Platelet-derived Endothelial Cell Growth Factor: Structure and function

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Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45-kDa single chain polypeptide, which stimulates the growth and chemotaxis of endothelial cells in vitro and angiogenesis in vivo. Purification from human platelets and cDNA cloning of PD-ECGF disclosed that it is a novel type of angiogenic factor without sequence similarity to hitherto known proteins. PD-ECGF is present in human platelets as well as in placenta. Amino acid sequencing of PD-ECGF from human placenta revealed that the placental form has an additional 5 amino acids at the N-terminus. In cultured cells, it is produced by normal fibroblasts as well as some transformed cell lines. PD-ECGF lacks a hydrophobic signal sequence and remains inside the producer cells. PD-ECGF may act at sites of injury as a wound hormone and thus play an important role under several physiological and pathological conditions.

Growth of endothelial cells is regulated by several growth factors and growth inhibitors, which are different from those for vascular smooth muscle cells and fibroblasts. The best-characterized endothelial growth factor may be basic fibroblast growth factor (FGF) (Table I) (for reviews, see Ref. 1, 2). Six other proteins have been reported to have similar structures to basic FGF, and, thus, they were denoted the FGF family of proteins. They have strong affinities for heparin, and the introduction of heparin affinity chromatography simplified their purification. Acidic and basic FGFs were initially purified from neural tissues; it turned out that they are produced in a variety of animal tissues including macrophages, endothelial cells and smooth muscle cells. However, there is no evidence indicating the presence of FGFs in human platelets. Another endothelial cell mitogen, which was recently purified and cloned, is vascular endothelial growth factor (VEGF) (Table I). VEGF is a 45-kDa dimeric protein, purified from bovine pituitary folliculostellate cells and monocytic leukemia cell lines. Interestingly, VEGF is structurally related to platelet-derived growth factor (PDGF), which does not stimulate the growth of endothelial cells.

Blood platelets are a rich source for growth factors, including PDGF, transforming growth factor (TGF)-β, epidermal growth factor (EGF)-like protein, connective tissue activating peptide (CTAP)-III, and insulin-like growth factor (IGF)-I and II. These growth factors have different target cell specificities and after platelet release reaction they may act as wound hormones at sites of injury. Experiments using intact platelets as well as crude platelet lysates have suggested the presence of mitogen(s) for endothelial cells in human platelets. The endothelial mitogenic activity in human platelets was well separated from those for fibroblasts by gel chromatography or isoelectric focusing.

Key words:
- Growth factor
- Platelet
- Endothelial cell
- PD-ECGF

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TABLE 1  COMPARISON OF ENDOTHELIAL CELL GROWTH FACTORS

<table>
<thead>
<tr>
<th>Designation</th>
<th>basic FGF</th>
<th>PD-ECGF</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>16–18 kDa*1</td>
<td>45kDa</td>
<td>45kDa</td>
</tr>
<tr>
<td>Structure</td>
<td>monomer</td>
<td>monomer</td>
<td>dimer</td>
</tr>
<tr>
<td>Target cells</td>
<td>EC*2, Epi, Fbl, etc</td>
<td>EC, Epi, etc</td>
<td>EC</td>
</tr>
<tr>
<td>Origin</td>
<td>neural tissues, EC, Mφ, SMC, etc</td>
<td>platelets, placenta</td>
<td>pituitary cells, monocyctic leukemia</td>
</tr>
<tr>
<td>Signal peptide</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Affinity for heparin</td>
<td>high (2.0 M NaCl)</td>
<td>low</td>
<td>high (&gt;0.9 M NaCl)</td>
</tr>
<tr>
<td>Related proteins</td>
<td>acidic FGF, hst-1, int-2, FGF-5, FGF-6, KGF</td>
<td>none</td>
<td>PDGF</td>
</tr>
</tbody>
</table>

*1: The 22 and 25 kDa, high molecular weight forms for basic FGF were also reported.  
*2: Abbreviation used are: EC, endothelial cells; Epi, epithelial cells; Fbl, fibroblasts; SMC, smooth muscle cells; and Mφ, macrophages.

Purification of PD-ECGF

Purification of PD-ECGF was performed by a six-step procedure from human platelet lysates\textsuperscript{10}. Growth promoting activity on endothelial cells was measured by the \textsuperscript{3}H-thymidine incorporation assay using endothelial cells from porcine aorta as target cells. Fig 1A shows the flow-chart for the purification of PD-ECGF from human platelet lysates. SDS-gel electrophoresis and silver staining disclosed that it is a 45-kDa single chain polypeptide. In the silver stained gel, we could observe doublet bands, which are probably due to the microheterogeneity of this protein. Analysis by chromatofocusing revealed that the pI of PD-ECGF is 4.8.

Recently, we found that human placenta contains large amounts of PD-ECGF\textsuperscript{11}. Purification of PD-ECGF from human placenta was achieved by a procedure similar to that for the purification from human platelets, with two additional, step (Fig. 1B). The molecular weight of the placental form of PD-ECGF was slightly larger than the platelet form (see below).

Biological activity of PD-ECGF

PD-ECGF stimulates the growth of vascular endothelial cells in vitro. The growth promoting activity on endothelial cells is not limited to the cells from large vessels; it also acts on capillary endothelial cells. Maximal stimulation of the growth of porcine aortic endothelial cells was obtained at 16 ng/ml (350 Pm) of PD-ECGF. It is noteworthy that the growth promoting activity declined at higher concentrations of PD-ECGF (more
than 100 ng/ml), and, thus, the dose-response curve was bell-shaped. Recent experiments have revealed that PD-ECGF is active on some other cell types, e.g., epithelial cells and choriocarcinoma cells (ref. 11 and Miyazono et al, unpublished observations), but most mesenchymal cells, such as fibroblasts and smooth muscle cells, did not respond to PD-ECGF.

