEGF-A, mPlGF) and human VEGF-A and PI GF (hVEGF-A, hPlGF).

The B16F10 melanoma model was selected on the basis of reports that human melanoma cells secrete hPlGF and also because the B16F10 model has been utilized to evaluate antibodies against PI GF in earlier reports (8, 17, 18, 22, 31). The A673 Ewing’s sarcoma xenograft model was employed since previous studies investigated antiangiogenic agents that target VEGF-A in this model and/or the contribution of host stroma to mVEGF production (36, 37). We exposed mouse B16F10 melanoma and human Ewing’s sarcoma A673 cells to sFLT01 in vitro to assess the phenotypic changes resulting from the neutralization of both VEGF-A and PI GF. To assess antitumor activity, sFLT01 was administered to mice bearing syngeneic B16F10 melanoma or human xenograft A673 sarcoma tumors. Given the clinical observation that VEGF-A and PI GF levels in circulation increased following antiangiogenic therapy, the serum levels of mPlGF, hPlGF, and hVEGF were quantified in the B16F10 and A673 tumor-bearing mice following the administration of sFLT01. Only mPlGF levels significantly increased at early time points when the tumors were responding to therapy and during late disease progression. Administration of sFLT01 to naive mice also produced upregulation of mPI GF, indicating a systemic host response. The increase in mPlGF in the host is consistent with reports that other antiangiogenic therapies delivered to naive mice elevated circulating cytokine levels (23). Thus, antiangiogenic therapies that target both PI GF and VEGF will likely not prevent upregulation of PI GF. Finally, our findings also indicate that resistance to anti-VEGF therapies may be attributed in part to a systemic host response and not exclusively to molecular changes in the malignant cells.

Materials and Methods

sFLT01 protein

CHO DXB11 cells were transfected with an expression vector containing the coding sequence of sFLT01 (34), using the Lipofectamine 2000 reagent (Invitrogen). Transfected cells were selected with increasing concentrations of methotrexate in MEM alpha, with no ribonucleosides or deoxyribonucleosides (Invitrogen), containing 10% dia-lyzed FBS (Invitrogen). The transfected pool was grown in 850-cm² roller bottles in 200 mL of selection medium. When the cells reached confluence, the selection medium was removed and replaced with IS CHO CD medium (Irvine Scientific). The conditioned medium was harvested...
3 to 4 days later, filtered using a 0.2-μm filter, and loaded onto a Protein A column that was preequilibrated with 0.7 mol/L NaCl, 20 mmol/L Tris, pH 7.0. The column was washed with equilibration buffer and eluted with 50 mmol/L glycine, pH 2.5. The eluted peak fraction containing sFLT01 protein was adjusted to pH 6.5 to 6.7 with 0.3 mol/L Na2HPO4 (dibasic). The sFLT01 was concentrated onto a Protein A column that was preequilibrated with 0.7 mol/L NaCl, 20 mmol/L Tris, pH 7.0. The column was washed with equilibration buffer and eluted with 50 mmol/L NaCl; 0.05% P20 surfactant; 3 mmol/L EDTA). The flow rate was 50 μL/min for both association and dissociation (10 minutes running buffer) steps. After each cycle, the cytokine surfaces were regenerated using 10 mmol/L glycine, pH 1.5, for 40 seconds at 30 μL/min. Biacore T100 evaluation software (v1.1.1) was used to analyze binding kinetics. Blank flow cells and 0 nmol/L samples were double-reference subtracted from data. All samples were fit to a 1:1 binding model.

Surface plasmon resonance binding analysis
A Biacore T100 instrument was utilized to perform affinity analysis. Biacore CM5 Series S sensor chips (GE Healthcare) were directly immobilized with 300RIUs hVEGF, mVEGF, hPIGF, and mPIGF (R&D Systems). Binding of sFLT01 was tested at 0, 7.5, 15, 30, 60, and 120 mmol/L concentrations in duplicate. All samples were diluted in HBS-EP + running buffer (10 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl; 0.05% P20 surfactant; 3 mmol/L EDTA). The flow rate was 50 μL/min for both association (5 minutes per sample injection) and dissociation (10 minutes running buffer) steps. After each cycle, the cytokine surfaces were regenerated using 10 mmol/L glycine, pH 1.5, for 40 seconds at 30 μL/min. Biacore T100 evaluation software (v1.1.1) was used to analyze binding kinetics. Blank flow cells and 0 nmol/L samples were double-reference subtracted from data. All samples were fit to a 1:1 binding model.

