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The discovery of placenta growth factor and its biological activity

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Abbreviations: BF-2, forkhead/winged helix transcription factor FoxD1; eNos, enodothelial nitric oxide synthase; HIFs, hypoxia inducible factors; HRE, hypoxia responsive element; MTF-1, metal transcription factor 1; NRP1 and NRP2, neuropilin 1 and 2; PlGF, placenta growth factor; TACE, TNF-α converting enzyme; TAM, tumor-associated macrophages; TIMP-3, tissue inhibitor of metalloproteinases; TK, tyrosine kinase; VEGFR-1 and VEGFR-2, VEGF receptor 1 and 2

Abstract

Angiogenesis is a complex biological phenomenon crucial for a correct embryonic development and for post-natal growth. In adult life, it is a tightly regulated process confined to the uterus and ovary during the different phases of the menstrual cycle and to the heart and skeletal muscles after prolonged and sustained physical exercise. Conversly, angiogenesis is one of the major pathological changes associated with several complex diseases like cancer, atherosclerosis, arthritis, diabetic retinopathy and age-related macular degeneration. Among the several molecular players involved in angiogenesis, some members of VEGF family, VEGF-A, VEGF-B and placenta growth factor (PlGF), and the related receptors VEGF receptor 1 (VEGFR-1, also known as Flt-1) and VEGF receptor 2 (VEGFR-2, also known as Flk-1 in mice and KDR in human) have a decisive role. In this review, we describe the discovery and molecular characteristics of PlGF, and discuss the biological role of this growth factor in physiological and pathological conditions.

Keywords: angiogenesis inducing agents; anoxia; endothelial cells; hypoxia; macrophages; neovascularization, pathologic; placenta growth factor; vascular endothelial growth factors

PlGF gene and protein

Placenta growth factor (PlGF) has been the second member of VEGF family discovered. The name refers to placenta since it was cloned from a human placental cDNA library (Maglione *et al*., 1991). The human *plgf* gene mapped to chromosome 14q24, whereas mouse gene is located on chromosome 12qD. Both genes are formed by seven exons spanning 13.7 kb in human and 10.4 kb in mouse, excluding the upstream and downstream regulatory sequences (Maglione *et al*., 1993a; DiPalma *et al*., 1996).

Like the others members of VEGF family (Ferrara *et al*., 2003; Takahashi and Shibuya, 2005), different isoforms due to alternative splicing are encoded by human *plgf* gene. It encodes four isoforms, PlGF 1-4 (Maglione *et al*., 1993a; Cao *et al*., 1997; Yang *et al*., 2003), composed by 131, 152, 203 and 224 amino acids after the removal of signal peptide (18 amino acids residues in length), respectively.

The primary difference between the four isoforms is that PlGF-1 and PlGF-3 are non-heparin binding diffusible isoforms while PlGF-2 and PlGF-4 have additional (highly basic 21 amino acids) heparin binding domains (Hauser and Weich, 1993; Maglione *et al*., 1993a; Yang *et al*., 2003). Conversely, mouse *plgf* gene encodes for the single isoform PlGF-2, able to bind heparin and composed by 140 amino acids in its mature form (DiPalma *et al*., 1996).

PlGF is secreted as a glycosylated homodimer. The most well-known structural feature of PlGF is due to six cysteine residues of each monomer that are engaged to form three intra-chain disulfide bonds, generating a particular three-dimensional structure known as cystine-knot motif. Two other cysteine residues of each monomer are engaged to form two inter-chain disulfide bonds necessary for the formation of the homodimer. Each homodimer shows two cystine-knot motif located at the opposite poles of the molecule. Despite the human PlGF shows only 42% amino acid sequence identity with the most active member of VEGF family, the VEGF-A, its three-dimensional structure elucidated at 2.0 Å resolution and compared with that of VEGF-A has evidenced a remarkable topological identity between the two proteins (Muller *et al*., 1997; Iyer *et al*., 2001).

The PlGF-1 dimer consists of two α-helices and

seven β-strands per monomer, which are covalently linked by two inter-chain disulphide bonds in an anti-parallel fashion. Structural and mutagenesis analyses (Errico *et al*., 2004) indicated that two negatively charged residues located in the β3-β4 loop (Asp72 and Glu73) are critical for receptor binding. Other residues crucial for receptor recognition are located in the N-terminal α-helix as well as on the β6 strand. The mutation of one (Asn84) of the two glycosylated residues of PlGF determines reduced binding activity indicating that, unlike in VEGF-A, glycosylation plays an important role in receptor binding.

