Angiogenesis during human extraembryonic development involves the spatiotemporal control of PDGF ligand and receptor gene expression

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Summary

We have examined the role of platelet-derived growth factor (PDGF) ligand and receptor genes in the angiogenic process of the developing human placenta. In situ hybridization analysis of first trimester placentae showed that most microcapillary endothelial cells coexpress the PDGF-B and PDGF \( \alpha \)-receptor genes. This observation indicates that PDGF-B may participate in placental angiogenesis by forming autostimulatory loops in capillary endothelial cells to promote cell proliferation. Endothelial cells of macro blood vessels maintained high PDGF-B expression, whereas PDGF \( \beta \)-receptor mRNA was not detectable. In contrast, PDGF \( \beta \)-receptor mRNA was readily detectable in fibroblast-like cells and smooth muscle cells in the surrounding intima of intermediate and macro blood vessels. Taken together, these data suggest that the PDGF-B signalling pathway appears to switch from an autocrine to a paracrine mechanism to stimulate growth of surrounding PDGF \( \beta \)-receptor-positive mesenchymal stromal cells. Smooth muscle cells of the blood vessel intima also expressed the PDGF-A gene, the protein product of which is presumably targeted to the fibroblast-like cells of the mesenchymal stroma as these cells were the only ones expressing the PDGF \( \alpha \)-receptor. PDGF-A expression was also detected in columnar cytotrophoblasts where it may have a potential role in stimulating mesenchymal cell growth at the base of the growing placental villi. We discuss the possibility that the regulation of the PDGF-B and \( \beta \)-receptor gene expression might represent the potential targets for primary angiogenic factors.

Key words: human placenta, angiogenesis, endothelial cells, platelet-derived growth factor.

Introduction

A crucial step in early human gestation is the establishment of an efficient nutrient-waste exchange between fetal and maternal blood circulation. To achieve this specialized function while maintaining a developmental potential, the human placenta exhibits a number of 'tumour-like' properties, such as immune privilege, extensive proliferation, invasion of the maternal tissue and extreme vascularization (Hamilton and Hamilton, 1977; Ohlsson, 1989; Ohlsson et al. 1991). In fact, the term 'angiogenesis' was coined by Hertig in 1935 to describe the rapid blood vessel formation in the developing placenta. The angiogenic process in the human placenta is initiated at approximately 3 weeks post conception when an arterial-capillary-venous system carrying embryonic blood in the placental villi is established. Embryonic microcapillaries are formed in close proximity of the trophoblast epithelium which constitutes a physical barrier between the extraembryonic tissue and maternal circulation (Hamilton and Hamilton, 1977; Ohlsson, 1989; Ohlsson et al. 1991). Normal and tumour cells have been shown to produce factors able to induce angiogenesis (Folkman, 1986). Some of these factors, such as some members of the fibroblast growth factor gene family, have been shown not only to stimulate endothelial cell proliferation but also to promote protease secretion and chemotaxis (Gospodarowicz et al. 1986). It seems that the capacity to induce blood vessel formation and the ability to induce endothelial cell proliferation and motility may not necessarily be connected. For example, angiogenin induces neovascularization without being either mitogenic or chemotactic (Folkman and Klagsbrun, 1987). It is likely, therefore, that multiple factors cooperate to control blood vessel formation. To address these issues at the molecular level, the developing placenta seems to be a suitable model system. Apart from displaying a marked and persistent angiogenic process, placental tissue has a relatively simple architecture and cell-type composition, which is convenient for studying angiogenesis under normal conditions.

Platelet-derived growth factor (PDGF) is a major serum mitogen that has been implicated in wound healing and in the etiology of atherosclerosis, although no evidence of any direct angiogenic activity has so far been reported (Ross et al. 1986; Heldin and Wester-
In vitro, PDGF has been shown not only to be a potent growth factor for cells of mesenchymal origin, but also to be able to stimulate cell movement through chemotaxis (Westmark et al. 1990). PDGF consists of homo- and heterodimers of two subunits, PDGF-A and PDGF-B. These isomers are encoded by two distinct but related genes, one of which (PDGF-B) is the cellular equivalent of the v-sis oncogene (Doolittle et al. 1983; Waterfield et al. 1983). The two molecules differ in their secretion pattern, as the PDGF-B chain remains cell associated, whereas the A chain is efficiently secreted (Beckmann et al. 1988; Östman et al. 1988). Moreover, while the A-chain only binds the PDGF α-receptor, the B-chain binds both the α- and β-receptor with similar affinity (Heldin and Westmark, 1989; Williams, 1989).

