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Expression of placenta growth factor is regulated by both VEGF and hyperglycaemia via VEGFR-2

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Abstract

Placenta growth factor (PlGF) has been implicated in both physiological and pathological angiogenesis; however, little is known about what regulates its expression. In this study, retinal microvascular endothelial cells and pericytes were exposed to varying concentrations of VEGF and glucose and PlGF expression measured by RT-PCR and Western blotting. Both PlGF mRNA and protein were observed in unstimulated microvascular endothelial cells with only weak expression in pericytes. In endothelial cells, VEGF (100 ng/ml) and glucose (15 mM) induced an increase in expression of PlGF at both the mRNA and protein level while no effect was observed for pericytes. The increase in PlGF expression could be totally abolished by blocking VEGFR-2, and in the case of glucose by neutralising VEGF. VEGF-stimulated PlGF expression was largely inhibited by PD 98059, an inhibitor of mitogen-activated protein kinase (MAPK) and partially by GF 109203X, an inhibitor of protein kinase C (PKC), indicating that VEGF up-regulates PlGF expression via the MAPK signalling pathway and partially through PKC. Taken together, our findings suggest that VEGF orchestrates the contribution of PlGF in angiogenesis via more than one intracellular pathway and that hyperglycaemia, as occurs in diabetes, is an important regulator of PlGF expression via VEGF up-regulation.

Keywords: Placenta growth factor; Angiogenesis; Vascular endothelial growth factor; Vascular endothelial growth factor receptor 2; Hyperglycaemia; Proliferative diabetic retinopathy; Pericytes; Endothelial cells

Introduction

Aberrant capillary formation is a devastating complication of microvascular diseases (Boulton et al., 1988; Cai and Boulton, 2002). A number of growth factors have been implicated in these diseases of which the VEGF family is considered of major importance (Neufeld et al., 1999). There is now increasing evidence that placenta growth factor (PLGF), in addition to VEGF, plays a critical role in eliciting and sustaining a newly forming vasculature (Cai et al., 2003; Khaliq et al., 1998; Luttun et al., 2002a).

Placenta growth factor (PlGF) is a polypeptide growth factor that shares 53% amino acid sequence homology with the platelet-derived growth factor domain of VEGF (Maglione et al., 1991). This member of the VEGF family occurs at least as three isoforms PlGF-1, PlGF-2 and PlGF-3, which are derived from the same gene via alternate splicing (Cao et al., 1997; Maglione et al., 1993). PlGF-2 differs from PlGF-1 in that it has an additional 21 basic amino acid domain that confers a strong heparin binding site similar to that of $VEGF_{165}$ (Cao et al., 1997). Little is known about the structure and function of PlGF-3. Unlike VEGF, which acts by binding both to VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), PlGF is believed to bind only to VEGFR-1 (Park et al., 1994). VEGFR-1 is a high-affinity

Abbreviations: PlGF, placenta growth factor; VEGF, vascular endothelial growth factor; VEGFR-1 (Flt-1), vascular endothelial growth factor receptor-1 (fms-like tyrosine kinase); VEGFR-2 (KDR/Flk-1), vascular endothelial growth factor receptor-2 (human kinase-insert-domain-containing receptor/fetal liver kinase-1 of mouse); MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PI3K, phosphotidylinositol 3-kinase; PLC-γ, Phospholipase C-γ; ERK, extracellular signal regulated kinase.

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tyrosine kinase receptor, which regulates metabolic activity and more recently has been shown to be implicated in angiogenesis (Bussolati et al., 2001; Luttun et al., 2002b; Weng and Usman, 2001). However, its role in intracellular signalling is not fully understood. Although PlGF alone has been previously reported to have a weak mitogenic effect on cultured microvascular endothelial cells, it is now evident that PlGF plays a crucial role either by potentiating the angiogenic effects of VEGF by displacing VEGF from the 'VEGFR-1 decoy' (Park et al., 1994) and/or by forming heterodimers with VEGF that can activate the VEGFR-2 (DiSalvo et al., 1995). Furthermore, we recently demonstrated that PlGF via VEGFR-1 contributes to pathological angiogenesis by prolonging cell survival signals and maintaining vascular networks (Cai et al., 2003). Additionally, in mPlGF null mice, the lack of PlGF induced regression of neovascular complexes (Carmeliet et al., 2001).

PlGF expression was originally thought to be restricted to the primary cytotrophoblasts of the placenta (Shore et al., 1997), but is now known to be widely distributed in many nonplacental cells including cells of the microvasculature during pathological angiogenesis. PlGF is highly expressed in the capillaries associated with tumors, wound healing, proliferative diabetic retinopathy and rheumatoid arthritis (Bottomley et al., 2000; Carmeliet et al., 2001; Failla et al., 2000; Nomura et al., 1998; Yamashita et al., 1999). However, it is unclear which microvascular cells produce PlGF nor what regulates its expression. PlGF, unlike VEGF, is considered not to be strongly hypoxia-inducible, suggesting that other factors must be important in its regulation.

In this study, we examined the expression of PlGF at both the gene and protein level in retinal microvascular endothelial cells and pericytes in response to VEGF and hyperglycaemia (a predictor of ocular angiogenesis associated with diabetes). We further investigated the role of VEGFR and the intracellular signalling pathways involved in the regulation of PlGF expression.