The pro-angiogenic activity of VEGF family members is exerted through the binding and activation of two tyrosine kinase (TK) receptors, which were initially identified as receptors for VEGF-A: VEGFR-1 (de Vries *et al*., 1992) and VEGFR-2 (Terman *et al*., 1992). These receptors consist of seven extracellular Ig-like domains, a transmembrane domain and an intracellular TK domain. The binding of ligands induces receptor dimerization and phosphorylation. Despite the three-dimensional similarity with VEGF-A, PlGF has the property to bind exclusively VEGFR-1 receptor (Park *et al*., 1994), with high affinity compared to VEGF-A and to VEGF-B, the other members of the family able to specifically bind VEGFR-1 (Olofsson *et al*., 1998). The minimal receptor domain required for the binding of VEGF-A, VEGF-B and PlGF is the Ig-like domain two, as well documented by co-crystal three-dimensional studies (Wiesmann *et al*., 1997; Christinger *et al*., 2004; Iyer *et al*., 2010). It is relevant to highlight that for PlGF binding to VEGFR-1, the Ig-like domain 3 plays an important role. As for VEGF-A (Keyt *et al*., 1996), VEGFR-1 domains 2 and 3 are necessary and sufficient for the binding of PlGF with near-native affinity. However, whereas the deletion of domain 3 causes a 50-fold decrease in VEGF binding, the effect on PlGF is more consistent resulting in about 500-fold reduction of binding of PlGF to the domain 2 (Davis-Smyth *et al*., 1998).

Despite the specificity of binding to VEGFR-1, PlGF may indirectly activate also VEGFR-2 in alternative ways. One possibility is represented by the ability of PlGF to bind VEGFR-1 displacing VEGF-A from this receptor and making VEGF-A available for the binding to VEGFR-2 (Carmeliet *et al*., 2001). Moreover, if coexpressed in the same cell, PlGF and VEGF-A may generate heterodimer form (DiSalvo *et al*., 1995) that is able to bind and activate VEGFR-1 but also to induce VEGFR-1/VEGFR-2 dimerization, if both receptors are expressed on cell surface (Tarallo *et al*., 2010). In addition, it has been reported that once PlGF has activated VEGFR-1 receptor, VEGFR-2 may be activated by transphosphorylation mechanism (Autiero *et al*., 2003).

Furthermore, like other isoforms of VEGF family members able to bind heparin, PlGF-2 is able to bind the two coreceptors Neuropilin 1 and 2 (NRP1 and NRP2), discovered as coreceptors of class 3 semaphorins, *via* the recognition of their b1b2 domain (Migdal *et al*., 1998; Mamluk *et al*., 2002; Gaur *et al*., 2009). The interactions of PlGF isoforms and PlGF/VEGF-A heterodimer with receptors are summarized in Figure 1.

PlGF expression

PlGF is highly expressed in placenta throughout all stages of gestation. It has been proposed to control trophoblast growth and differentiation (Maglione *et al*., 1993a; Khaliq *et al*., 1996), thus suggesting a role for the protein during invasion of the trophoblast into the maternal decidua (Vuorela *et al*., 1997).

Immunohistochemistry analyses revealed the presence of PlGF in the vasculosyncytial membrane and in the media of large blood vessels of the

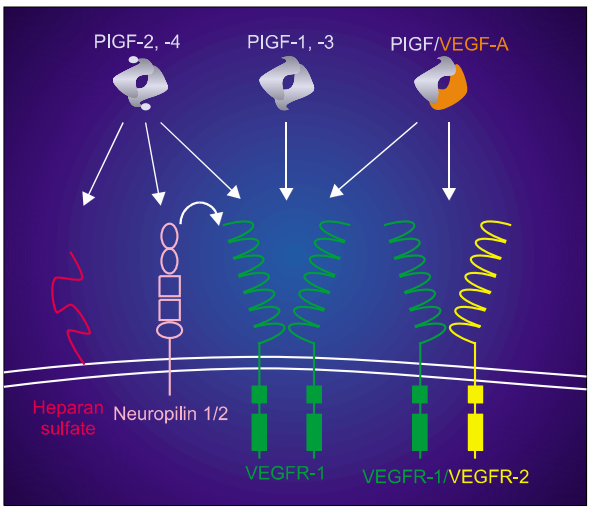

Figure 1. Schematic representation of binding properties of PlGF isoforms and PlGF/VEGF heterodimer. The possible dimers formed by PlGF monomer (gray) are represented. In the case of PlGF isoforms 2 and 4, the heparin-binding domain is represented by additional filled oval. For the heterodimer, the VEGF moiety is in orange. For VEGF receptors (green VEGFR-1, yellow VEGFR-2) the seven Ig-like domains are represented as half ovals, whereas filled rectangles represent the intracellular TK domains. The extracellular Neuropilins receptor 1 and 2 domains are represented as vertical ovals (domains ai, a2), square (b1, b2) and an horizontal oval (domain c) (Mamluk *et al*., 2002). Heparan sulfate is represented in red.

placenta. In situ hybridization analysis showed the presence of PlGF in the villous trophoblast while in this context VEGF-A is expressed in cells of mesenchymal origin within the chorionic plate, thus not in placenta cells (Khaliq *et al*., 1996; Vuorela *et al*., 1997).

