Chromosomal Location, Exon Structure, and Vascular Expression Patterns of the Human PDGFC and PDGFD Genes

Marko Uutela, MSc; Juha Laurén, BMed; Erika Bergsten, MSc; Xuri Li, PhD; Nina Horelli-Kuitunen, PhD; Ulf Eriksson, PhD; Kari Alitalo, MD, PhD

Background—Platelet-derived growth factor (PDGF), which is a major mitogen for vascular smooth muscle cells and has been implicated in the pathogenesis of arteriosclerosis, is composed of dimers of PDGF-A and PDGF-B polypeptide chains, encoded by different genes. Here, we have analyzed the chromosomal localization, structure, and expression of 2 newly identified human genes of the PDGF family, called PDGFC and PDGFD.

Methods and Results—We used fluorescence in situ hybridization to locate PDGFC and PDGFD in chromosomes 4q32 and 11q22.3 to 23.2, respectively. Exon structures of PDGFC and PDGFD were determined by sequencing from genomic DNA clones. The coding region of PDGFC consists of 6 and PDGFD of 7 exons, of which the last 2 encode the C-terminal PDGF cysteine knot growth factor homology domain. An N-terminal CUB domain is encoded by exons 2 and 3 of both genes, and a region of proteolytic cleavage involved in releasing and activating the growth factor domain is located in exon 4 in PDGFC and exon 5 in PDGFD. PDGFC was expressed predominantly in smooth muscle cells and PDGFD in fibroblastic adventitial cells, and both genes were active in cultured endothelial cells and in a variety of tumor cell lines. Both PDGFC-C and PDGFD-D also stimulated human coronary artery smooth muscle cells.

Conclusions—PDGFC and PDGFD have similar genomic structures, which resemble those of the PDGFA and PDGFB genes. Their expression in the arterial wall and cultured vascular cells suggests that they can transduce proliferation/migration signals to pericytes and smooth muscle cells. (Circulation. 2001;103:2242-2247.)

Key Words: platelet-derived factors • genes • muscle, smooth

Platelet-derived growth factor (PDGF) is a major mitogen for fibroblasts, smooth muscle cells (SMCs), and other cells of mesenchymal origin. Originally, PDGF was purified from human platelets, but recent studies have shown that the 2 PDGF genes, PDGFA and PDGFB, can be transcribed by a number of different cell types. The encoded polypeptides form disulfide-bonded homodimers (PDGF-AA and PDGF-BB) or heterodimers (PDGF-AB), which bind to and activate 2 structurally related protein tyrosine kinase receptors, PDGFR-α and PDGFR-β. The α-receptor binds both the A and B chains of PDGF, but the β-receptor binds only the B chain.

Targeted mutagenesis studies of PDGFs and their receptor genes show evidence of the importance of these genes during embryonic development. Approximately 50% of the PDGF-A–null mice die in utero before embryonic day 10, and the surviving mice have a complex postnatal phenotype affecting the lungs, the skin, and the nervous system (reviewed by Betsholtz and Raines). The PDGFR-α–deficient mice have incomplete cephalic closure, impaired neural crest development, cardiovascular and skeletal defects, and edemas and die at embryonic days 8 to 16. Both PDGFB– and PDGFR-β-deficient mice have severely disrupted kidney development and defective development of blood vessels, with a dilated aorta and bleeding, leading in most cases to death during embryonic days 17 to 19. All muscle cell lineages are severely affected by the lack of PDGFR-β in chimeric embryos. PDGF has also been shown to be involved in human diseases, for example, in arteriosclerosis and cancer (see Betsholtz and Raines for references).

We have identified 2 novel PDGFs and shown that PDGFC binds to PDGFR-α and PDGFD to PDGFR-β. Here, we report the structures and chromosomal localizations of the PDGFC and PDGFD genes and show that they are expressed in cells of the vascular wall, suggesting that these genes can participate in vascular development and pathology.
Methods
Isolation and Analysis of Genomic DNA Clones and Fluorescence In Situ Hybridization

Human genomic clones for PDGFC and PDGFD were isolated by polymerase chain reaction–based screening from P1 bacteriophage and P1 artificial chromosome human diploid genome libraries (Genome Systems). The primer pairs used for PDGF were 5'-GAGGTAAGATTATACAGTGCACAC-3' (sense) and 5'-AGTAACTTT GCTTGGGACACATT-3' (antisense), and those for PDGFD were 5'-GCCAACGTTTACAGTTGCACAT-3' (sense) and 5'-CAGCGTTTTTCCCTGAACTTC-3' (antisense), resulting in the amplification of 177- and 176-bp DNA fragments, respectively. The clones were verified by restriction-enzyme mapping, Southern blotting, and sequencing. Genomic organization of PDGFC and PDGFD was determined by sequencing the whole coding sequence and identifying the exon-intron junctions from the genomic clones.

