Placental Growth Factor Is a Survival Factor for Tumor Endothelial Cells and Macrophages

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Abstract

The vascular endothelial growth factor (VEGF)-related factor, placental growth factor (PlGF), has been shown recently to play an important role in pathological VEGF-driven angiogenesis. In this study, we examine the effects of mPlGF/PlGF-2 overexpression in tumors grown from glioma cells containing a tetracycline-regulated mPlGF cDNA. Overexpression of mPlGF leads to increased tumor growth and vascular survival. When tetracycline is used to abruptly withdraw mPlGF overexpression, we see increased apoptosis in both vascular cells and macrophages. In addition, PlGF-2 induces survival gene expression and inhibits apoptosis in vitro. Thus, we propose that PlGF-2 contributes to tumor angiogenesis by providing increased survival function to endothelial cells and macrophages.

Introduction

VEGF-A is the founding member of a family of proteins that now includes VEGFs B, C, D, E, and PlGF. VEGF-A is essential for normal vascular development and promotes angiogenesis in a variety of in vitro and in vivo settings by modulating proliferation, permeability, and survival. The molecular nature of VEGF-A survival function and the signaling steps involved have been investigated in cultured endothelial cells. VEGF-A signaling leads to the phosphorylation of the survival kinase Akt/protein kinase B (1, 2). This appears to be dependent on functional VE-cadherin and VEGFR-2 (3). Additionally, VEGF-A has been shown to lead directly to increased expression of several antiapoptotic genes, such as bcl-2, survivin, and COX-2 (4–8). We and others have demonstrated that tumor blood vessels have decreased pericyte coverage or abnormal interactions between pericytes and endothelial cells (9, 10). Our previous studies in tumors and in normal development, and more recent work by others, have pointed to an extended dependency on soluble VEGF-A for endothelial cell survival to compensate for the scarcity of perivascular support cells (9, 11, 12).

Understanding more about how tumors promote survival of their vasculature is important for the identification of molecular targets that may be used to induce vascular regression during antiangiogenesis therapy. A more recently described member of the VEGF family, PGF, is widely expressed in tumors but not in normal adult or developing tissues except for placenta (reviewed in Ref. 13). Consistent with its lack of expression in the developing embryo, mPlGF null mice initially appeared to lack a phenotype. However, subsequent experiments have demonstrated that mPlGF is critical for VEGF-A-induced angiogenesis in tumors and in a variety of other types of pathological angiogenesis (14). Although mPlGF is unique among other angiogenic factors in having an essential role only in pathological settings, exactly how mPlGF functions is still unclear. It has been suggested that mPlGF may function, at least in part, by synergistically amplifying VEGF-A functions or by directly up-regulating VEGF-A (15).

An even more complicated situation likely exists in humans compared with mice, where the knock-out studies were performed. Although mice have only one PlGF gene, humans have two, PlGF-1 and PlGF-2. These differ in their ability to bind neuropilin. Recent work showed that PlGF-1 acts as a natural antagonist to VEGF-A when coexpressed in tumor cells. The mechanism was shown to be the formation of inactive PlGF-1/VEGF-A heterodimers (16). The mouse gene (mPlGF) is homologous to hPlGF-2, and it is this isoform that we have analyzed in the studies described below. In this study, we provide evidence that not only does mPlGF (PlGF-2) expression benefit the tumor vasculature and lead to increased vascularity and tumor growth, but that withdrawal of mPlGF leads to increased apoptosis and necrosis. In particular, both vascular cells and tumor macrophages appear dependent on mPlGF for survival. We show further evidence that one potential mechanism to explain mPlGF survival function is the up-regulation of survivin in endothelial cells.

Materials and Methods

Expression Vectors. PlGF mouse cDNA was cloned by RT-PCR from mouse embryos and confirmed by DNA sequencing. The cDNA was ligated into the pTETSPLICE vector. The pTET-TAK vector containing the tTA gene was cotransfected along with a neomycin vector for selection into the C6 glioma cells from American Type Culture Collection. Individual colonies were picked and expanded, and tetracycline regulation of PlGF was confirmed by Northern blot analysis using standard methods.