PlGF is expressed during early embryonic development. Indeed, transcripts encoding mouse PlGF were abundant in trophoblastic giant cells associated with the parietal yolk sac at early stages of embryogenesis suggesting a role to coordinate vascularization in the deciduum and placenta during early embryogenesis (Achen *et al*., 1997). In addition PlGF is expressed at a low level in several other organs including the heart, lung, thyroid, skeletal muscle, and adipose tissue under normal physiological conditions (Viglietto *et al*., 1995; Persico *et al*., 1999; Voros *et al*., 2005).

At cellular level, the expression of PlGF was demonstrated in endothelial cells (Hauser and Weich, 1993; Yonekura *et al*., 1999), in thyroid cells (Viglietto *et al*., 1995), in immortalized or in transformed mouse embryonic fibroblasts and in NIH 3T3 cells (Carmeliet *et al*., 2001). Differently from VEGF-A, PlGF is expressed only in a limited number of tumor-derived cell lines (Persico *et al*., 1999; Cao, 2009).

Due to the main role that the hypoxic stimulus has in the upregulation of many pro-angiogenic factors when neo-vessels formation is required, studies to unveil the modulation of PlGF expression at molecular level have been executed mainly in hypoxic conditions. The main effectors of hypoxic stimulus are the transcriptional factors known as hypoxia inducible factors (HIFs) (Semenza, 1999). Although some reports indicated an upregulation of PlGF in cells exposed to hypoxia, the analysis of promoter/ enhancer region of PlGF did not show hypoxia responsive element (HRE) sequence, as observed for VEGF-A and VEGFR-1 receptor (Green *et al*., 2001; Oura *et al*., 2003; Selvaraj *et al*., 2003).

In this region, the presence of many putative recognition sequences for metal transcription factor 1 (MTF-1) and for NF-κB were observed. Indeed, the involvement of MTF-1 in immortalized/Ras-transformed mouse embryonic fibroblast and in NIH 3T3 cells (Green *et al*., 2001), and the involvement of NF-κB in human embryonic kidney 293 cells (Cramer *et al*., 2005), has been demonstrated in the modulation of PlGF expression in hypoxic condition. However overexpression of HIF-1α in endothelial cells (Yamakawa *et al*., 2003) or in primary cardiac and vascular cells (Kelly *et al*., 2003) positively influences the expression of PlGF. These results indicated that HIFs might have a role in the mechanism of control of PlGF expression. Therefore, further studies are needed to definitively clarify the molecular basis of hypoxia-induced PlGF expression. Moreover, PlGF expression was shown to be modulated by the forkhead/winged helix transcription factor FoxD1 (BF-2) in the developing kidney stroma due to a conserved HNF3b binding site identified on PlGF promoter region (Zhang *et al*., 2003).

Finally, PlGF expression is also controlled at a post-transcriptional level with a mechanism already described for other growth factors and for many oncogenes (Kozak, 1987; Parkin *et al*., 1988; Muller and Witte, 1989; Arrick *et al*., 1991). The 5' untraslated region of PlGF mRNA contains a small open reading frame potentially coding for a peptide of 13/15 amino acids in human and five amino acids in mouse, whose deletion or mutation of potential initiator codons, substantially increase PlGF expression (Maglione *et al*., 1993b).

