RAPID COMMUNICATION

Cultured Human Endothelial Cells Express Platelet-Derived Growth Factor A Chain

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Four principal cell types involved in the pathophysiologic responses of the vessel wall—endothelial cells, smooth muscle cells, platelets, and monocyte/macrophages—secrete platelet-derived growth factor-like (PDGF-like) mitogenic activities. Extensive structural data on these activities exist only for the mitogen produced by platelets, which is a 30-kd dimeric protein composed of structurally related A and B polypeptide chains encoded by different genes. It was previously demonstrated that normal cultured endothelial cells transcribe mRNA encoding the B chain of PDGF from the *c-sis* gene. Here several new structural features of the mitogen produced by cultured vascular endothelial cells are shown. Hybridization analysis of RNA from normal cultured human umbilical vein endothelial (HUVE) cells revealed that they contain three PDGF A chain transcript species. These RNA species comigrated with and appeared to have the same relative abundance as the three RNA species previously identified in RNA from two human tumor cell lines. A chain transcripts were not identified in RNA from a strain of bovine aortic endothelial cells or in human dermal fibroblasts. The A chain transcripts in HUVE had the same relative abundance as the B chain transcripts. Immunoprecipitation of metabolically labeled endothelial conditioned medium with anti-PDGF antiserum revealed a 31-kd species which was split by reduction and alkylation into two species of 16.5 and 17 kd. Thus, endothelial cells secrete a dimeric mitogen antigenically related to PDGF, with a structure identical to previously isolated PDGF A-chain homodimer. These findings are consistent with the possibility that secretion of PDGF by human endothelial cells may be regulated independently of B-chain expression. (Am J Pathol 1987, 127:7–12)

THE ENDOTHELIAL LINING of blood vessels is uniquely positioned to interact with components of the blood as well as with the underlying smooth muscle cells of the vascular media. In this location products produced by vascular endothelial cells (ECs) may play an important role in pathophysiologic processes such as inflammation, wound healing, thrombosis, and atherosclerosis.[1–3] ECs in culture produce a platelet-derived growth factor-like (PDGF-like) mitogenic activity that stimulates growth of fibroblasts and smooth muscle cells and competes with PDGF for cell surface receptor binding,[4] but its structural relationship to PDGF is unknown. It has previously been demonstrated that cultured ECs transcribe mRNA

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species that hybridize with probes for the *c-sis* homologous B chain of PDGF.[5,6] Additionally, direct sequence analysis of a cloned cDNA from human EC cDNA libraries has confirmed that the *c-sis* homologous transcript made by ECs encodes PDGF B chain.[5] Recently a cDNA sequence for the A chain of PDGF was obtained from a human glioma cell line cDNA library.[7] We have utilized the cloned A-chain cDNA from the glioma cell line as a probe to demonstrate that cultured ECs transcribe mRNA species encoding the A chain of PDGF as well. Additionally, immunoprecipitations were performed to characterize the secreted mitogen. The metabolically labeled growth factor from cultured human umbilical vein endothelial conditioned medium, defined by anti-PDGF antisera, consisted of a 31-kd species that under reducing conditions was resolved into two species of 16.5 and 17 kd.

Materials and Methods

Cell Culture

Human endothelial cells (HUVEs) were harvested from two to six umbilical cord veins and established in primary culture as previously described.[8] Cultures were serially passaged under the conditions of Maciag et al.[9] as modified by Thornton et al.[10] Medium 199 and antibiotics were from M.A. Bioproducts (Walkersville, Md); tissue culture plastic dishes were from Corning (Corning, NY), and fetal calf serum was from GIBCO (Grand Island, NY). Endothelial cell growth supplement (ECGS) was a gift from Dr. Thomas Maciag (Revlon Biotechnology Research Center, Rockville, Md), and porcine heparin was purchased from Sigma Chemical Company (St. Louis, Mo).

The SV-40 transformed endothelial cell line (SV-HEC, line F) was maintained as previously described.[11] Bovine aortic endothelial cells (BAECs) were cultured from calf thoracic aorta as previously described,[8] and RNA from a single strain (11-BAEC) was isolated at Passage 17. A human dermal fibroblast (HDF) strain[12] was a gift of Dr. James Rheinwald (Dana Farber Cancer Institute, Boston, Mass). Human osteosarcoma (HOS) cells were obtained from the American Type Tissue Culture Collection, Rockville, Maryland.

RNA Analysis

Total cytoplasmic RNA or poly(A^+) RNA was prepared from confluent cultures of cells as described elsewhere.[13,14] The RNAs were denatured with formaldehyde, electrophoresed in a 1% agarose gel, blotted onto nitrocellulose, and probed with ^{32}P-labeled purified restriction fragments containing either PDGF A chain cDNA[7] or PDGF B chain (*c-sis*) cDNA,[5] with the use of standard techniques as described elsewhere.[14] The filters were washed to 0.5 × SSC at 65 C and exposed to x-ray film for 3 days.