Materials and methods

Reagents

Recombinant $VEGF_{165}$ and neutralising antibodies raised against the extracellular domains of VEGFR-1, VEGFR-2 and VEGFR-3 were obtained from R&D Systems (R&D Systems Europe Ltd., Abingdon, UK). Anti-phospho-MAP kinase/Erk1/2 polyclonal antibody was purchased from Upstate (Upstate, UK). Glucose was from BDH (BDH, UK). The antibody against PlGF was from Santa Cruz (UK). PD 98059 and GF 109203X were bought from LC Laboratory (Boston, MA). TRIzol Reagent was from Invitrogen (Glasgow, UK), Reverse-iT™ and PCR ReddyMix™ were purchased from Abgene (UK). All other materials were from Sigma unless otherwise stated.

Cell culture

Primary cultures of bovine retinal microvascular endothelial cells and pericytes were isolated from freshly isolated bovine eyes by homogenisation and a series of filtration steps, as described previously (Wong et al., 1987). Endothelial cells were maintained in endothelial cell basal medium with growth supplement (TCS Works Ltd., Buckingham, UK). Endothelial cells were characterised by their cobblestone appearance and expression of factor VIII antigen (Wong et al., 1987). Pericytes were cultured in Eagle's Minimal Essential Medium (GibcoBRL, UK) containing 10% fetal calf serum (FCS) and identified and distinguished from endothelial cells by their size and irregular morphology (Wong et al., 1987). Both types of cells were used between passages 1 and 3 for all experiments. Upon reaching near confluence, the medium was replaced with medium containing either VEGF at 10 and 100 ng/ml or glucose at 15 and 25 mM for 24 and 48 h (these time points were identified as optional from preliminary experiments). Medium alone (containing 5.6 mM glucose) served as the control for all experiments.

RT-PCR

Total RNA was isolated from cells that had been cultured under various concentrations of VEGF or glucose, using TRIzol Reagent (Invitrogen), and then analysed by RT-PCR using Reverse-iT™ and PCR ReddyMix™ (ABgene) according to the manufacturer's protocol. The RNA concentration was determined by the spectrophotometer from GeneQuant II (Pharmacia Biotech, England) and equal quantity of total RNA was used from different samples. The primers for PlGF were designed according to the sequences of PlGF-1 and -2 mRNA from the GenBank™. The oligonucleotide primers used for the amplification of bovine PlGF cDNA were 5′-GTG CCC TTC CAG CAA GTG T-3′ and 5′-TCT TCC GAG GGA CTG GTT AC-3′. The resultant PCR products were 347 and 410 bp for PlGF-1 and PlGF-2, respectively. The cDNA was amplified using the PCR ReddyMix™, each cycle consisting of 20 s at 94°C, 30 s at 55°C and 60 s at 72°C. All the samples were amplified in a linear amplification range established using a serial cDNA dilution and varying the number of cycles. PCR products were electrophoresed onto a 1.2% agarose gel containing ethidium bromide and visualised under UV light. The relative intensities of the bands were quantified by densitometric analysis.

Immunoprecipitation and Western blotting

For detection of PlGF, conditioned medium was centrifuged at 200 × *g* for 5 min to remove any debris.

The protein concentration in the medium was determined by the BCA protein assay (Pierce, UK) using bovine serum albumin as standard according to the manufacturer's instructions. The medium was immunoprecipitated by incubation with an anti-PlGF antibody with reactivity against both PlGF-1 and -2 and then protein A/G-agarose (Santa Cruz Biotechnology, USA). The immunoprecipitates were subjected to SDS-PAGE and the resolved proteins transferred onto nitrocellulose membranes. The membranes were probed with an anti-PlGF antibody followed by incubation with a secondary antibody conjugated with HRP. The ECL system (Santa Cruz) was used to visualise the bands. For detection of phosphorylation of ERK1/2, endothelial cells were pretreated with or without 25 μM PD98059 for 20 min before administration of VEGF100 ng/ml. Vehicle only was as a control. After incubation for 24 h, endothelial cells were harvested and lysed with RIPA buffer (50 mM Tris–HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM of EDTA, PMSF, Na_3VO_4, NaF each) containing protease inhibitors (1 μg/ml each of aprotinin, leupeptin, pepstatin). Western blotting were performed and the phosphorylation of ERK1/2 was detected with an anti-phospho-MAP kinase/Erk1/2 polyclonal antibody.

Inhibition studies

To identify which VEGFR is involved in the regulation of PlGF expression, total RNA was isolated from cultures treated with 60 ng neutralising antibody to VEGFR-1, -2, or -3 per milliliter of culture medium for 1 h before incubation with 100 ng/ml VEGF for 24 h. PlGF mRNA expression was determined using RT-PCR. To determine if glucose regulates PlGF expression via the production of VEGF, total RNA was isolated at 24 h after stimulation of cells by 15 mM glucose with or without 200 ng/ml anti-VEGF antibody.

Intracellular signalling studies

To investigate the intracellular signalling pathways involved in the regulation of PlGF expression, endothelial cells were incubated with either PD 98059 (an inhibitor of MAPK) at 25 μM, or GF 109203X (an inhibitor of PKC) at 5 μM, or Wortmannin (an inhibitor of PI3 kinase) at 100 ng/ml, or medium containing the DMSO carrier alone for 20 min before the addition of 100 ng/ml VEGF. To rule out the inhibitors having an effect on the RT-PCR data, they were included in the positive control of the First-Strand Synthesis Kit. Neither inhibitor had an effect on the PCR results (data not shown).

Statistical analysis

The results represent the mean of at least three separate experiments. Statistical analysis was carried out by unpaired Student's *t* test. Values were measured by laser densitometry and expressed as mean ± SEM. Statistical significance was defined as $P < 0.05$.