Using the Boyden chamber method, we found that PD-ECGF stimulates the migration of endothelial cells; no migration was induced for vascular smooth muscle cells by PD-ECGF\textsuperscript{12}. Checkerboard analysis of the endothelial cell migration disclosed that the effect of PD-ECGF was due to the stimulation of the directed cell migration (chemotaxis), but not to the stimulation of random cell migration (chemokinesis).

The growth promoting effect of PD-ECGF was neutralized by an antitrypanosoma reagent, suramin. The effect of suramin is not limited to PD-ECGF, but can be generally observed in different types of growth factors.

When platelet lysate was prepared at neutral pH, it stimulated the growth of endothelial cells. On the other hand, platelet lysates treated with acid markedly inhibited the growth of endothelial cells, which is due to the TGF-\(\beta\) effect. TGF-\(\beta\), a potent inhibitor for endothelial cell growth, is present in human platelets in a large quantities, but most of it is present as a latent (inactive) form. The latent form of TGF-\(\beta\) is activated by transient acidification of platelet lysates (Fig. 2). The physiological mechanisms for the activation of TGF-\(\beta\) are not fully elucidated.

PD-ECGF stimulates angiogenesis \textit{in vivo}. In the chorioallantoic membrane (CAM) assay, a marked invasion of capillary vessels could be observed into the methylcellulose discs containing PD-ECGF. The angiogenic effect of PD-ECGF in the CAM assay was neutralized by antiserum against PD-ECGF\textsuperscript{12}.

cDNA cloning, expression and genomic organization of human PD-ECGF
cDNA cloning of PD-ECGF was performed by screening a human placental cDNA library with synthetic oligonucleotide probes, constructed based on the amino acid sequence of PD-ECGF. A clone of 1.8 kb was obtained; the nucleotide sequencing of this clone disclosed that it is identical with the results from amino acid sequencing of purified PD-ECGF\textsuperscript{12}.

The open reading frame of PD-ECGF is composed of 482 amino acids. PD-ECGF has no sequence similarity with hitherto known proteins. The N-terminal sequence of PD-ECGF from human platelets starts from the alanine residue at position 11, indication that processing has taken place at this position (Fig. 3). Interestingly, the N-terminal sequence of PD-ECGF from human placenta starts from the threonine residue at position 6, indicating that placenta-derived material has five additional amino acids at

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the N-terminus. There are seven cysteine residues in PD-ECGF; thus, PD-ECGF contains at least one free thiol group. PD-ECGF does not have an N-terminal signal sequence, which is also a property of basic and acidic FGFs. These results indicated that PD-ECGF is not secreted from producer cells by the classical pathway; instead, it has a tendency to remain inside the cells.

When the cDNA for PD-ECGF was transfected into NIH 3T3 cells, the cells started to produce bioactive PD-ECGF in vitro; a major part was, however, observed in the cell lysates. Moreover, when the cDNA for PD-ECGF was transfected into a1-1 cells, i.e., the NIH 3T3 cells transformed by activated H-ras gene, and injected to nude mice, the tumors obtained after two weeks showed a highly vascularized structure. These results again indicated that PD-ECGF has an angiogenic activity in vivo.

The human PD-ECGF gene was recently obtained. It is composed of 10 exons, dispersed over a 4.3 kb region (Fig. 3). The PD-ECGF promoter lacks “TATA” box and “CCAAT” box, but a cluster of six copies of potential Spl binding sites was observed just upstream of the transcription start site. PD-ECGF gene was localized to chromosome.

Production and localization of PD-ECGF

In normal animal tissues, the richest sources for PD-ECGF are platelets and placenta. In cultured cells, PD-ECGF was shown to be produced in human foreskin fibroblasts, vascular smooth muscle cells, and some transformed cells lines (Table II). Among those, we found that two anaplastic thyroid carcinoma cell lines and one breast carcinoma cell line produce large quantities of PD-ECGF. Also in these cells, PD-ECGF remained in the cells, and secreted only slowly to the conditioned media.

Localization of PD-ECGF in human platelets appears to be important for its in vivo function. Thrombin treatment of platelets led to release of growth factors in platelet α-granules, such as PDGF, TGF-β and IGFs. In contrast, PD-ECGF was not secreted from platelets by thrombin treatment. Subcellular localization study of PD-ECGF by high pressure disruption of platelets followed by sucrose gradient centrifugation, indicated that PD-ECGF is present in the cytosol fraction of human platelets. These results indicate that PD-ECGF is released from platelets under different conditions from PDGF and other growth factors in platelet α-granules.

In placenta, PD-ECGF is present in the stromal tissues. Thus, PD-ECGF may play important roles in angiogenesis as well as trophoblast structures in the developing placenta, probably in a paracrine fashion.

Perspectives

Recent experiments on PD-ECGF have disclosed the structure and some biological functions of this molecule. Future research for PD-ECGF may be directed towards understanding the release mechanisms of this molecule, the identification of the cell surface receptors for PD-ECGF, and the study of the in vivo function of PD-ECGF in different clinical disorders.

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