Immunoprecipitation

HUVEs reaching confluence in 150-cm flasks were labeled for 5 hours with 0.5 mCi ^{35}S-cysteine (1200 Ci/mmol) in 5 ml cysteine-free F-10 medium and chased for 1.5 hours with 5 ml Eagle's minimal essential medium (MEM) per flask. The pulse and chase medium were pooled and made 0.5% phenylmethylsulfonyl floride, 2.5% aprotinin, and 0.02% Triton X-100. The conditioned medium was incubated with 50 μl of normal rabbit serum overnight at +4 C. One hundred microliters of packed protein A Sepharose (Pharmacia) beads were added and the incubation prolonged for 2 hours. The beads were pelleted by centrifugation for 10 minutes at 2000 rpm, and 50 μl of PDGF-antiserum was added to the supernatant for an overnight incubation at +4 C. Protein A–Sepharose beads were added and incubated for 2 hours prior to being washed four times with 0.5 M NaCl, 10 mM Tris buffer, pH 7.4, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 2 mg/ml bovine serum albumin (BSA), and one time with 10 mM Tris buffer, pH 7.4. The beads were eluted with 100 μl of 3.6% SDS, 80 mM Tris buffer, pH 8.8, 0.01% bromophenol blue, and heated at 95 C for 3 minutes. The supernatants were divided into two equal portions, one of which was reduced by incubation with 10 mM dithiothreitol for 3 minutes at 95 C and alkylated with 50 mM iodoacetamide, and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 13–18% gradient gels.

Results

A purified 1.3 kb restriction fragment containing the cloned PDGF A chain coding region and portions of the 5′ and 3′ untranslated regions was used as a probe for Northern blot analysis; three transcript species were detected in cytoplasmic RNA of cultured HUVEs and an SV-40 transformed human EC line (Figure 1A). The probe did not identify A-chain transcripts in cytoplasmic RNA from a strain of bovine aortic ECs or from human dermal fibroblasts. Failure to detect A-chain expression in bovine ECs with a human probe may reflect extensive polymorphic differences between species, loss of the locus entirely, or diminished expression of the gene by this strain of

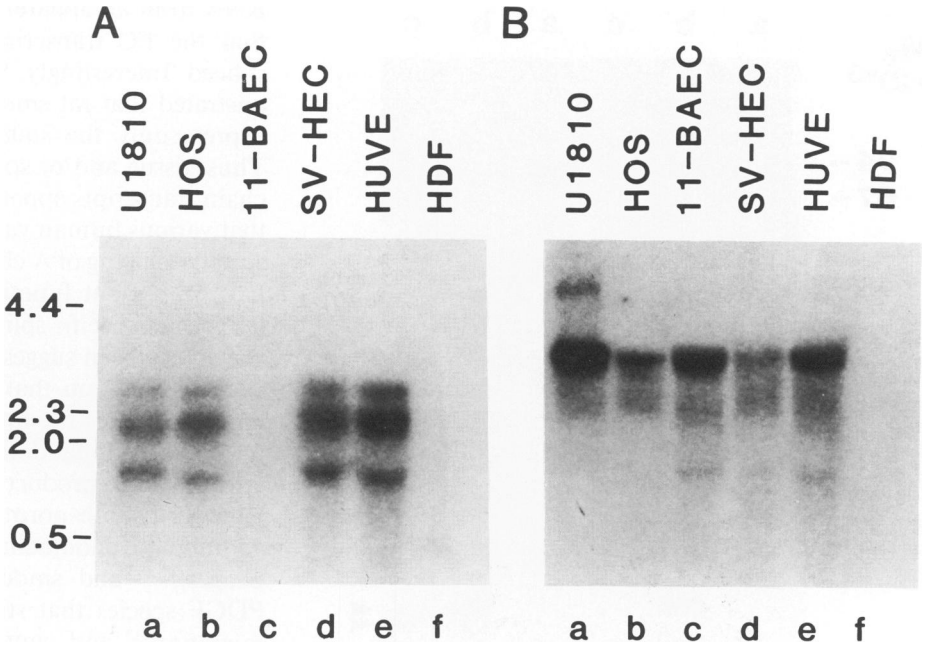

Figure 1—Expression of PDGF transcripts by cultured vascular cells. **A**—Expression of PDGF A-chain transcripts. The molecular sizes of ^{32}P-3′ end-labeled Hind III-digested phage DNA, processed in parallel with the RNA samples, are indicated on the left. **B**—Expression of PDGF B chain (*c-sis*) transcripts.

bovine ECs during passage in culture. The three EC A-chain transcripts found in human ECs comigrated with and appeared to be in about the same relative abundance as the three species found in osteosarcoma cell lines HOS and U-1810 (Beltsholtz et al, unpublished data); the transcripts were about 2.8, 2.2, and 1.4 kb in molecular weight. Densitometric analysis of the Northern blots indicates that the ratios of the transcript species in both the transformed osteosarcoma cell lines and the normal cultured HUVEs are similar, with the intermediate (2.2 kb) species being about twice as prevalent as either the large (2.8 kb) or small (1.4 kb) species. The exact extent of homology between the transcripts produced by the normal ECs and those found in the transformed osteosarcoma cell lines must be determined by direct sequence analysis of the cDNAs corresponding to these transcripts. In contrast to the results with the A-chain probe, a B-chain (*c-sis*) probe identified a 3.5 kb transcript from replicate blots in all three EC types but not in human dermal fibroblasts (Figure 1B). The size of the B-chain transcript is consistent with that predicted from B-chain genomic sequence analysis[15] and by direct sequencing of overlapping endothelial B-chain cDNA clones.[5] In HUVEs the A-chain and B-chain transcripts appeared to have about the same relative abundance.