Results

Expression of PlGF mRNA in microvascular cells

PlGF-1 and PlGF-2 mRNA expression was observed in both retinal microvascular endothelial cells and pericytes under normal culture conditions (Fig. 1). The bovine primers gave an intense band for PlGF-2 at the expected 410 bp and a very weak signal for PlGF-1 at 347 bp. The signal intensity for PlGF-2 mRNA from the microvascular endothelial cells was 3.5 times stronger than that of the pericytes.

The effect of VEGF and glucose on PlGF expression in endothelial cells

Exposure of microvascular endothelial cells to 10 and 100 ng/ml VEGF or 15 mM glucose for 24 h resulted in a significant increase in PlGF-2 mRNA expression compared to untreated control (Fig. 2A). This increase was greatest at 100 ng/ml VEGF and 15 mM glucose. Exposure to 25 mM glucose did not affect PlGF-2 mRNA expression compared to control. Ten-millimolar glucose was similar to control while 20 mM glucose was similar to that with 15 mM glucose on the expression of PlGF. A similar result was observed following immunoprecipitation and immunoblotting of the conditioned

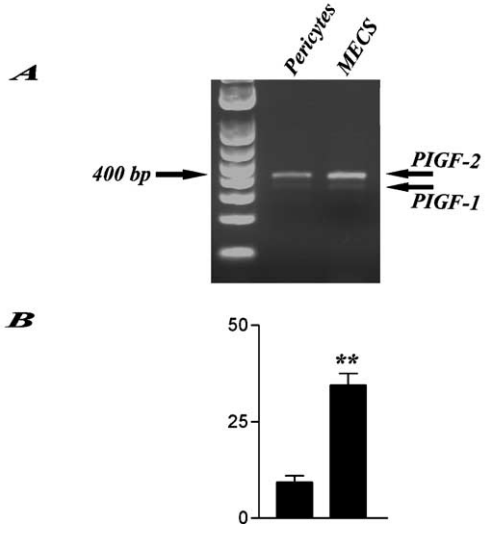

Fig. 1. RT-PCR analysis of PlGF mRNA expression in microvascular endothelial cells and pericytes. Total RNA was isolated from cultured bovine retinal microvascular endothelial cells and pericytes. After reverse transcription, cDNA was amplified using bovine-specific primers. (A) The RT-PCR products were analyzed by agarose gel electrophoresis. The PlGF-1 and PlGF-2 bands ran at 347 and 410 bp, respectively. (B) Band intensity (arbitrary units) for PlGF-2 was quantified by laser densitometry from at least three separate experiments. The vertical bar represent SEM; $^{**}P < 0.01$.

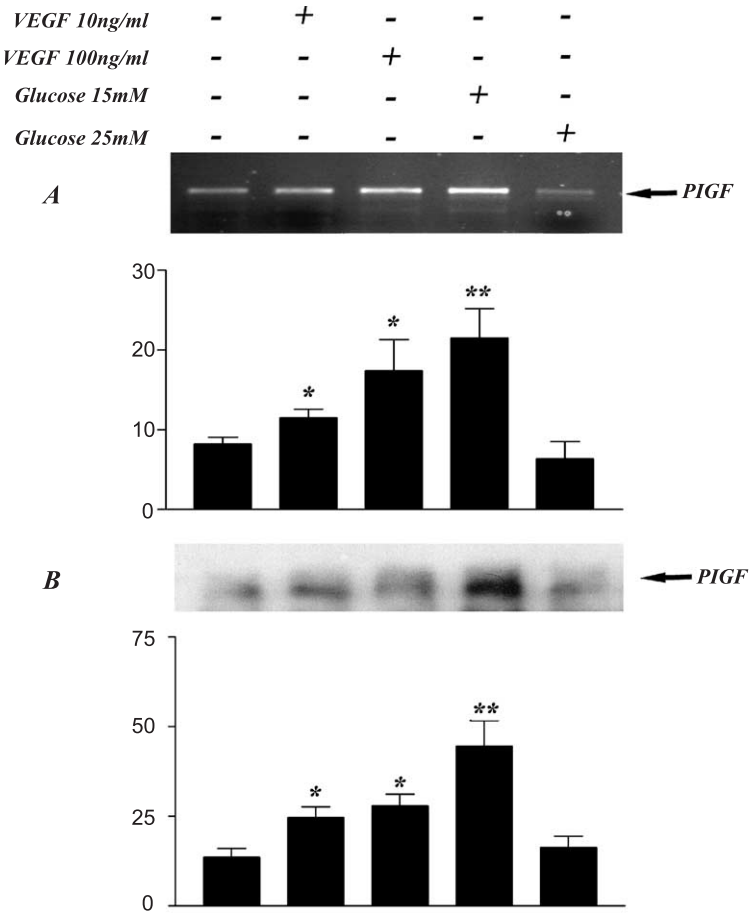

Fig. 2. The expression of PlGF mRNA and protein in microvascular endothelial cells under different conditions. Microvascular endothelial cells were treated with VEGF 10 and 100 ng/ml, glucose 15 mM or glucose 25 mM, or exposed to medium alone (5.6 mM glucose) for 24 h. (A) Total RNA was extracted and RT-PCR performed to determine changes in PlGF mRNA expression. PCR products were analyzed by agarose gel electrophoresis and intensity of the PlGF-2 bands was quantified by laser densitometry from at least three separate experiments. (B) Conditioned media were immunoprecipitated using a polyclonal antibody raised against PlGF and the immunoprecipitates electrophoresed on a 12% SDS-PAGE followed by blotting on a nitrocellulose membrane. Positive bands were visualized by an ECL detection system. Band intensity (arbitrary units) for PlGF was quantified by laser densitometry from at least three separate experiments. Vertical bars represent ± SEM; P values (*$P < 00.5$, **$P < 0.01$) indicate the significant difference between VEGF/glucose stimulation and untreated control.