Tumor Growth. Anesthetized Nude mice were given either a slow-release, 21-day tetracycline pellet from Innovative America or given a mock surgery and suture. One million cells from the best-regulated C6:TET-PlGF clone were injected s.c. into the mice. Tumors were followed until the group without tetracycline had reached 0.5 cm. Some tumors from each group were harvested for histological examination. Tumors were measured, and the animals without tetracycline pellets were given tetracycline in the drinking water as described previously (reference).

Histology. Formalin-fixed tumors were embedded in paraffin and cut into 4–5-μm sections for histology. Antibodies used included lectin BS-1 to detect blood vessels and macrophages, antialaminin to visualize the basement membranes, and anti-α-smooth muscle actin to visualize pericytes, smooth muscle, and myofibroblasts. TUNEL analysis was done with the In Situ Cell Death Detection, POD (Roche Molecular Biochemicals).

Real-Time RT-PCR. Human dermal microvascular endothelial cells (Clonetics) were grown to confluence and starved overnight in serum-free medium. After stimulation with PlGF (R&D) for 7 h, unstimulated and stimulated cells were harvested for RNA and RT-PCR. RNA was isolated with RNEasy kit as recommended by the manufacturer (Qiagen). One μg of RNA was taken to RT-PCR reaction. Real-time PCR reactions were conducted in 50 μl contain-
Results and Discussion

To investigate the function of \textit{mPlGF} in glioma tumors, we created a tetracycline-regulated expression system in C6 rat glioma cells. This system uses the tetracycline promoter driving a mouse cDNA of \textit{mPlGF}. The \textit{mPlGF} expression is controlled by coexpression of the \textit{tTA} transcription factor that uses tetracycline as a cofactor such that expression is “on” in the absence of tetracycline and “off” in the presence of tetracycline. After transfection of the \textit{tTA} plasmid and the expression plasmid to C6 cells and isolation of several clonal lines, confirmation of controlled expression was done by Northern analysis of cells growing with or without tetracycline (Fig. 1A). Compensatory changes in the endogenous VEGF-A levels were not seen by Northern analysis, suggesting that at least this very related and potent angiogenic factor is not likely to be directly mediating the effects we see in tumors grown from these cells (Fig. 1B).

One million tumor cells were inoculated s.c. into Nude mice that either contained a slow-release tetracycline pellet or had a mock surgery to mimic pellet implantation. In the animals with systemic tetracycline, tumor growth was markedly delayed compared with the tumors without tetracycline (Fig. 1C). The experiment was repeated three times, and consistent differences in tumor growth were seen each time. Thus, the overexpression of \textit{mPlGF} in these tumors increased tumor growth. Tumors were intentionally harvested at ~0.5 cm\(^3\), prior to their becoming large and necrotic to avoid as much as possible extensive VEGF hypoxic induction. In addition, the blood vessels in these tumors were immature, as marked by abnormalities in vessel structure. These abnormalities included gaps and loss of polarity such that laminin surrounded some endothelial cells rather than demarcating the abluminal interior of blood vessels with and without tetracycline demonstrates that \textit{mPlGF} cDNA (0.65 kb) is highly induced in the absence of tetracycline. The 28S RNA is shown as a loading control. \textit{B}, VEGF-A endogenous expression (4.5 kb) is unaffected by cDNA expression in the induced cell line compared with the parental cells. \textit{C}, tumor growth is increased by \textit{mPlGF} cDNA expression when the animals, as evidenced by the mean tumor volume, are not given tetracycline (\(P < 0.0001\); bars, SD. The data shown are from a single experiment with 5 (+TET) and 7 (−TET) animals.