Based upon the observation that the PDGF-B gene was coexpressed with the myc protooncogene in proliferative cytotrophoblasts, we have previously proposed that PDGF is a placentally derived factor that influences the 'pseudomalignant' phenotype of the early human placenta (Goustein et al. 1985). The deduction that PDGF is a growth factor for cytotrophoblasts was later proven to be correct in in vitro assays, using cytotrophoblasts purified from term human placenta (L. Holmgren, unpublished data). In this study, we have analyzed the spatial distribution of active PDGF ligand and receptor genes in both early and late stages of human placental development. We have found that the PDGF-B gene is expressed at particularly high levels in endothelial cells in placentae of late first and third trimester pregnancies. Since the PDGF-B and β-receptor genes are extensively coexpressed in capillary endothelial cells, a role in the continuous angiogenesis of human extraembryonic tissues is proposed. Endothelial cells of larger blood vessels maintained high PDGF-B gene expression, whereas no PDGF β-receptor mRNA could be detected, suggesting that the angiogenic process is developmentally controlled through the spatiotemporal pattern of PDGF ligand and receptor gene expression.

Materials and methods

Human placental tissue

First trimester and term human placentas were obtained from legal elective abortions and Caesarean sections performed at the Huddinge University Hospital. Due to ethical and practical considerations, the second trimester and a major part of the third trimester have not been covered in this study. The placental tissue, which was staged according to ultrasound and patient history records, was either quickly frozen in liquid nitrogen, or fixed in buffered 4% paraformaldehyde for 20 h prior to dehydration and paraffin embedding.

Immunohistochemistry

Paraffin sections were incubated with 0.3% H₂O₂ for 15 min prior to overnight incubation with anti-vimentin mouse monoclonal antibodies (Dakopatts, Inc.) at 4°C. Positive staining was visualized with an avidin–biotin peroxidase enzyme kit (Vectastain, Inc.) reaction according to the protocol of the manufacturer.

Hybridization analysis

For northern blot analysis, total cellular RNA was prepared as previously described (Pfifer–Ohlsson et al. 1984). RNA was denatured and electrophoresed through a 0.9% agarose–formaldehyde gel. RNA was transferred to a Hybond nylon membrane (Amersham), hybridized at 45°C for 36 h and washed in 0.1×SSC containing 0.5% SDS at 52°C (Pfifer–Ohlsson et al. 1984). Filters were hybridized with cDNA probes labelled by nick translation according to the protocol of the manufacturer (Amersham), to a specific activity of 1×10⁶ cts min⁻¹ ng⁻¹ DNA. The following probes were used in this study: PDGF-A and PDGF-B (Betsholtz et al. 1986; C. Betsholtz, unpublished reagent) and PDGF β-receptor clone pSV7d15.1 (Claesson-Welsh et al. 1989) covering the coding sequence for northern blot analysis. For in situ hybridization analysis, a probe covering 800 base pairs of the region coding for the extracellular part of the PDGF α-receptor, was used (K. Funa, unpublished reagent) along with PDGF β-receptor clone 2A3 (Claesson-Welsh et al. 1988). β-tubulin mRNA levels were used for standardizing the amount of RNA in each lane. In situ hybridization analysis was performed on 5 μm formaldehyde-fixed paraffin sections of human placenta, as previously described (Ohlsson et al. 1989). Sections were hybridized with 35S-labeled riboprobes transcribed from supercoiled plasmid templates, yielding probes with similar specific activity and size. Receptor probes were directed against the extracellular region of the molecules, in order to avoid cross hybridization with mRNA encoding other tyrosine kinases. Sections were hybridized at 56°C overnight and washed stringently prior to RNAase treatment, as described previously (Ohlsson et al. 1989). Following application of Ilford K5 photographic emulsion (diluted 1:1 in 2% glycerol in H₂O) and exposure at 4°C for 1–3 weeks, sections were developed and counterstained with Mayer's hematoxylin and mounted. Each in situ hybridization reported here has been independently repeated with at least three different placental specimens, with the same result.