medium for PlGF peptide (Fig. 2B). The secreted peptide was present at higher amounts in the medium of cells exposed to 10 and 100 ng/ml VEGF or 15 mM glucose compared to either control cultures or cells exposed to 25 mM glucose. A faint PCR band could be observed at 347 bp (PlGF-1), which appeared to be more intense at 100 ng/ml VEGF and 15 mM glucose than any of the other conditions tested. There was little difference between PlGF expression at 24 and 48 h post exposure apart from a slight increase at 10 ng/ml VEGF at the later time point (data not shown). Neither VEGF nor glucose appeared to have any effect on the regulation of PlGF expression in pericytes at either 24 or 48 h post exposure (data not shown).

Identification of the receptor mediating VEGF-induced PlGF expression

To identify the receptor(s) responsible for the VEGF-induced expression of PlGF, VEGFR-1, -2 and -3 were blocked by neutralising antibodies before addition of VEGF. The stimulatory effect of VEGF on PlGF expression was completely inhibited by blocking VEGFR-2 (Fig. 3B) while blockade of either VEGFR-1 or -3 had no effect on the expression of PlGF mRNA (Figs. 3A,C). Blocking VEGFR-2 also blocked PlGF peptide release into the conditioned medium (Fig. 3D).

We also investigated the mechanism by which 15 mM glucose can up-regulate the expression of PlGF-2 mRNA. PlGF expression induced by 15 mM glucose was decreased by greater than 85% in microvascular endothelial cells treated with an anti-$VEGF_{165}$ antibody compared to cells exposed to glucose alone (Fig. 4).

The intracellular signalling pathways involved in PlGF expression

Because previous reports have shown that MAPK, PKC and PI3K have significant roles in VEGF-induced intra-

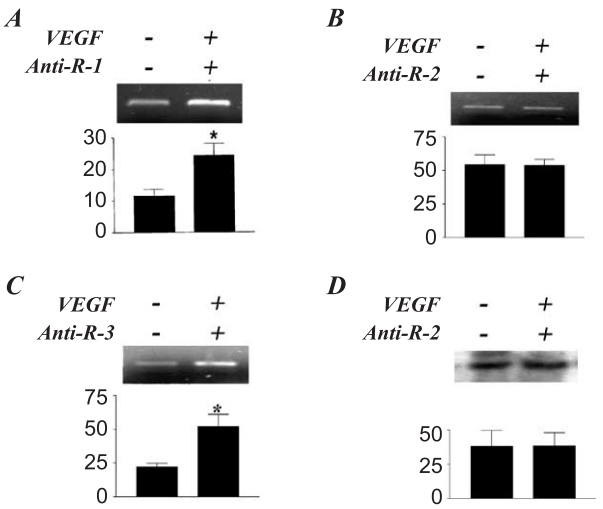

Fig. 3. The effects of blocking VEGF receptors on the expression of PlGF mRNA and protein. Microvascular endothelial cells were treated with neutralizing antibodies for VEGFR-1 (A), VEGFR-2 (B, D) and VEGFR-3 (C) for 1 h before incubation with 100 ng/ml VEGF. Medium alone acted as the control. After incubation for 24 h, the cells were harvested for RT-PCR (A–C) and the culture medium for PlGF peptide analysis (D). The PCR product for PlGF-2 and the PlGF peptide were analyzed electrophoretically and the band intensity (arbitrary units) quantified by laser densitometry from at least three separate experiments. The vertical bars represent the SEM; *$P < 00.5$.

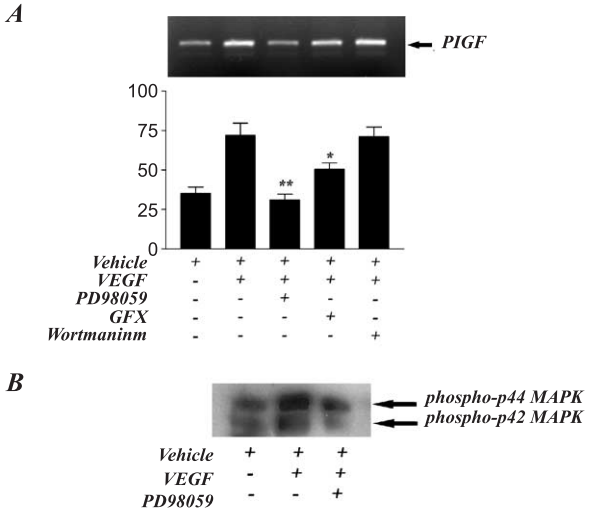

Fig. 5. VEGF up-regulates the expression of PlGF mRNA mainly via the MAPK pathway. (A) Microvascular endothelial cells were pretreated with either MAPK inhibitor, PD 98059; PKC inhibitor, GF 109203X; or a PI3K inhibitor, Wortmannin or the DMSO vehicle for 1 h before stimulation with 100 ng/ml VEGF for 24 h. Total RNA was isolated and RT-PCR was conducted. Band intensity (arbitrary units) for PlGF-2 was quantified by laser densitometry from at least three separate experiments. Vertical bars represent ± SEM; P values (*$P < 00.5$, **$P < 0.01$) indicate the significant difference between VEGF stimulation with and without inhibitor. (B) Microvascular endothelial cells were treated with either 100 ng/ml VEGF or 100 ng/ml VEGF plus PD 98059 for 24 h, after which the cells were lysed by RIPA buffer. The phosphorylation of ERK was detected with anti-phospho-Erk1/2 polyclonal antibody. Positive bands were visualized by an ECL detection system. A representative from three separate experiments is shown.