Cell culture
Mouse B16F10 melanoma and human A673 Ewing’s sarcoma cell lines (American Type Culture Collection) were grown in RPMI 1640/10% FBS (Invitrogen) ± 150 μg/mL sFLT01 for 9 to 28 days. At several time points, aliquots of the cells were grown to confluency in a T25 flask and were washed twice with serum-free RPMI 1640. The cells were overlayed with 4 mL of serum-free medium for 24 hours in the absence of sFLT01. The conditioned medium was collected and centrifuged to remove any loose cells and transferred to a new tube. The remaining cells were collected by trypsin/EDTA digestions (Invitrogen), total cells were counted, and the resulting cell pellet was lysed with lysis buffer (Roche Diagnostics) and 2 mmol/L sodium orthovanadate (New England Biolabs). The conditioned medium and lyed cell pellets were analyzed for secreted and intracellular levels of PlGF and VEGF-A by ELISA (R&D Systems).

In vivo models
Mouse B16F10 melanoma or human A673 sarcoma cell lines were cultured as describe earlier. For the B16F10 melanoma tumor model, C57Bl/6 mice (Charles River) were implanted subcutaneously with 5 × 106 cells mixed 1:1 with Matrigel in RPMI in a 200 μL volume (n = 12 per group). For the human A673 Ewing’s sarcoma tumor model, beige nude (C57Bl/6 × C3H)F1 mice (Charles River) were implanted subcutaneously with 9 × 106 cells mixed 1:1 with Matrigel in RPMI in a 200 μL volume (n = 10 per group). sFLT01 (10 or 25 mg/kg) or vehicle was delivered by intraperitoneal injection 2 or 3 times per week. Tumors were measured with calipers, and dosing was initiated when tumors were ~100 mm³ in volume. Tumor volume was calculated using the following formula: width² × length × 0.52. Mice were euthanized when tumors measured 20 mm in diameter or became ulcerated. Studies were blinded.

Non–tumor-bearing mice (C57Bl/6 or beige nude) were administered an intraperitoneal injection of 0, 10, or 25 mg/kg sFLT01 in a 0.9% saline vehicle on days 0, 4, and 7. Blood was collected from the ocular sinus at 4, 24, and 48 hours post-injection on days 0, 4, and 7. Blood was also collected on days 14 and 21. Serum samples were assayed using PlGF and VEGF ELISA (R&D Systems). All procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee in accordance with the Federal Animal Welfare Act (9 CFR, 1992) and were conducted in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care)-accredited facility.

Immunohistochemistry
A673 and B16F10 subcutaneous tumors were fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin. Tumor sections (5 μm) were incubated with a rat anti-mouse CD31 antibody (Fitzgerald Industries). A biotinylated anti-rat antibody (Jackson Immunochemicals), ABC-Elite peroxidase (cat. no. PK-6100; Vector Laboratories), and 3,3'-diaminobenzidine (DAB; Dako) chromagen were applied for detection. Tumors were counterstained with hematoxylin, and whole image scans were generated at 20× magnification with the Aperio SCANSCOPE XT scanning system. Microvessel density (MVD) represents the total number of CD31⁺ vessels/mm² of tissue area and was quantified using Aperio’s MVD algorithm. Additional morphometric measurements such as vessel lumen size and perimeter were also generated using the Aperio MVD algorithm and are reported in μm and μm², respectively.

A673 tumors were collected 1 day after the third (day 11) or sixth (day 21) sFLT01 dose, were flash frozen in optimal cutting temperature compound, and cut into 4- to 5-μm sections. Samples were fixed in NBF for 10 minutes and then incubated with a rat anti-mouse CD31 antibody (BD Biosciences) or rabbit anti-mouse NG2 antibody (Chemicon). Secondary goat anti-rat-Cy3 or goat anti-rabbit-Cy2 antibodies (Jackson Immunochemicals) were applied for detection.