Results

Expression of PDGF ligands and receptors during placental development

Early first trimester placental development is characterized by extensive proliferation of the cytotrophoblast cells in order to establish pregnancy followed by the initiation of vascularization of the placental villus to establish an efficient exchange between the embryonic and maternal blood circulatory systems. At term pregnancy, the placenta has undergone extensive vascularization, while the vast majority of the cytotrophoblast cells have differentiated and lost their proliferative potential (Hamilton and Hamilton, 1977; Ohlsson et al. 1991). To analyze whether the expression of the PDGF ligand and receptor genes were temporally restricted to the early remodeling stage of the late maintenance stage of placental development, we examined mRNA levels by northern blot hybridization. Fig. 1 shows a northern blot comparing total cellular RNA samples extracted from placentae at 4.5–4.6 weeks and 37–40 weeks of gestation. There is no significant difference in the expression of PDGF-A mRNA between first trimester and term placenta, while the PDGF-B gene is expressed at considerably higher
Fig. 1. Northern blot analysis of PDGF-ligand and receptor expression during human placental development. Each lane was loaded with 10 μg of total cellular RNA. The PDGF-A signal results from a three day exposure, the other signals were the result of an overnight exposure. The filter was hybridized with a β-tubulin probe to verify that similar amounts of RNA were loaded in each lane. HSF, human foreskin fibroblast.

levels in term placenta than in first trimester placenta. Conversely, the expression of both the PDGF α- and β-receptor genes are expressed at higher levels in early placenta (Fig. 1). The detection of both PDGF ligand and receptor mRNAs in placenta, taken together with the short-range action of these molecules, indicates that PDGF functions within the placental tissue.

**PDGF-B ligand and β-receptor gene expression patterns are developmentally controlled in cells of the placental vasculatory system**

During the first trimester, placental capillaries impinge on the trophoblast epithelium and consist of 3–5 elongated endothelial cells surrounding the embryonic blood cells (Fig. 2A). To identify the producer and target cells that make up the potential in vivo cell–cell signalling pathway for PDGF, we employed in situ hybridization approaches. By using 35S-labelled antisense riboprobes under stringent hybridization conditions, the expression patterns of both PDGF ligand and receptor genes were compared in adjacent sections of human placenta. Endothelial cells of placental microcapillaries harboured very active PDGF-B genes. In fact, Fig. 3C, D show that endothelial cells, in comparison with both neighbouring cytotrophoblasts and nonendothelial mesenchymal stroma cells, expressed the PDGF-B gene at markedly higher levels. Moreover, we could not detect any PDGF-A expression in placental capillaries (Fig. 3A, B), suggesting that it is primarily the PDGF-BB homo-dimeric form that is produced in growing placental blood vessels. To identify the target cells for PDGF-B ligand, the spatial expression patterns of both the PDGF α- and β-receptor genes were analyzed. While the PDGF α-receptor expression was restricted to fibroblast-like cells not associated with any blood vessel of early placenta (Fig. 3E, F), the PDGF β-receptor gene was active primarily in endothelial cells of microcapillaries (Fig. 3G, H). These data show that the PDGF-B ligand and the PDGF β-receptor genes are extensively coexpressed in endothelial cells of placental capillaries and could, therefore, contribute to the angiogenesis of the human placenta by promoting endothelial cell proliferation in an autocrine fashion.

Larger blood vessels of first trimester placenta have a continuous endothelium surrounded by fibroblast-like cells, which do not show positive staining with α-actin antibodies (Fig. 2B, data not shown). These blood vessels were analyzed for PDGF ligand and receptor gene expression to determine if the coexpression of the PDGF-B and the PDGF β-receptor genes was maintained during later stages of blood vessel formation. Although high PDGF-B gene expression persisted in the endothelium, no positive hybridization signal could be detected with the PDGF β-receptor probe in these cells (Fig. 4A, B; see also model in Fig. 6). Conversely, fibroblast-like cells of the surrounding mesenchyme exhibited stronger PDGF β-receptor hybridization signal than the majority of the mesenchymal stroma, where no PDGF-B signal could be detected. This strongly suggests that endothelial cell-derived PDGF acts in a strict paracrine fashion in more mature blood vessels and is targeted to the surrounding stromal cells. It is likely that the down regulation of PDGF β-receptor gene expression in endothelial cells constitutes an important event in preventing these cells from receiving excessive proliferative stimuli. A later stage of blood vessel formation can be observed in term placenta, where macrovessels are intimately surrounded by smooth muscle cells staining positively with α-actin antibodies (Fig. 2C, data not shown). A subpopulation of α-actin-positive staining cells express the PDGF β-receptor gene, but not the PDGF-B ligand gene (Fig. 4E, F). This observation supports the supposition that endothelial cell-derived PDGF acts in a paracrine fashion to stimulate the formation of smooth muscle layers surrounding macro blood vessels (Majesky et al. 1990).