cellular signalling pathways, we determined which of these kinases was involved in the regulation of PlGF expression. Addition of the MAPK inhibitor PD 98059 completely abolished VEGF-induced up-regulation of PlGF expression while addition of the PI3K inhibitor Wortmannin had no effect. The PKC inhibitor GF 109203X only partially abolished the VEGF-induced expression of PlGF (Fig. 5A). We further demonstrated that VEGF 100 ng/ml significantly increased the phosphorylation of ERK 1/2 and that PD 98059 abrogated this phosphorylation induced by VEGF (Fig. 5B).

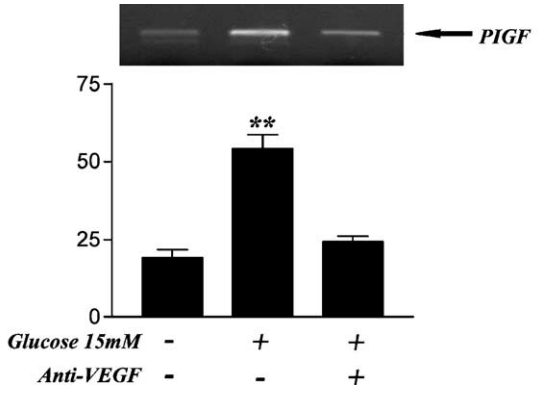

Fig. 4. Effect of $VEGF_{165}$ neutralizing antibody on glucose-induced PlGF mRNA expression. Microvascular endothelial cells were exposed to 15 mM glucose for 24 h in the presence or absence of 200 ng/ml neutralizing antibody for $VEGF_{165}$. Medium alone acted as the control. Total RNA was isolated and RT-PCR performed as described above. Band intensity for PlGF-2 was quantified by laser densitometry from at least three separate experiments. Vertical bars represent ± SEM. The expression of PlGF-2 mRNA in cells exposed to 15 mM glucose was significantly reduced (**$P < 0.01$) in the presence of a neutralizing antibody for $VEGF_{165}$.

Discussion

There is increasing evidence that PlGF plays a major role in pathological angiogenesis (Carmeliet et al., 2001; Luttun et al., 2002a) and that its function differs between different vascular beds. Furthermore, while PlGF has been shown to exert a weak angiogenic effect via binding to VEGFR-1 there is increasing evidence that it acts synergistically with VEGF-A, enhancing the latter's activity (Carmeliet et al., 2001). Activation of VEGFR-1 by elevating PlGF not only results in intermolecular transphosphorylation of VEGFR-2 but also produces a distinct biological effect (Autiero et al., 2003). Studies in PlGF-deficient mice have demonstrated that loss of PlGF reduces vascular leakage induced by skin wounding, allergens and neurogenic inflammation (Carmeliet et al., 2001; Luttun et al., 2002a). PlGF appears to exert its effect by enhancing the permeability-inducing activity of VEGF as application of endogenous PlGF does not increase vascular permeability in a mouse model (Luttun et al.,

2002a). Up-regulation of PlGF mRNA and peptide have been demonstrated during angiogenesis (Bottomley et al., 2000; Carmeliet et al., 2001; Khaliq et al., 1998; Nomura et al., 1998; Yamashita et al., 1999); however, temporal expression appears to be dependent on tissue type and the type of pathology. In tissues such as skin (Luttun et al., 2002a) and placenta (Achen et al., 1997), PlGF expression is observed early in the angiogenic process presumably associated with increasing vascular permeability while in the retina and certain tumors its expression is only observed during the later stages of angiogenesis (Carmeliet et al., 2001; Khaliq et al., 1998), suggesting it plays a role in sustaining VEGF-induced angiogenesis. Evidence is provided that PlGF also acts as a survival factor for microvascular endothelial cells (Adini et al., 2002; Cai et al., 2003) by preventing apoptosis.

Little is known about what regulates the expression of PlGF during pathologic neovascularisation. It is considered that, unlike VEGF-A, PlGF is not hypoxia-inducible (Cao et al., 1996; Khaliq et al., 1999; Simpson et al., 1999; Yonekura et al., 1999), although some studies have reported that both hypoxia and hyperoxia can up-regulate PlGF expression during angiogenesis in certain tissues (Khaliq et al., 1999; Nomura et al., 1998). To further investigate the regulation of PlGF expression, we have focussed on VEGF and hyperglycaemia; VEGF because of its prominent role in angiogenesis and its synergism with PlGF; hyperglycaemia because of its association with retinal neovascularisation in diabetes (Boulton et al., 1988; Cai and Boulton, 2002). We show in this study that both endothelial cells and pericytes, the principal cell types of the microvasculature, express PlGF-1 and -2. While PlGF-2 was the major transcript for both endothelial cells and pericytes, its expression was more than 3.5 times greater in endothelial cells compared to pericytes. This is in agreement with the observations of Yonekura et al. (1999) who studied human dermal microvascular endothelial cells and bovine retinal pericytes. The constitutive expression of PlGF-2 in untreated cells may play a role in tissue maintenance as PlGF is known to be expressed at low levels in a variety of tissues. However, the basal levels in cell culture may also reflect, at least in part, "activation" of the cells during the culture process. That VEGF is up-regulated by hypoxia may at least in part explain the reports that hypoxia can up-regulate PlGF. Interpretation from such studies will be highly dependent on the methodology used and the degree of hypoxia obtained (Boulton et al., 1994).