For PlGF immunohistochemistry (IHC), heat-induced epitope retrieval was done on deparaffinized tissue sections, using Dako citrate buffer (Dako), and then the sections were incubated with a 1:50 dilution of a rabbit polyclonal anti-PlGF antibody (cat. no. 250824; Abbiotec). Dako Envision kit (cat. no. K4011; Dako) and DAB as the chromogen were used. For VEGF-A IHC, a 1:200 dilution of a rabbit polyclonal anti-VEGFA antibody (cat. no. sc-152; Santa Cruz Biotechnology) was used as the primary
antibody, a biotinylated goat anti-rabbit IgG (cat. no. 111-065-144; Jackson Immunoresearch) was used as the secondary antibody (cat. no. 111-065-144), and ABC-Elite peroxidase and was used for detection with DAB as the chromogen. For IHC on negative control sections, the primary antibody was replaced with rabbit universal negative control antibody (cat. no. N1699; Dako).

Statistical analysis

In vitro data are expressed as mean ±SD. Comparisons between treatment and control groups were made using student’s t test. Tumor volumes are expressed as mean ± SEM and were analyzed by the ANOVA test. Kaplan–Meier survival curves were analyzed by the log-rank test. Graphpad Prism 5.0 (Graphpad Software, Inc.) was used for analysis. P < 0.05 was considered statistically significant.

Results

The ability of sFLT01 protein, expressed by transfected HEK293 cells into conditioned medium, to neutralize hVEGF-A was shown previously in vitro in binding and human umbilical vein endothelial cell proliferation assays (34). The binding affinity of the recombinant sFLT01 protein for mVEGF-A, mPIGF, hVEGF-A, and hPIGF was further characterized by surface plasmon resonance technology. mVEGF-A shares 89% amino acid homology with hVEGF-A, and mPIGF has a 65% homology to hPIGF (38, 39). sFLT01 bound to mVEGF in addition to hVEGF-A (Fig. 1A and B). sFLT01 also bound to both mPIGF and hPIGF (Fig. 1C and D). The kinetic analysis indicated that sFLT01 has strong binding interactions with these growth factors (Table 1). These results show that sFLT01 is cross-species reactive and can neutralize both PIGF and VEGF-A that are secreted by either human malignant cells or murine cells in xenograft or syngeneic tumor models.

The expression profile of VEGF-A and PIGF was investigated in mouse B16F10 melanoma cells and human A673 Ewing’s sarcoma cells in vitro. Comparisons were made between cells exposed to 150 μg/mL sFLT01 for 9 to 28 days and untreated (control) cells. At the end of the culture period, cells were transferred to serum-free medium containing no sFLT01, the supernatant was collected following a 24-hour incubation, and assayed by ELISA to determine secreted levels of VEGF-A and PIGF. Cell lysates were prepared to quantify by ELISA intracellular VEGF-A and PIGF levels. Higher levels of mVEGF-A were detected in the conditioned medium of untreated B16F10 cells than in the cell lysates (Fig. 2A). In contrast, when B16F10 cells were exposed to sFLT01, little or no secreted mVEGF-A was detected and the levels of mVEGF-A were higher in the cell lysates than in the conditioned medium with a decrease over time (Fig. 2A). Untreated B16F10 cells secreted higher levels of mVEGF than mPIGF (Fig. 2A and B). mPIGF secretion also decreased when B16F10 cells were exposed to sFLT01, but there was no increase in mPIGF in the cell lysates (Fig. 2B).

The human A673 Ewing’s sarcoma cells secreted hVEGF-A in culture but not following exposure to sFLT01 (Fig. 2C). Unlike the B16F10 cells, little or no VEGF-A was detected in

**Figure 1.** Surface plasmon resonance (Biacore) detection of the interaction between sFLT01 and VEGF or PIGF. The binding of sFLT01 to angiogenic growth factors was tested at 0, 7.5, 15, 30, 60, and 120 mmol/L concentrations. Sensorgrams indicate binding of sFLT01 to hVEGF-A (A) and mVEGF (B). sFLT01 also bound to hPIGF (C) and mPIGF (D).
In the subcutaneous B16F10 melanoma tumor model, mice were treated with vehicle (control) or 25 mg/kg sFLT01 twice per week by intraperitoneal injection beginning when tumors were approximately 100 mm³ in volume. Administration of sFLT01 resulted in a 14-day tumor growth delay determined when tumors reached 1,500 mm³ compared with the control group ($P < 0.0001$; Fig. 3A). Median survival significantly increased from 17 days in the control group to 28 days with sFLT01 treatment, an increase of approximately 60% ($P = 0.0021$; Fig. 3B). B16F10 tumors from treated and control mice were collected 1 day after the second sFLT01 dose to evaluate the vasculature. The blood vessels in the tumors from mice treated with sFLT01 were notably more immature and less developed than those from tumors in the control group that presented patent lumens and greater length (Fig. 3C). Morphometric analysis of the blood vessels indicated that although the MVD and vessel wall thickness were not significantly reduced at the time the tumors were collected, the mean vessel area, perimeter, lumen size, and vascular area were significantly reduced by sFLT01 administration ($P < 0.05$; Fig. 3D).