The PDGF-A gene is expressed in columnar cytotrophoblasts and smooth muscle cells during placental development

In an attempt to establish the functional significance of the general expression of the PDGF α-receptor gene in the mesenchyme of both the first trimester and term
human placenta, excluding the endothelial cells, we examined the pattern of PDGF-A gene expression. Interestingly, the PDGF-A gene of the term placenta is expressed at high levels in smooth muscle cells of macro blood vessels (Fig. 4C). The target cells of smooth muscle cell-derived PDGF-A ligand are likely to be the mesenchymal stroma cells in the periphery of large blood vessels (see model in Fig. 6). In the early placenta (first trimester), by far the highest levels of PDGF-A gene expression were localized instead to the cytotrophoblasts at the base of the growing tips of placental villi (Fig. 5). These cells also expressed the PDGF-B gene which, in contrast to the PDGF-A gene, is generally expressed in the most proliferative cytotrophoblasts (Goustin et al. 1985; L. Holmgren, unpublished data). As expression of the PDGF α-receptor gene could only be detected in mesenchymal cells of the underlying stroma, it is conceivable that cytotrophoblast-derived PDGF-A may stimulate cell growth in a paracrine fashion, in order to renew the mesenchyme at the base of the growing villus during first trimester of pregnancy. (Fig. 5, see also Fig. 3).

**Discussion**

Although PDGF has been implicated in a number of pathological conditions like atherosclerosis, tissue fibrosis and tumorigenesis, little is still known about the in vivo function of PDGF. To understand the normal biological role of PDGF in development, we have used the human placenta as a model system. The establishment of the human placenta involves many of the properties that are associated with the pathological conditions in the adult in which PDGF is implicated, such as rapid cell proliferation and extensive angiogenesis (Ohlsson, 1989; Ohlsson et al. 1990). We have previously shown that the human placental cytotrophoblasts coexpresses both PDGF ligand and receptor genes, implicating a role in the extensive proliferation of cytotrophoblasts during the initial stages of pregnancy (Goustin et al. 1985). In this paper, we have studied PDGF gene expression in late first trimester and third trimester placentas. Essentially all microcapillary endothelial cells of first and third trimester placentas show extensive coexpression of the PDGF-B and the PDGF-β receptor genes (see model, Fig. 5A). A similar expression pattern has been found in capillaries in the mesenchyme surrounding human carcinomas and glioblastomas (Hermansson et al. 1988; Funa et al. 1990). Endothelial cells have previously been thought to be unresponsive to PDGF (Heldin et al. 1981; Kazlauskasa and DiCorleto, 1985) but recent results show that purified human and bovine microvascular cells express PDGF receptors and also elicit a mitogenic response to PDGF (Bar et al. 1989; Beitz et al. 1991). Although no reports have shown PDGF to be angiogenic per se, it is likely that PDGF-B plays an
Fig. 2. Three distinct stages of placental blood vessel formation in the human placenta. First trimester (10 weeks) placental villus with microcapillaries juxtaposed to the trophoblast epithelium (A). Blood vessel of first trimester placenta with a continuous endothelium surrounded by fibroblast-like cells (B). Third trimester macro blood vessel with surrounding smooth muscle cells (C). Sections were immunohistochemically stained with a mesenchymal cell-specific vimentin antibody. mb, maternal blood; mc, microcapillary; str, mesenchymal stroma; tb, trophoblast epithelium; ec, endothelial cells; sm, smooth muscle cells. Magnifications: x165 (A) and x134 (B, C).