Both VEGF and hyperglycaemia (15 mM glucose) were able to up-regulate PlGF expression at the gene and protein level. Microvascular endothelial cells abundantly express VEGFR-1 and VEGFR-2 (de Vries et al., 1992; Terman et al., 1992) while pericytes express VEGFR-1 only (Takagi et al., 1996). Our observation that VEGF-induced up-regulation of PlGF occurs via VEGFR-2 has several important implications for angiogenesis. First, VEGF up-regulates PlGF via VEGFR-2; in turn, PlGF enhances VEGF-driven angiogenesis through the unique cross-talking between VEGFR-1 and VEGFR-2. Second, VEGFR-2 is up-regulated in the endothelial cells of new vessels, making them more susceptible to VEGF stimulation. Third, regulation of PlGF via VEGFR-2 will ensure availability of VEGFR-1 for ligand binding. Fourth, this effect is relatively endothelial specific since cells without VEGFR-2, e.g., pericytes, do not up-regulate PlGF in response to exogenous VEGF. However, PlGF may also regulate hematopoietic stem cells (Katoh et al., 1995), retinal progenitor cells (Yang and Cepko, 1996), neurons (Jin et al., 2002) and osteoblasts (Deckers et al., 2002), which all express VEGFR-2. Fifth, PlGF has both an autocrine and paracrine role although the importance of the latter is unclear. Elevated glucose is known to modify endothelial cell and pericyte behavior (Asakawa et al., 1996; Beltramo et al., 2002; Okuda et al., 1996; Romeo et al., 2002; Sato et al., 2001; Williams et al., 1997) including attachment, proliferation, metabolic activity, apoptosis, nitric oxide synthesis and growth factor production. One such growth factor to be up-regulated by elevated glucose is VEGF (De Vriese et al., 2001; Tilton et al., 1997; Williams et al., 1997). Our observation that neutralisation of VEGF inhibits the glucose-induced up-regulation of PlGF supports the observations that (i) glucose up-regulates VEGF in microvascular endothelial cells and (ii) glucose up-regulates PlGF via VEGF and VEGFR-2. Clinically, one of the criteria to diagnose diabetes is if plasma glucose reaches or exceeds 11.1 mM (Edwards et al., 1995). Fifteen millimolars of glucose is therefore within the hyperglycaemic range associated with diabetes while 25 mM glucose is towards the highest levels associate with diabetes and can cause cell cytoxicity and make cells lose their compensation ability. We conclude that 25 mM glucose has no effect on VEGF production and therefore does not up-regulate PlGF in our test system. This is in general agreement with studies on other cell types that report that glucose levels up to 20 mM up-regulate VEGF with little or no effect at higher concentrations (De Vriese et al., 2001; Sone et al., 1996; Tilton et al., 1997; Williams et al., 1997).

Activation of VEGFR-2 can initiate several intracellular pathways involving PLC-γ, PKC, MAPK and PI3K (Ortega et al., 1999; Zachary and Gliki, 2001). In our study, we were able to demonstrate that VEGF greatly increased the phosphorylation of ERK1/2 and PD98059, an inhibitor of MAPK, abrogated this effect and abolished the expression of PlGF induced by VEGF. However, blocking PKC also resulted in a slight reduction in VEGF-induced PlGF expression, suggesting that there is cross-talk between intracellular signaling pathways. Furthermore, the reported PlGF-specific activation of the ERK pathway in porcine aortic endothelial cells suggests that interactions between the VEGFR-1 and VEGFR-2 may serve to regulate both PlGF expression and VEGF action (Landgren et al., 1998).

This study clearly demonstrates that VEGF may play a critical role in the up-regulation of PlGF, which can promote pathological angiogenesis in the following ways: (i)

activated by PlGF, VEGFR-1 transactivates VEGFR-2 and thereby enhances the response to VEGF-A (Autiero et al., 2003); (ii) increasing levels of PlGF will compete for VEGF-A binding to the high-affinity receptor VEGFR-1, which will, in turn, allow VEGF-A to access the lower affinity VEGFR-2. Thus, PlGF can make and keep endothelial cells more sensitive to lower concentrations of VEGF-A that could be supplied by surrounding cells and/or endothelial cells themselves; (iii) PlGF:VEGF heterodimers may supplement low levels of VEGF, thereby contributing to the stimulation of endothelial cell growth (DiSalvo et al., 1995); (iv) the production of the heparin-binding form of PlGF may cause retrieval of sequestered heparin-binding angiogenic factors such as VEGF-A_{165} in a soluble form (Park et al., 1994); (v) PlGF could recruit VEGFR-1-positive bone marrow-derived cells, which have been recently shown to contribute to pathological angiogenesis (Luttun et al., 2002b); (vi) PlGF induces VEGFR-1 signalling independently of cross talk with VEGFR-2 (Autiero et al., 2003; Cai et al., 2003).

Acknowledgments

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References

Achen, M.G., Gad, J.M., Stacker, S.A., Wilks, A.F., 1997. Placenta growth factor and vascular endothelial growth factor are co-expressed during early embryonic development. Growth Factors 15, 69–80.