IHC was done on formalin-fixed, paraffin-embedded sections of subcutaneous B16F10 control tumors to investigate the tumor microenvironment. It revealed mPlGF and mVEGF staining in malignant cells and in the adjacent fibroblasts (Fig. 4A). Similarly, mPlGF and mVEGF-A staining was observed in B16F10 cells and in fibroblasts in tumors from mice treated with sFLT01. A negative control rabbit antibody applied to tumor sections did not show background staining. These results indicate that the stromal cells such as fibroblasts in addition to the B16F10 malignant cells can produce and secrete mPlGF and mVEGF-A.

Blood was collected from mice at 2 time points during the B16F10 efficacy study to quantify mPlGF in circulation. On day 12 post-tumor cell implant, blood was collected 24 hours after the second dose of sFLT01 or vehicle when tumors were approximately 180 or 315 mm³ in volume, respectively. Blood was also collected on days 18 to 25 when tumors reached the endpoint volume of more than 2,000 mm³ and mice were removed from the study. Mice had received a total of 4 to 5 doses and were euthanized approximately 3 days after the last sFLT01 dose.

The circulating levels of mPlGF in the control group were undetectable on day 12 when the average tumor volume was 315.3 ± 49.2 mm³ and increased to 406 ± 205.7 pg/mL when tumors were 2,753.6 ± 1,562.8 mm³ (Fig. 4B). In contrast, the circulating levels of mPlGF in the sFLT01 treatment group were significantly elevated on day 12 ($7,038.6 ± 1,325.6$ pg/mL) when tumors were an average volume of $177.9 ± 24.5$ mm³ ($P < 0.0001$). The circulating mPlGF levels remained higher ($8,584.3 ± 1,575.2$ pg/mL) than those in the control group ($406.0 ± 205.7$) throughout the study until animals reached euthanasia criteria ($P < 0.0001$). The mPlGF ELISA detected only free or unbound mPlGF. We were unable to determine the levels of mVEGF because sFLT01 interfered with the mVEGF ELISAs from 2 vendors we tested and generated artifactual results. The results shown in Figure 4B indicate that mPlGF was upregulated not only when sFLT01 was efficacious and slowed tumor growth but also when the tumors no longer responded to treatment.

To further investigate the efficacy of sFLT01 treatment and the in vivo source of circulating PI GF following sFLT01 treatment (i.e., malignant cells or host tissue), we utilized the human A673 Ewing’s sarcoma xenograft model. Mice were treated with vehicle (control) or 20 mg/kg sFLT01 3 times per week by intraperitoneal injection beginning when tumors reached approximately 100 mm³ in volume. Administration of sFLT01 resulted in a 28-day tumor growth delay determined when tumors reached 1,500 mm³ compared with control group ($P < 0.0001$; Fig. 5A). Median survival significantly increased from 20 days in the control group to 50 days following sFLT01 treatment ($P < 0.0001$; Fig. 5B). Double immunofluorescent staining for pericytes, using an anti-NG2 antibody, and for endothelial cells, using an anti-CD31 antibody, revealed that at 2 and 3 weeks the blood vessels in the sFLT01-treated A673 tumors were immature and underdeveloped compared with control (Fig. 5C). The images also suggest that sFLT01 treatment may delay pericyte coverage of blood vessels in the tumor microenvironment. sFLT01 treatment resulted in a significant reduction in MVD at 2 weeks post–tumor cell implantation following 3 doses of sFLT01 ($P = 0.0007$; Fig. 5D). However, there