For fluorescence in situ hybridization, genomic DNA clones were nick translated with biotin 11-dUTP (Sigma) and hybridized on metaphase chromosomes derived from normal human peripheral blood lymphocytes, which were treated with 5-bromodeoxyuridine at late replicating phase to induce banding pattern. The hybridization was carried out in 50% formamide/10% dextran sulfate in 2× SSC, and the signals were detected as described earlier. A multicolor image analysis was used for the acquisition, display, and quantification of the hybridization signals. The identification of the chromosomes was based on 4',6'-diamidino-2-phenylinole banding pattern, which resembles G bands, after 5-bromodeoxyuridine incorporation at the early replicating phase.

Cell Culture and Isolation and Analysis of RNA

Human umbilical vein and microvascular endothelial cells and human coronary artery SMCs (HCASMCs) were purchased from Promocell and cultured in passages 2 to 5, as recommended by the supplier. The Wi-38 fetal lung fibroblasts and human tumor cell lines were obtained from American Type Culture Collection. The isolation of polyadenylated RNA was by oligo-dT cellulose chromatography; 5 μg was electrophoresed in 1% agarose gels containing formaldehyde and blotted onto Hybond-N filters (Amersham), which were used for hybridization with 32P-labeled PDGFC and PDGFD cDNA fragments.

Cell Proliferation Analysis

The core domain PDGF-CC was generated as previously described. A His-tagged (6× His) version of full-length PDGFD was produced in baculovirus-infected SF9 insect cells. This protein contained a specific cleavage site for the factor Xa protease in the hinge region in front of the core domain (cleavage site IEGR×2, replacing amino acid residues 251 to 258). The introduced factor Xa cleavage site does not affect the ability of the PDGF-D chains to form dimers. The factor Xa cleaved protein product was used in cell proliferation analysis. HCASMCs were cultured on a 96-well plate for 24 hours. The culture medium was then replaced with 100 μL of 10% SDS/10 mmol/L HCl overnight at 37°C and absorbance was measured at 540 nm.

Statistical Analysis

For statistical analysis, an unpaired t test was performed comparing PDGF-CC, PDGF-DD, and PDGF-BB stimulation against the starvation medium control. Analysis was done with the t test calculator at GraphPad.com.

Antibodies

Rabbit anti–human PDGFD antisemur was produced in rabbits by immunization with the MAP peptide SKVLDRLNDDAKRYS (residues 257 to 272; Genbank accession number AF336376). For affinity purification, the peptide was coupled to epoxy-activated Sepharose 6B (Pharmacia). Bound antibodies were eluted with 1% acetic acid and 20 mmol/L sodium hydroxide, and eluates were pooled and dialyzed against PBS. Rabbit anti–human PDGFC antibodies were produced as described.

Immunohistochemical Staining

Tissues were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated, embedded in paraffin, cut at 5 μm, and treated with trypsin for 20 minutes at 37°C. The sections were then stained with the PDGF-C and PDGFD specific antibodies by use of the TSA-kit (NEL Life Sciences) according to the manufacturer’s instructions. Biotinylated anti-rabbit IgG (Vector Laboratories, diluted 1:300 before use) was used for detection. Sections were counterstained with hematoxylin. Antigen-specific blocking of the antibodies was carried out with a 10× molar excess of the immunogen for 12 hours at 4°C.

Results

Gene Structure

As can be seen from the schematic comparison of the exon-intron organization of PDGF family genes in Figure 1A, PDGFC consists of 6 exons containing coding sequences. Exon 1 has a long untranslated region and encodes the signal peptide. Exons 2 and 3 encode the so-called CUB domain found in both PDGF-C and PDGFD. Exons 5 and 6 encode the PDGFD-homology domain, and exon 4 encodes a hinge region between the two. PDGFD has 7 exons; a comparison of the PDGFC and PDGFD amino acid sequences is shown in Figure 1B (arrowheads mark the sites of the introns). Exon 1 contains poorly conserved sequences, whereas exons 2– and 3– encoded CUB domains are ~58% identical, as are exon 6– and 7– encoded PDGFD-homology domains (57%). As can be seen from the comparisons, exon 4– encoded sequences of PDGFD cannot be identified in PDGFC, whereas the exon 5 (PDGFD exon 4)–encoded hinge region contains a conserved basic sequence motif, RKSR/K (arrowheads and shaded area), which may be one target of the proteolytic cleavage that separates the PDGFD domain from the rest of the propeptide and activates PDGFC and PDGFD biological activity. Such cleavage peptides are located at the N-terminal of the cystine knot growth factor domain in PDGFD-A and PDGFD-B as well.