Adini, A., Kornaga, T., Firoozbakht, F., Benjamin, L.E., 2002. Placental growth factor is a survival factor for tumor endothelial cells and macrophages. Cancer Res. 62, 2749–2752.

Asakawa, H., Miyagawa, J., Higashiyama, S., Goishi, K., Hanafusa, T., Kuwajima, M., Taniguchi, N., Matsuzawa, Y., 1996. High glucose and hyperosmolarity increase heparin-binding epidermal growth factor-like growth factor (HB-EGF) production in cultured human aortic endothelial cells. Cell Biochem. Funct. 14, 181–186.

Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., Kroll, J., Plaisance, S., De Mol, M., Bono, F., et al., 2003. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat. Med. 9, 936–943.

Beltramo, E., Pomero, F., Allione, A., D'Alu, F., Ponte, E., Porta, M., 2002. Pericyte adhesion is impaired on extracellular matrix produced by endothelial cells in high hexose concentrations. Diabetologia 45, 416–419.

Bottomley, M.J., Webb, N.J., Watson, C.J., Holt, L., Bukhari, M., Denton, J., Freemont, A.J., Brenchley, P.E., 2000. Placenta growth factor (PlGF) induces vascular endothelial growth factor (VEGF) secretion from mononuclear cells and is co-expressed with VEGF in synovial fluid. Clin. Exp. Immunol. 119, 182–188.

Boulton, M.E., McLeod, D., Garner, A., 1988. Vasoproliferative retinopathies: clinical, morphogenetic and modulatory aspects. Eye 2, S124–S139 (Suppl.).

Boulton, M., Khaliq, A., McLeod, D., Moriarty, P., 1994. Effect of 'hypoxia' on the proliferation of microvascular cells in vitro. Exp. Eye Res. 59, 243–246.

Bussolati, B., Dunk, C., Grohman, M., Kontos, C.D., Mason, J., Ahmed, A., 2001. Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide. Am. J. Pathol. 159, 993–1008.

Cai, J., Boulton, M., 2002. The pathogenesis of diabetic retinopathy: old concepts and new questions. Eye 16, 242–260.

Cai, J., Ahmad, S., Jiang, W.G., Huang, J., Kontos, C.D., Boulton, M., Ahmed, A., 2003. Activation of vascular endothelial growth factor receptor-1 sustains angiogenesis and Bcl-2 expression via the phosphatidylinositol 3-kinase pathway in endothelial cells. Diabetes 52, 2959–2968.

Cao, Y., Linden, P., Shima, D., Browne, F., Folkman, J., 1996. In vivo angiogenic activity and hypoxia induction of heterodimers of placenta growth factor/vascular endothelial growth factor. J. Clin. Invest. 98, 2507–2511.

Cao, Y., Ji, W.R., Qi, P., Rosin, A., 1997. Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. Biochem. Biophys. Res. Commun. 235, 493–498.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H., et al., 2001. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat. Med. 7, 575–583.

de Vries, C., Escobedo, J.A., Ueno, H., Houck, K., Ferrara, N., Williams, L.T., 1992. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255, 989–991.

De Vriese, A.S., Tilton, R.G., Stephan, C.C., Lameire, N.H., 2001. Vascular endothelial growth factor is essential for hyperglycemia-induced structural and functional alterations of the peritoneal membrane. J. Am. Soc. Nephrol. 12, 1734–1741.

Deckers, M.M., van Bezooijen, R.L., van der Horst, G., Hoogendam, J., van Der Bent, C., Papapoulos, S.E., Lowik, C.W., 2002. Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. Endocrinology 143, 1545–1553.

DiSalvo, J., Bayne, M.L., Conn, G., Kwok, P.W., Trivedi, P.G., Soderman, D.D., Palisi, T.M., Sullivan, K.A., Thomas, K.A., 1995. Purification and characterization of a naturally occurring vascular endothelial growth factor:placenta growth factor heterodimer. J. Biol. Chem. 270, 7717–7723.

Edwards, C.R.W., Bouchier, I.A.D., Haslett, C., 1995. Endocrine and metabolic diseases. Davidson's Principles and Practice of Medicine. Churchill Livingstone.

Failla, C.M., Odorisio, T., Cianfarani, F., Schietroma, C., Puddu, P., Zambruno, G., 2000. Placenta growth factor is induced in human keratinocytes during wound healing. J. Invest. Dermatol. 115, 388–395.

Jin, K., Zhu, Y., Sun, Y., Mao, X.O., Xie, L., Greenberg, D.A., 2002. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc. Natl. Acad. Sci. U. S. A. 99, 11946–11950.

Katoh, O., Tauchi, H., Kawaishi, K., Kimura, A., Satow, Y., 1995. Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation. Cancer Res. 55, 5687–5692.

Khaliq, A., Foreman, D., Ahmed, A., Weich, H., Gregor, Z., McLeod, D., Boulton, M., 1998. Increased expression of placenta growth factor in proliferative diabetic retinopathy. Lab. Invest. 78, 109–116.

Khaliq, A., Dunk, C., Jiang, J., Shams, M., Li, X.F., Acevedo, C., Weich, H., Whittle, M., Ahmed, A., 1999. Hypoxia down-regulates placenta growth factor, whereas fetal growth restriction up-regulates placenta growth factor expression: molecular evidence for "placental hyperoxia" in intrauterine growth restriction. Lab. Invest. 79, 151–170.