Role of PlGF in angiogenesis

The first evidence of PlGF as pro-angiogenic factor was reported in 1997. Ziche *et al*. (1997a) demonstrated that PlGF-1 induced a dose-dependent angiogenic response in the rabbit cornea and in the chick embryo chorioallantoic membrane. Subsequently, the generation and the analysis of *plgf* knock out mouse model have had a central role to unveil the biological functions of PlGF. Despite the high level of expression in placenta, the absence of PlGF did not compromise the normal embryonic development of the mice. Indeed, *plgf* null mice born at a Mendelian frequency are healthy and fertile (Carmeliet *et al*., 2001). PlGF is also dispensable for physiological angiogenesis induced in the heart and muscle by exercise (Gigante *et al*., 2004). This indicates that PlGF is redundant for vascular development and physiological vessel maintenance in healthy adults. However, in the adult, the knock out of *plgf* impairs angiogenesis and arteriogenesis during pathological conditions such as tumor growth, heart, limb and ocular ischemia, (Carmeliet *et al*., 2001; Luttun *et al*., 2002; Pipp *et al*., 2003; Rakic *et al*., 2003). Another mouse model, the double knock out for *plgf* and enodothelial nitric oxide synthase (eNos), has further evidenced the importance of PlGF in pathological angiogenesis. eNOS and its final by-product nitric oxide (NO) represent a downstream target for the angiogenic response elicited by VEGF-A (Papapetropoulos *et al*., 1997; Ziche *et al*., 1997b). eNos -/- mice, like *plgf* -/-, showed a reduced neo-angiogenesis in pathological conditions

(Murohara *et al*., 1998). The mouse carrying the combined deletion of the two genes showed, in mild hind limb ischemia model, a heterogeneous ischemic phenotype ranging from cyanosis of finger-tip to self-amputation and increased death rate occurring in 47% of the animals undergoing the surgical procedure.

This model has represented the first experimental animal model of defective angiogenesis that allows individuating a functional link between PlGF and eNOS (Gigante *et al*., 2006). These experiments of loss-of-function clearly indicated that the activity of PlGF seems to be confined to the pathological conditions.

The involvement of PlGF in stimulating angiogenesis was also confirmed in gain-of-function studies. Transgenic mice overexpressing *plgf* in skin under the control of keratin-14 promoter showed a substantial increase in number, branching and size of dermal blood vessels, with a significant increase of mature smooth muscle-coated vessels, together with enhanced vascular leakiness (Odorisio *et al*., 2002). Accordingly, adenovirus-mediated PlGF transfer in the ischemic heart and limb was able to elicit a strong angiogenic response, giving rise to numerous larger vessels, with an efficacy almost comparable to that of VEGF-A (Luttun *et al*., 2002). The same approach of delivery in xenograft tumors did not show an increase in terms of tumor volume and vessel density but generated an increase in terms of vessel lumen, inflammatory infiltrate and vessel maturation (Tarallo *et al*., 2010). Delivery of recombinant PlGF homodimer or PlGF/VEGF-A heterodimer significantly promoted angiogenesis in ischemic conditions (Luttun *et al*., 2002; Autiero *et al*., 2003).

Gain and loss of function experiments have clearly indicated that PlGF promotes pathological angiogenesis acting at different levels. Indeed, it may directly stimulate vessel growth by acting on the growth, migration and survival of endothelial cells (Ziche *et al*., 1997a; Carmeliet *et al*., 2001; Adini *et al*., 2002; Fischer *et al*., 2007) and vessel maturation, by increasing the proliferation and recruitment of smooth-muscle cells and supporting the proliferation of fibroblasts (Yonekura *et al*., 1999; Bellik *et al*., 2005). Moreover PlGF is crucial for the recruitment and maturation of bone marrow-derived progenitors involved in angiogenic process (Hattori *et al*., 2002; Rafii *et al*., 2003) and to promote differentiation and activation of monocyte-macrophage lineage that are able to further support the angiogenic stimulus (Clauss *et al*., 1996; Scholz *et al*., 2003; Selvaraj *et al*., 2003).

The wide spectrum of paracrine action of PlGF is directly correlated to the expression of VEGFR-1 receptor on many cell lineages (Fischer *et al*., 2008). The specific role of PlGF in pathological conditions was further confirmed by the observation that during pathological angiogenesis cells having a role in this biological phenomenon, like endothelial cells (Yonekura *et al*., 1999; Ponticelli *et al*., 2008; Tarallo *et al*., 2010), smooth muscle cells (Yonekura *et al*., 1999), fibroblasts (Green *et al*., 2001), bone-marrow progenitors (Lyden *et al*., 2001; Hattori *et al*., 2002), over-express or start to express PlGF. Since these cells also express VEGFR-1 receptor, PlGF exerts also autocrine activity to sustain angiogenesis.

Role of PlGF in different diseases

The study of PlGF in pathological angiogenesis has allowed to assign to PlGF/VEGFR-1 axis a central role in the activation and sustainment of the inflammatory switch associated with neo-angiogenesis. Furthermore, many other cell types express PlGF in pathological conditions, such as keratinocytes (Odorisio *et al*., 2006), cardiomyocytes (Luttun *et al*., 2002), retinal pigment epithelial cells (Hollborn *et al*., 2006; Miyamoto *et al*., 2007), bronchial epithelial cells (Mohammed *et al*., 2007) and tumour cells (Parr *et al*., 2005; Wei *et al*., 2005; Fischer *et al*., 2007). This upregulation is due not only to hypoxia but also to other stimulus including nitric oxide (Mohammed *et al*., 2007), cytokines, as interleukin 1 and tumour necrosis factor-α (De Ceuninck *et al*., 2004), growth factors, as transforming growth factor-β1 (Yao *et al*., 2005), and oncogenes (Larcher *et al*., 2003). VEGFR-1 is positively modulated by hypoxia in pathological conditions (Larcher *et al*., 2003).