Chromosomal Locations of the PDGFC and PDGFD Genes

In fluorescence in situ hybridization analysis for the PDGFC gene, 30 metaphases out of 43 (70%) showed specific hybridization signals on human chromosome 4q32 and 34 metaphases out of 40 (85%) showed specific PDGFD hybridization signals on chromosome 11q22.3 to 23.2 (Figure 2A).

Expression of PDGFC and PDGFD in Human Vascular Cells in Culture

Northern blotting and hybridization analysis demonstrated signals for PDGFC mRNA and even stronger signals for PDGFD mRNA in human umbilical vein endothelial and microvascular endothelial cells. In HCASMCs, PDGFC mRNA was much more abundant than PDGFD mRNA (Figure 2B). Conversely, in the Wi-38 human fibroblast cell line, PDGFD mRNA was considerably more abundant than PDGFC mRNA.
Only very small amounts of PDGF-C mRNA were expressed in the 293T human kidney cells (Figure 2B). Essentially no regulation of either mRNA, however, was observed in hypoxic conditions or when the human microvascular endothelial cells were stimulated by vascular endothelial growth factor-C, fibroblast growth factor-1, or tumor necrosis factor-α (data not shown).

The Table shows a summary of the results of mRNA expression analysis in the human vascular cells.

**PDGF-C and PDGF-D Expression in Tumor Cell Lines**

Both novel PDGF mRNAs were also expressed in several tumor cell lines (Figure 2C and Table). Specifically, the 3.8- and 2.8-kb transcripts of PDGF-C were present in fibrosarcoma (HT-1080), breast cancer (BT-474), and prostate cancer (PC-3) cell lines, whereas only the 3.8-kb transcript was observed in erythroleukemia cells (HEL). The 4.0-kb PDGF-D mRNA was present in lung alveolar carcinoma (A549) and in breast and prostate carcinoma cells. Interestingly, comparison with the expression of PDGF-A and PDGF-B genes indicated that all 4 family members are independently regulated in tumor cell lines (Table).

**Localization of PDGF-C and PDGF-D Antigens in the Arterial Wall**

Immunoperoxidase staining of sections from the suprarenal artery showed weak signals for PDGF-C in the SMC layer (Figure 3A), and stronger staining for PDGF-D was located in the adventitial connective tissue layer (Figure 3C), consistent with their patterns of expression in cultured SMCs and fibroblasts, respectively.

**PDGF-C and PDGF-D Stimulate Proliferation of Smooth Muscle Cells**

Cultured serum-starved HCASMCs were incubated with purified recombinant PDGF-CC, PDGF-DD, PDGF-BB, or starvation medium for 3 days, then lysed, and the numbers of...
viable cells were analyzed by use of the MTT assay. The relative MTT values indicated that the numbers of viable cells were significantly higher in wells incubated with either PDGF-CC, PDGF-DD, or PDGF-BB than in wells with only starvation medium (Figure 4). Although the detailed dose responses in such experiments remain to be determined, these results support the hypothesis that both PDGF-CC and PDGF-DD stimulate SMC proliferation/survival.

**Discussion**

Here, we report the structures and chromosomal localizations of 2 novel members of the PDGF gene family, the PDGFC and PDGFD genes. The evolutionary relatedness of the 4 PDGF genes was apparent in the examination of their exon structures. In all cases, the coding sequences for the cystine knot growth factor domain are similarly divided into 2 exons, which are located in the 3' region of these genes. All also contain similar putative proteolytic cleavage sites in homologous positions. In PDGFC and PDGFD, however, the 5' exons encode the additional CUB domain, which is not present in PDGF-A or PDGF-B. We have not found any alternatively spliced forms of the novel PDGF genes yet, although we have detected a variant-length PDGF-D mRNA in stomach tissue (M.U., unpublished data, 2000). The promoter region and complete expression patterns of PDGFC and PDGFD remain to be elucidated.