Landgren, E., Schiller, P., Cao, Y., Claesson-Welsh, L., 1998. Placenta growth factor stimulates MAP kinase and mitogenicity but not phospholipase C-gamma and migration of endothelial cells expressing Flt 1. Oncogene 16, 359–367.

Luttun, A., Brusselmans, K., Fukao, H., Tjwa, M., Ueshima, S., Herbert, J.M., Matsuo, O., Collen, D., Carmeliet, P., Moons, L., 2002a. Loss of placental growth factor protects mice against vascular permeability in pathological conditions. Biochem. Biophys. Res. Commun. 295, 428–434.

Luttun, A., Tjwa, M., Moons, L., Wu, Y., Angelillo-Scherrer, A., Liao, F., Nagy, J.A., Hooper, A., Priller, J., De Klerck, B., et al., 2002b. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat. Med. 8, 831–840.

Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P., Persico, M.G., 1991. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc. Natl. Acad. Sci. U. S. A. 88, 9267–9271.

Maglione, D., Guerriero, V., Viglietto, G., Ferraro, M.G., Aprelikova, O., Alitalo, K., Del Vecchio, S., Lei, K.J., Chou, J.Y., Persico, M.G., 1993. Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. Oncogene 8, 925–931.

Neufeld, G., Cohen, T., Gengrinovitch, S., Poltorak, Z., 1999. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 13, 9–22.

Nomura, M., Yamagishi, S., Harada, S., Yamashima, T., Yamashita, J., Yamamoto, H., 1998. Placenta growth factor (PlGF) mRNA expression in brain tumors. J. Neurooncol. 40, 123–130.

Okuda, Y., Adrogue, H.J., Nakajima, T., Mizutani, M., Asano, M., Tachi, Y., Suzuki, S., Yamashita, K., 1996. Increased production of PDGF by angiotensin and high glucose in human vascular endothelium. Life Sci. 59, 1455–1461.

Ortega, F., Marti, E., Cascante, M., 1999. New insights into metabolic pathway optimization by analogy with industrial manufacturing processes. Biochem. Soc. Trans. 27, 276–281.

Park, J.E., Chen, H.H., Winer, J., Houck, K.A., Ferrara, N., 1994. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J. Biol. Chem. 269, 25646–25654.

Romeo, G., Liu, W.H., Asnaghi, V., Kern, T.S., Lorenzi, M., 2002. Activation of nuclear factor-kappaB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. Diabetes 51, 2241–2248.

Sato, T., Paryani, G., Kao, R.S., Li, A.F., Roy, S., 2001. Antisense oligonucleotides modulate high glucose-induced laminin overexpression and cell proliferation: a potential for therapeutic application in diabetic microangiopathy. Antisense Nucleic Acid Drug Dev. 11, 387–394.

Shore, V.H., Wang, T.H., Wang, C.L., Torry, R.J., Caudle, M.R., Torry, D.S., 1997. Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast. Placenta 18, 657–665.

Simpson, D.A., Murphy, G.M., Bhaduri, T., Gardiner, T.A., Archer, D.B., Stitt, A.W., 1999. Expression of the VEGF gene family during retinal vaso-obliteration and hypoxia. Biochem. Biophys. Res. Commun. 262, 333–340.

Sone, H., Kawakami, Y., Okuda, Y., Kondo, S., Hanatani, M., Suzuki, H., Yamashita, K., 1996. Vascular endothelial growth factor is induced by long-term high glucose concentration and up-regulated by acute glucose deprivation in cultured bovine retinal pigmented epithelial cells. Biochem. Biophys. Res. Commun. 221, 193–198.

Takagi, H., King, G.L., Aiello, L.P., 1996. Identification and characterization of vascular endothelial growth factor receptor (Flt) in bovine retinal pericytes. Diabetes 45, 1016–1023.

Terman, B.I., Dougher-Vermazen, M., Carrion, M.E., Dimitrov, D., Armellino, D.C., Gospodarowicz, D., Bohlen, P., 1992. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem. Biophys. Res. Commun. 187, 1579–1586.

Tilton, R.G., Kawamura, T., Chang, K.C., Ido, Y., Bjercke, R.J., Stephan, C.C., Brock, T.A., Williamson, J.R., 1997. Vascular dysfunction induced by elevated glucose levels in rats is mediated by vascular endothelial growth factor. J. Clin. Invest. 99, 2192–2202.

Weng, D.E., Usman, N., 2001. Angiozyme: a novel angiogenesis inhibitor. Curr. Oncol. Rep. 3, 141–146.

Williams, B., Gallacher, B., Patel, H., Orme, C., 1997. Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro. Diabetes 46, 1497–1503.

Wong, H.C., Boulton, M., Marshall, J., Clark, P., 1987. Growth of retinal capillary endothelia using pericyte conditioned medium. Invest. Ophthalmol. Visual Sci. 28, 1767–1775.

Yamashita, H., Eguchi, S., Watanabe, K., Takeuchi, S., Yamashita, T., Miura, M., 1999. Expression of placenta growth factor (PIGF) in ischaemic retinal diseases. Eye 13, 372–374.

Yang, K., Cepko, C.L., 1996. Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. J. Neurosci. 16, 6089–6099.

Yonekura, H., Sakurai, S., Liu, X., Migita, H., Wang, H., Yamagishi, S., Nomura, M., Abedin, M.J., Unoki, H., Yamamoto, Y., Yamamoto, H., 1999. Placenta growth factor and vascular endothelial growth factor B and C expression in microvascular endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis. J. Biol. Chem. 274, 35172–35178.

Zachary, I., Gliki, G., 2001. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc. Res. 49, 568–581.