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>$k_{u}, \text{1/(mol/L s)}$</th>
<th>$K_{u}, \text{s}$</th>
<th>$K_{d}, \text{mol/L}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hVEGF-A</td>
<td>2.03E+05</td>
<td>3.82E-04</td>
<td>1.89E-09</td>
</tr>
<tr>
<td>mVEGF-A</td>
<td>1.52E+05</td>
<td>4.34E-04</td>
<td>2.93E-09</td>
</tr>
<tr>
<td>hPIGF</td>
<td>6.96E+05</td>
<td>8.17E-03</td>
<td>1.18E-08</td>
</tr>
<tr>
<td>mPIGF</td>
<td>2.10E+05</td>
<td>9.63E-04</td>
<td>4.59E-09</td>
</tr>
</tbody>
</table>

NOTE: The binding affinity of sFLT01 to hVEGF-A, mVEGF-A, hPIGF, and mPIGF was measured by surface plasmon resonance (Biacore) binding assays. sFLT01 had comparable binding affinities to the mouse and human homologues.
Figure 2. VEGF-A and PlGF production by B16F10 and A673 cells. Conditioned medium and lysates from cells in culture were assayed for VEGF-A and PlGF levels by ELISA. Serum-free conditioned medium was collected over a 24-hour period and contained no sFLT01. B16F10 melanoma cells secreted lower levels of VEGF-A and PlGF following exposure to sFLT01 (A) but intracellular levels did not change significantly (B). A673 sarcoma cells exposed to sFLT01 also decreased secretion of VEGF-A and intracellular levels were not significantly altered (C). sFLT01 altered the intracellular levels of PlGF over time in the A673 cells but did not substantially change the secretion pattern (D).
was no reduction in MVD at 3 weeks after 6 doses of sFLT01 (P = 0.9619). At 3 weeks, the MVD in the sFLT01 group was similar to the MVD at 2 weeks (P = 0.4174) but the MVD in the control was significantly lower because of the formation of fewer but larger blood vessels with patent lumens (P = 0.0012).

Human A673 Ewing’s sarcoma cells secreted 10-fold higher levels of hVEGF than levels of hPlGF in cell culture (Fig. 2). In contrast, in control mice bearing human A673 xenograft tumors (Fig. 6A), there were generally higher levels of circulating hPlGF (524.7 ± 585.9 pg/mL) than hVEGF (85.0 ± 36.5 pg/mL), although the levels of circulating hPlGF varied within the group (data not shown). Higher levels of circulating hPlGF than hVEGF in mice bearing A673 tumor model are in direct contrast to the pattern of growth factor secretion by the same cells in vitro. Differences in the secretion patterns of growth factors by malignant cells in vitro and in vivo have been observed in other tumor models (40). The serum levels of hVEGF (93.7 ± 45.3 pg/mL) and hPlGF (831.2 ± 837.3 pg/mL) following sFLT01 administration were comparable with the serum levels in the control group (P = 0.7497 and P = 0.3912, respectively). However, the serum levels of mPlGF (10,449.2 ± 11,488.0 pg/mL) following sFLT01 treatment were significantly higher than the levels of mPlGF in the control group (16.4 ± 49.2 pg/mL; P < 0.0001; Fig. 6A). The ability to selectively quantify hPlGF secreted by the human malignant cells and mPlGF secreted by the host, both of which are neutralized by sFLT01, allowed clear distinction between PlGF secreted by the mouse normal tissues or tumor stroma and secretion by the human cancer cells. These results indicate that while sFLT01 can bind and neutralize PlGF, treatment with sFLT01 stimulates excess production of PlGF by the host which can be detected in the circulation.
To further investigate the role of host tissue in the secretion of mPlGF into circulation in mice bearing A673 Ewing’s sarcoma xenografts, sFLT01 (10 or 25 mg/kg) was administered to naive, non–tumor-bearing mice on days 0, 4, and 7. The circulating mPlGF levels increased over time between 4 and 48 hours after injection of sFLT01 on days 0 and 7 (Fig. 6B). In contrast, circulating mPlGF was not detectable in the control group. This effect was not mouse strain specific and was also observed in non–tumor-bearing immunocompetent C57Bl/6 mice (Fig. 6B). The levels of mPlGF decreased from the time of sFLT01 injection and returned to baseline levels within 2 weeks after the last injection of sFLT01 on day 21.