**Summary of the Expression of PDGF mRNAs in Various Human Tumor Cell Lines**

<table>
<thead>
<tr>
<th>Tumor Cell Line</th>
<th>PDGF-C</th>
<th>PDGF-D</th>
<th>PDGF-A</th>
<th>PDGF-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-1080</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T47D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BT-474</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SW480</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A-549</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PC-3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A498</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WiDr</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G401</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jar-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JEG</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U-937</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HL-60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ML-2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CHRF</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DAMI</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TF-1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K-562</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HEL</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MOLT</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

When overexpressed in the heart, PDGF-C was shown to enhance myocardial fibroblast proliferation.9a Because of the involvement of the PDGF genes in blood vessel development and in the pathogenesis of arteriosclerosis,2,14 it was of interest to find expression of the PDGF-C and PDGF-D
mRNAs in cultured microvascular endothelial cells. PDGF-D was found in the adventitial connective tissue surrounding the suprarenal artery, suggesting that it provides a paracrine ligand for the arterial SMCs, which have been shown to express PDGFR-β. The biological activity of recombinant PDGF-CC and PDGF-DD was confirmed in HCASMC cultures, in which both of these factors stimulated cell proliferation/survival. Thus, although these factors bind to distinct receptors, PDGF-α and PDGFR-β, respectively, they appear to share at least one redundant effect via these 2 receptors. In previous studies, both PDGF-AA, which transduces signals via PDGFR-α, and PDGF-BB, which transduces signals via both PDGFR-α and PDGFR-β and via their heterodimers, have been shown to mediate SMC proliferation stimuli (for references, see Reference 1). Several antagonists specific for PDGF or its receptors have recently been developed and shown to inhibit intimal hyperplasia formation in various animal models, predominantly via increased SMC apoptosis and possibly also via interference with SMC migration. Thus, it can be speculated that via their ability to stimulate the PDGFRs, PDGF-CC and PDGF-DD could also play a role in arteriosclerosis.

The PDGF-C and PDGF-D mRNAs were also expressed along with PDGF-A and PDGF-B mRNAs in several tumor cell lines, but the variable expression patterns seen in a subset of the cell lines indicate that these genes are differently regulated. The role of PDGF-D in tumors needs further study, because the v-sis oncogene of simian sarcoma virus and the dermatofibrosarcoma protuberans–associated fusion gene encode functional PDGF-BB homologues capable of binding all known PDGFs. No chromosomal translocations or inherited tumor loci, however, have as yet been mapped to the regions in which we located the novel genes. The PDGFs secreted by tumor cells could be responsible for some of the stromal proliferative or so-called desmoplastic reactions around tumors. Furthermore, because fibroblasts have the ability to contract collagen gels in response to PDGF, the novel genes could be involved in modulating the tension between cells and extracellular matrix structures and thus the regulation of interstitial fluid pressure and homeostasis in the connective tissue matrix.

Interaction between endothelial cells and mural cells (pericytes and SMCs) is essential for vascular development and maintenance. Recent results by Yamashita and colleagues show that vascular endothelial growth factor receptor-2–expressing cells derived from embryonic stem cells can differentiate into both endothelial and mural cells and can reproduce the vascular organization process. In this process, vascular endothelial growth factor promotes endothelial cell differentiation, whereas mural cells are induced by PDGF-BB. A possible role for PDGF-DD in the induction of mural cells remains to be studied. Although the physiological and also possible pathological significance of PDGF-CC and PDGF-DD is unclear, however, at least PDGF-DD could possibly have a role in pericyte migration during angiogenesis, and perhaps both PDGF-CC and PDGF-DD could be involved in the intimal SMC accumulation in arteriosclerosis. Such a possibility deserves careful further analysis of these novel genes.

Acknowledgments
This study was supported by the Finnish Academy of Sciences, the University of Helsinki Hospital (TYH 8105), the State Technology Development Center, EU Biomed program BMH-98–3380, the Finnish Cancer Organization, the Sigrid Juselius Foundation, the Swedish Medical Research Council, and the Novo Nordisk Foundation. We thank Dr Aarno Palotie for collaboration in fluorescence in situ hybridization; Joni Turunen, Tapio Tainola, Mari Helanterä, Pipsa Ylikantala, and Maritta Putkiran for excellent technical assistance; and Dr Carl-Henrik Heldin and Dr Christer Betsholtz for kind comments on the manuscript.

References

Figure 4. Effect of PDGF-CC, PDGF-DD, and PDGF-BB on HCASMC proliferation/survival. Approximately 100 ng/mL of growth factors was used in assay. Relative MTT values and SDs from 3 triplicate determinations are shown, cells incubated with only starvation media having value 1 (**P<0.001 vs starvation control). SM indicates starvation medium.


Chromosomal Location, Exon Structure, and Vascular Expression Patterns of the Human PDGFC and PDGFD Genes
Marko Uutela, Juha Laurén, Erika Bergsten, Xuri Li, Nina Horelli-Kuitunen, Ulf Eriksson and Kari Alitalo

_Circulation._ 2001;103:2242-2247
doi: 10.1161/01.CIR.103.18.2242

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/103/18/2242

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/