Fig. 3. Pattern of PDGF ligand and receptor gene expression in a placental villus at 10 weeks gestation. Adjacent sections (5 μm) of a first trimester placental villus were hybridized with 35S-labeled riboprobes for PDGF-A (A, B), PDGF-B (C, D), PDGF α-receptor (E, F) and PDGF β-receptor (G, H), respectively. Results of experiments are shown in a double exposure of bright-field and dark-field, where positive hybridization signal is represented by the red colour. Arrows denote the location of microcapillaries. High levels of PDGF-B (C, D) and β-receptor mRNA were detected in capillary endothelial cells (G, H). Fibroblast-like cells expressed both types of PDGF receptor genes (E, F) (G, H) but neither of the PDGF-ligands (A, B) (C, D). Abbreviations are the same as in Fig. 2. Magnifications: x73 (A, C, E and G), x281 (B, D, F and H).
Fig. 4. *In situ* hybridization analysis of a first trimester intermediate bloodvessel surrounded by stromal fibroblasts (A, B) and a third trimester macro bloodvessel surrounded by smooth muscle cells (C–F). Sections were hybridized with probes for PDGF-A (C), PDGF α-receptor (D), PDGF-B (A, E) and PDGF β-receptor (B, F). Endothelial cells of intermediate and macro bloodvessels expressed the PDGF-B gene whereas no positive hybridization with PDGF β-receptor probe could be detected. PDGF β-receptor mRNA was highly expressed in stromal fibroblasts and smooth muscle cells surrounding blood vessels. Smooth muscle cells also expressed the PDGF-A gene. Abbreviations are the same as in Fig. 2. Magnification: ×281.

Fig. 6. Hypothetical model depicting the patterns of PDGF ligand and receptor gene expression and cell–cell signalling pathways in microcapillary (A), intermediate (B) and macro (C) blood vessels in the human placenta. Arrows indicate producer/target cells for PDGF. Endothelial cells (blue colour), smooth muscle cells (orange) and fibroblast-like cells (yellow). AA, PDGF-AA; BB, PDGF-BB.
important role in blood vessel formation via stimulation of capillary endothelial cell growth. The suppression of the proliferative endothelial cell phenotype may have important roles both in restricting angiogenesis and as an initiating step in the formation of larger blood vessels. It is interesting to note, therefore, that PDGF β-receptor gene expression in endothelial cells is below the detection limits in large blood vessels that have established a continuous endothelium, but are not yet surrounded by smooth muscle cells. We propose that expression of the PDGF β-receptor gene is a limiting factor controlling PDGF involvement in endothelial cell growth during angiogenesis. The presumed autocrine action of endothelial cell-derived PDGF-B is probably switched to a paracrine action, which may entail the multiplication of undifferentiated mesenchymal stroma cells in earlier stages and smooth muscle cells in later stages of blood vessel formation. Our observation that the PDGF β-receptor gene is very actively expressed in the surrounding fibroblast-like cells and in the smooth muscle intima of large blood vessels of third trimester placentae suggests a dependency on factors produced from the endothelial cell layer. It is interesting to note that proliferation of capillary endothelial cells is inhibited by cocultivation with smooth muscle cells and pericytes indicating a complex feedback pattern between endothelial cells and the surrounding intima (D’Amore and Thompson, 1987). Smooth muscle cells surrounding blood vessels and cytrophoblasts of the tip of growing villi express the PDGF-A but not the PDGF-B gene. Most fibroblast-like cells of the mesenchymal stroma expressed the PDGF α-receptor gene, which indicates that PDGF-A may stimulate cell growth of these cells in a paracrine fashion but only in very distinct regions of the mesenchyme.

Based upon the observations reported here, we conclude that PDGF-B is probably involved in the angiogenesis of the developing human placenta, by forming autostimulatory loops in capillary blood vessels. Since larger blood vessels do not express the PDGF β-receptor, this PDGF-B-dependent loop is probably restricted at the PDGF β-receptor level. We further conclude that the expression of PDGF ligand and receptor genes during neovascularization in neoplasia and cell repair after blood vessel injury is consistent with the expression pattern during the establishment and growth of the blood circulatory system during human development. The possibility that the transcriptional control of the PDGF-B and β-receptor genes represent general and convergent focus for primary angiogenic factors warrants further study.

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References


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