**DNA Content Analysis.** Primary bovine retina endothelial cells were starved 24 h, and then 50 ng of \textit{PIGF} or VEGF-A\(_{165}\) were added for an additional 48 h. Cells without added growth factor or grown in full medium were used as negative and positive controls, respectively. The cells were trypsinized, washed twice with PBS, and resuspended in cold 100% ethanol (drop by drop). After overnight incubation at −20°C, the cells were washed twice with PBS and resuspended in 500 \(\mu\)l of buffer [0.1% Triton X-100 (Sigma), 0.1 mm EDTA (pH 7.4), 0.05 mg/ml RNase (Promega), and 50 \(\mu\)g/ml propidium iodide] and incubated for 1 h in the dark before FACS analysis of DNA content.

![Fig. 2. Double immunohistochemistry for lectin staining of vascular cells in the \textit{mPlGF}-overexpressing tumors (A) compared with antilaminin staining of the same field (B) shows the irregular tumor blood vessels and abundant macrophages in these tumors. White arrows highlight blood vessels, and blue arrows illustrate tumor macrophages. Double staining for lectin (C) and anti-SMA (D) demonstrates that although many tumor SMA-positive cells are present, they are not making tight association with the blood vessels. White arrows highlight a large vessel with a single SMA-positive cell in close apposition, whereas yellow arrows illustrate many SMA-positive cells not in apposition to blood vessels. A and B are shown at a higher magnification than C and D. Erythrocytes are autofluorescent.](cancerres.aacrjournals.org/download/fig_2.png)
contacts (Fig. 2, tumor blood vessels without normal pericyte/smooth muscle cell detected without tight association to blood vessels, leaving many stroma. In addition, smooth muscle cells or myofibroblasts were evident as lectin-positive, laminin-negative single cells in the tumor blue arrows illustrate tumor macrophages. Tumor macrophages are minal face of the vessel. White arrows highlight blood vessels, and blue arrows illustrate tumor macrophages. Tumor macrophages are evident as lectin-positive, laminin-negative single cells in the tumor stroma. In addition, smooth muscle cells or myofibroblasts were detected without tight association to blood vessels, leaving many tumor blood vessels without normal pericyte/smooth muscle cell contacts (Fig. 2, C and D). Again, white arrows highlight blood vessels (note this large vessel has one detectable SMA-positive cell in contact with it), and blue arrows illustrate the smooth muscle actin-positive cells not in close apposition to blood vessels. Normal blood vessels are wrapped in pericytes or smooth muscle such that vessels as large as arterioles or venules (or the vessel highlighted in Fig. 2, C and D) have a clear layer of SMA-positive cells surrounding the endo- thelium. Lack of perivascular smooth muscle actin-positive cells has been previously correlated to an extended dependency on survival factors (12, 14, 15).

Analysis of mPlGF survival function was performed as reported previously for VEGF-A (17). In this assay, tumors are allowed to grow with expression of the TET:mPlGF cDNA in the absence of tetracycline. After giving these animals tetracycline, tumors are harvested after 20- and 36-h intervals and analyzed for signs of apoptosis by TUNEL staining. In control tumors, little apoptosis is evident at these relatively early stages of tumor growth (0.5 cm diameter; Fig. 3A). However, after mPlGF withdrawal by tetracycline, apoptosis is significant (Fig. 3, C and E) and localized mostly to cells that express the PlGF receptor (both endothelial cells and macrophages; Fig. 3E; Refs. 18–20). Macrophages were identified as lectin-positive cells not associated with a vessel-like structure or in contact with other lectin-positive cells and had a characteristic motile morphology with multiple cell processes. Tumors from parental C6 cells without mPlGF tetracycline vectors did not show increased levels of apoptosis after tetracycline. Thus, the loss of PlGF leads to apoptosis in a manner consistent with the hypothesis that mPlGF provides survival signals for VEGFR1-expressing cells. Fig. 3E, a magnification and overlay of the lower part of Fig. 3, B and C, shows most clearly that the apoptotic (red) cells are part of vascular structures or macrophages (yellow) that stain with the lectin BS-1. Quantitation of apoptosis in the tumors treated for 36 h with tetracycline compared with controls is shown in Fig. 3E. In addition to morphology, an antimacrophage antibody (F4) was used to identify the lectin-positive cells that responded to PlGF withdrawal as macrophages. Fig. 3G shows lectin staining, and [3H] shows the same field stained with antimacrophage F4. Together, these data show that both endothelial cells and macrophages die after mPlGF withdrawal.