Discussion

PlGF contributes to angiogenesis during normal physiologic events and in pathologic conditions such as ischemia and cancer (7). It may have prognostic value in several human cancers including those of the breast (19, 41), lung (14, 42), and colon (43). Overexpression in human tumors and proangiogenic activity has identified PlGF as a potential target for therapeutic intervention (30). Higher circulating levels of PlGF have been detected in patients following antiangiogenic therapy with sunitinib and bevacizumab (26–29). The effect is not limited to PlGF, as increased circulating levels of VEGF, sVEGFR-2, and sVEGFR-3 were also observed in RCC patients being treated with sunitinib (44). There are 2 protein therapeutics in clinical trials that neutralize PlGF: aflibercept is a fusion protein that binds to soluble VEGF-A and PlGF, and TB403 is a monoclonal antibody against PlGF (30, 45). Multi-targeted small molecule tyrosine kinase inhibitors that have been shown to antagonize VEGFR-1/Flt-1 in the clinic or in preclinical development also block PlGF signaling. sFLT01 is a novel, soluble decoy receptor which is composed of the VEGF/PlGF binding domain of VEGFR-1/Flt-1. We explored the phenotypic changes of cells exposed to...
sFLT01 in vitro and investigated PlGF secretion patterns in 2 preclinical tumors models following administration of sFLT01.

sFLT01 neutralizes the murine and human homologues of VEGF-A and PlGF, thereby facilitating investigations in preclinical xenograft models in which the tumor mass is composed of human malignant cells and murine stromal cells, vasculature, and infiltrating cells. The antitumor efficacy of sFLT01 and host response were evaluated and we determined whether the source of VEGF and PlGF was human cancer cells and/or host stroma in xenograft tumor models and in syngeneic models in which the angiogenic growth factors are solely murine-derived. sFLT01 was efficacious in slowing tumor growth, increasing survival, and reducing intratumoral MVD in the B16F10 melanoma and in the A673 sarcoma models. As has been observed in the clinic with VEGF inhibitors, sFLT01 administration to tumor-bearing mice resulted in an acute increase in the circulating levels of mPlGF when sFLT01 was efficacious. However, high blood levels of mPlGF persisted when tumors no longer responded to therapy. In addition, circulating mPlGF levels were acutely elevated in non–tumor-bearing mice on administration of sFLT01 and returned to baseline levels over time showing dissociation between mPlGF levels and antitumor efficacy. Changes in circulating mVEGF levels could not be determined in the presence of sFLT01 by the mVEGF ELISA.
sFLT01 exposure altered the expression patterns of m/hVEGF-A and m/hPlGF secretion by cancer cell lines in vitro. However, mouse B16F10 melanoma cells did not upregulate mPlGF secretion after exposure to sFLT01 in culture, suggesting that host normal cells were likely the primary source of circulating mPlGF in vivo, although IHC revealed mPlGF expression in the malignant cells of B16F10 tumors. Although this does not exclude the possibility that the malignant cells in vivo could be a source of elevated serum mPlGF levels, PlGF production by fibroblasts has also been detected in the fibroblasts of the B16F10 tumors and in fibroblasts under hypoxic conditions (9). Thus, normal or tumor-associated fibroblasts may have contributed to the increased circulating mPlGF in mice bearing B16F10 melanoma tumors or A673 Ewing’s sarcoma tumors. Importantly, higher serum mPlGF levels occurred in mice bearing human A673 tumor cells and in non–tumor-bearing mice following sFLT01 administration. Our results further expand on the findings of investigators who have observed that the induction of angiogenic growth factors is a host response to antiangiogenic therapy following the administration of sunitinib or anti-VEGF-A in non–tumor-bearing mice (23, 31).

Although PlGF levels increased in some patient populations following antiangiogenic therapy, there are conflicting reports regarding PlGF increases in circulation and in the tumor microenvironment in mice following treatments that target the VEGF pathway (22, 31). Fischer and colleagues observed increases in PlGF in circulation and PIGF mRNA in B16F10, CT26, and Panc02 tumors. In contrast, Bais and colleagues reported that anti-VEGF-A treatment did not increase PlGF levels in several murine tumor-bearing strains. However, these discrepancies may be due to differences in the specific antiangiogenic therapies used, the tumor models employed, or the timing and duration of treatment. Further studies are needed to elucidate the molecular mechanisms underlying PlGF upregulation in response to antiangiogenic therapy.
syngeneic models including B16F10. The anti-VEGF-A treatment did, however, produce increased circulating PlGF levels in non–tumor-bearing mice. Although our findings do not distinguish whether an anti-PlGF treatment will be equally or more effective than anti-VEGF-A treatment, or whether neutralization of PlGF, VEGF-A, or both, by sFLT01 leads to the PlGF host response, the results reported here imply that upregulation of PlGF may be initiated by other antiangiogenic therapies and will not be prevented with a dual VEGF-A/PlGF antagonists using a protein therapeutic.