These data have prompted to investigate whether PlGF has a role in other pathologies and once again the *plgf* knock out mouse has been crucial for these studies. Indeed it has been reported that PlGF plays a role also in atherosclerosis, cutaneous delayed-type hypersensitivity, obesity, cartilage and bone repair and in rheumatoid arthritis (Carmeliet *et al*., 2001; Oura *et al*., 2003; Lijnen *et al*., 2006; Maes *et al*., 2006; Yoo *et al*., 2009). In all pathological models studied, the absence of PlGF impaired the associated inflammation and/ or the angiogenesis determining a general reduction of pathological status. In addition, in the model of fracture repair it has been demonstrated that PlGF is able to activate also unexpected mechanisms. It induced proliferation and osteogenic differentiation of mesenchymal progenitors stimulating cartilage turnover as well as the remodeling of the newly formed bone by stimulating osteoclasts differen-

tiation. As expected, all the cell types involved in these biological processes express VEGFR-1 receptor.

Recent insights

Two new important functions have been recently described for PlGF. The first concerns the polarization status of tumor-associated macrophages (TAM). In non-progressing or regressing tumors, TAMs present a classic M1-like macrophage activation program, characterized by proinflammatory activity, antigen presentation and tumor lysis. In malignant tumors, TAMs show M2-type activation that determines increased angiogenesis and tumor cell intra/extravasation and growth. In this status they suppress antitumor immunity by preventing activation of dendritic cells, CTLs, and NK cells (Mantovani and Sica, 2010; Qian and Pollard, 2010). Ronly *et al*. (2011) have reported that host-produced histidine-rich glycoprotein promotes the antitumor immune response and vessel normalization, effects known to decrease tumor growth and metastasis and to enhance chemotherapy, by skewing TAM polarization away from the M2- to M1-like phenotype. This effect was obtained by down-regulation of PlGF. Therefore PlGF is important to sustain the pro- angiogenic M2-type phenotype.

The second concerns the response necessary for adaptive cardiac remodeling during transverse aortic constriction (Carnevale *et al*., 2011). The cardiac remodeling proceeds by an early adaptive hypertrophic response, characterized by coordinated cardiomyocyte growth, angiogenesis and inflammation (Hunter and Chien, 1999; Frey and Olson, 2003). The absence of PlGF entailed a dysregulation of cardiac remodeling that negatively affects muscle growth, mainly ascribable to a failure in establishment of adequate inflammatory response. At molecular level, an impaired activity of TNF-α converting enzyme (TACE) due to a strong increase of its main natural inhibitor, tissue inhibitor of metalloproteinases (TIMP)-3 has been observed (Vanhoutte and Heymans, 2010). TACE is essential to activate TNF-α from a membrane-bound form, one of the earliest inflammatory events in overloaded hearts (Wang et al., 2009; Ding *et al*., 2010). Therefore, PlGF finely tunes a balanced regulation of TIMP-3/TACE axis, allowing the establishment of an inflammatory response necessary for adaptive cardiac remodeling.

Concluding remarks

PlGF is a multitasking cytokine able to stimulate angiogenesis by direct or indirect mechanisms thanks to its ability to bind and activate VEGFR-1 receptor expressed in many cell types involved in this biological process. Although initially controversial data have been reported on the pro-angiogenic role of PlGF (De Falco *et al*., 2002; Carmeliet and Jain, 2011), the numerous studies of the last decade undoubtedly support its role in angiogenesis. Furthermore, these studied have clearly evidenced the crucial role of PlGF in modulating the inflammation associated not only to pathological angiogenesis but also to other diseases. These data have strongly stimulated the search for inhibitor of PlGF for therapeutic approaches. Once again controversial data have produced (Bais *et al*., 2010; Van de Veire *et al*., 2010), nonetheless a neutralizing anti-PlGF antibody is now in phase two of clinical trials (Martinsson-Niskanen *et al*., 2011). Considering the therapeutic perspective, the search for a physiological function of endogenous PlGF still continues because the elucidation of its physiological role became crucial to predict the possible adverse affects of PlGF inhibitors.

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