To support the interpretation of the in vivo experiments, we also investigated PlGF survival functions in vitro. We compared the ability of 50 ng of PlGF-2 and 50 ng of VEGF-A to prevent cell death in serum-free medium (Fig. 4). FACS analysis of DNA content was used to quantitate the protective functions. PlGF-2 was able to reduce the number of cells in the sub-G₁ fraction from 23.22 to 7.39%. VEGF-A was able to reduce the dying cell number even further to 2.56%. Both of these factors are dimers and were administered in serum-free medium, independently confirming the ability of PlGF-2 to protect endothelial cells from apoptosis in a similar manner as VEGF-A.

Finally, PlGF-2 induction of endothelial cell survival gene expres-

![Fig. 3. Double staining for TUNEL and blood vessels is shown. Analysis of apoptosis in tumors expressing high levels of mPlGF (A) and the blood vessels of the same field (B) compared with apoptosis in tumors 36 h after mPlGF withdrawal (C) and the blood vessels and macrophages that are responding (D). An overlay and increased magnification of C and D is shown in E where the apoptotic cells are dark red and the vascular cells are yellow. F, the quantitation of apoptosis in controls and tumors after mPlGF withdrawal for 36 h (means, 4.37 in −TET control, and 24.19 in the 36-h mPlGF withdrawal) per high power field (P < 0.0001). Bars, SD. G, lectin staining of the same field as antimacrophage F4 staining (H).](cancerres.aacrjournals.org)
Using human dermal microvascular endothelial cells stimulated 7 h with 50 ng/ml of PlGF-2 (R&D), we saw reproducible increases in mPlGF expression as detected by real-time RT-PCR. GAPDH was used as an internal control. A, raw data (Ct values) are shown as averages of triplicate experiments. B, the Ct values were converted to absolute values using standard curves for each primer pair. These were first normalized to GAPDH and then to control cells.

Fig. 5. The results of endothelial cell survivin gene expression after 8-h stimulation with PlGF-2. The data shown are representative of multiple independent experiments performed. A, raw data (Ct values) are shown as averages of triplicate experiments. B, the Ct values were converted to absolute values using standard curves for each primer pair. They were first normalized to GAPDH and then to control cells.

Although previous studies have clearly defined an important role for mPlGF in pathological angiogenesis, the mechanisms used by mPlGF in these settings have not been fully elucidated. It has been suggested that mPlGF may function to promote pathological angiogenesis by potentiating the effects of VEGF-A on endothelial cells. Our studies of VEGF-A expression in the mPlGF tumor cells indicate that compensatory up-regulation of VEGF-A is not likely to be the mechanism of mPlGF "potentiation" of VEGF-A. However, in vivo we cannot rule out other forms of collaboration that may include enhanced signaling through the VEGF-A receptors or an increase in VEGFR-2 bioavailability attributable to saturation of VEGFR-1 by mPlGF. The in vitro experiments that demonstrate PlGF-2-mediated antiangiogenic gene expression, however, do strongly suggest that mPlGF has a direct effect on the survival of endothelial cells and extravascular tumor macrophages, even in the absence of exogenous VEGF-A.

These studies suggest that targeting PlGF-2 in tumor angiogenesis therapy may induce tumor vessel regression and apoptosis. Because mPlGF is not essential for growth or maintenance of physiological angiogenesis, it may be more advantageous to target PlGF-2 rather than VEGF-A in tumor angiogenesis. Moreover, in settings where promotion of new blood vessels is the goal, such as following myocardial or peripheral ischemia, PlGF-2 may promote stability of a newly formed vasculature via induction of endothelial cell antiapoptotic gene expression without promoting further vessel formation or hyperpermeability.

References
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