The potential of PlGF as a marker can be viewed in the context of prognosis, efficacy, or pharmacodynamics. (19, 40–42). In patients with RCC treated with sunitinib, circulating PlGF levels were higher toward the end of the 4 week-drug cycle and returned to baseline levels 2 weeks thereafter, suggesting that PlGF could be a putative pharmacodynamic indicator (44). PlGF has been assessed as a marker following antiangiogenic therapy with bevacizumab in rectal cancer patients and in RCC patients treated with sunitinib (27, 44, 46), and it has been suggested that serum PlGF may be a sign of tumor escape when patients no longer respond to therapy (26). However, our results indicate that following administration of sFLT01, circulating PlGF was elevated in A673 tumor–bearing mice when the tumor was responding and during disease progression. The increase in circulating mPlGF levels likely reflects a systemic host response to sFLT01 and may limit the utility of serum PlGF as an indicator of efficacy or loss thereof.

The increased secretion of PlGF into circulation upon administration of antiangiogenic therapies could potentially promote more aggressive disease. PlGF can recruit VEGFR-1+ progenitor cells from the bone marrow, thereby promoting hematopoiesis (47). Hematopoietic stem cells, in turn, can then repopulate tumor vasculature and, in part, support angiogenesis and tumor regrowth. Similar to VEGF, PlGF promoted adult vasculogenesis by enhancing endothelial precursor cell recruitment and vessel formation at the site of neovascularization in B16F10 tumors (11). PlGF overexpression in an engineered rat C6 brain tumor line implanted in nude mice conferred protection against apoptosis and induced a survival phenotype in tumor endothelial cells and macrophages (10). PlGF enhanced the mobilization of endothelial cells and tumor cell invasion in the B16-BL6 melanoma model (48). Thus, PlGF can exert effects on numerous cell types that comprise the tumor microenvironment and may therefore promote malignant disease.

Indeed, controversy exists as to whether antiangiogenic therapies can actually accelerate metastasis. Sunitinib accelerated metastasis and decreased survival in mice when 231/LM2-4LUC+ cancer cells were delivered intravenously either before or after sunitinib treatment and also following the removal of primary xenograft tumors (49). A more invasive phenotype developed in the RIP1-Tag2 model when mice were treated with an anti–VEGFR-2 antibody or with sunitinib (50). Although mice initially responded to the therapy, the progression to end-stage disease was more rapid with the tumors becoming more invasive within the tumor microenvironment and resulting in an increase in distant metastasis. Furthermore, preclinical studies may incorporate the antiangiogenic agent in the absence of chemotherapy, as is the treatment regimen in the clinic. The factors that may contribute to antiangiogenic resistance and tumor escape are not clearly defined and have been the subject of several review articles (51–53). In addition to PlGF, cytokines such as FGF, SDF-1, IL-8, and PDGF are a few examples of putative compensatory agents that have been discussed. Stromal host cells and tumor-infiltrating cells such as myeloid cells and progenitors have also been implicated, whereas the malignant cells cannot be excluded.

The data we have generated using a novel fusion protein, sFLT01, that binds with great affinity to mouse and human PlGF and VEGF show that simultaneously neutralizing these 2 angiogenic growth factors in preclinical tumor models is effective and similar therapeutic protein strategies may offer some clinical benefit in treating cancer. The administration of sFLT01 resulted in elevated serum mPlGF levels both in tumor-bearing and non–tumor-bearing mice indicating a systemic host response. These results hold implications for therapies that simultaneously target PlGF and VEGF pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 5, 2010; revised November 15, 2010; accepted December 1, 2010; published OnlineFirst February 22, 2011.


Placental Growth Factor Upregulation Is a Host Response to Antiangiogenic Therapy


Clin Cancer Res 2011;17:976-988. Published OnlineFirst February 22, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-10-2687

Cited articles
This article cites 50 articles, 19 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/17/5/976.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/17/5/976.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.