To characterize the EC-derived mitogen, HUVEs were metabolically labeled, and rabbit anti-human PDGF antiserum was used for immunoprecipitation. A 31-kd protein was specifically precipitated from the culture medium and analyzed by SDS-PAGE (Figure 2, asterisk). Upon reduction and alkylation, the 31-kd band was replaced by two closely migrating 16.5 and 17-kd species (Figure 2, arrowheads). The apparent conversion of the EC-derived protein upon reduction and alkylation suggests that, like PDGF, it has a dimer structure.

Discussion

The PDGF-like activities synthesized by human tumor cells have been specifically precipitated by the same anti-PDGF antiserum used in this study, permitting structural comparisons. Some human tumor cell lines (eg, U-2 OS [osteosarcoma], U-4 SS [synovial sarcoma], B-5GT [giant cell sarcoma], U-343 MGaCl2 [glioma], RD [rhabdomyosarcoma],[7] and WM 266-4 [melanoma][16]) secrete PDGF-like mitogens of 31-kd, which are split by reduction into 17- and 16.5-kd components identical to those observed with ECs. This pattern of precipitation is distinct from that observed with PDGF from human platelets (where a 17-kd and multiple smaller species are observed upon reduction),[17-21] and with the B-chain homodimer isolated from cells acutely transformed by simian sarcoma virus (where p28^{sis} undergoes dimer formation and proteolytic modification yielding a 24-kd cell associated form[22,23]). The PDGF-like growth factor produced by U-2 OS has been shown to be an A-chain homodimer.[24] In addition, several of the tumor cell lines secreting the 31-kd growth factor

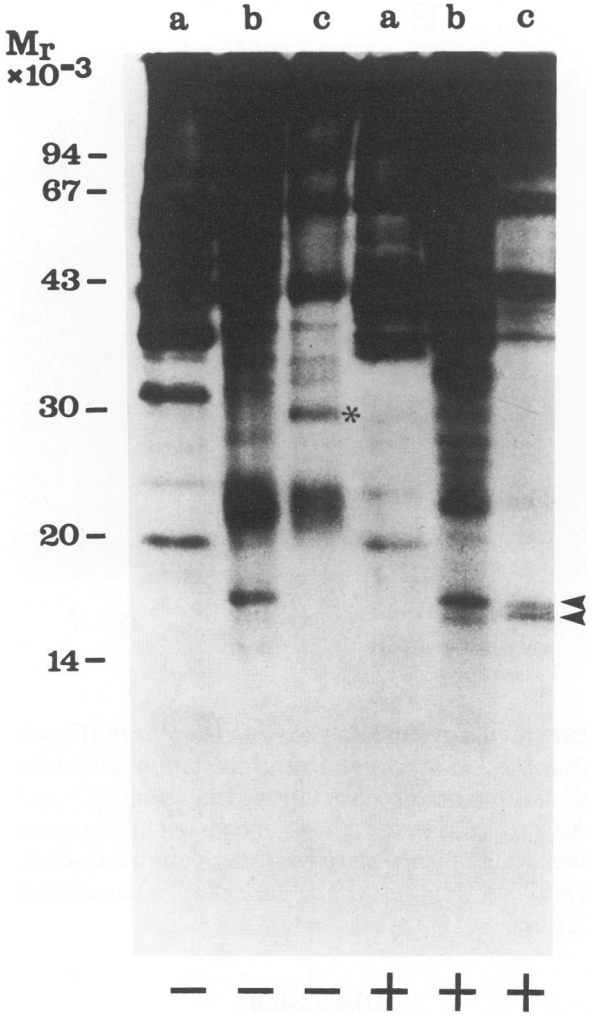

Figure 2 — Immunoprecipitation of metabolically labeled PDGF-like activities produced by human umbilical vein endothelial cells.

Lanes a–c are precipitations of HUVE-conditioned medium; a is labeling controls; b is precipitations with rabbit serum; c is immunoprecipitation with PDGF antiserum. The samples reduced and alkylated are indicated by a "+" at the bottom of the gel.

(RD, B5GT, SKLMS, and WM 266-4) express only the A-chain mRNA.[7,16] It is therefore possible that the EC-derived growth factor is an A-chain homodimer, in spite of expression of B-chain mRNA,[5,6] a situation analogous to that prevailing in U-2 OS.[7,24]

If the endothelial mitogen is an A-chain homodimer, several alternatives exist to explain why the EC mitogen behaves as a doublet after reduction. First, because the A chain possesses one possible site for N-linked glycosylation,[7] this apparent molecular weight difference might reflect differences in glycosylation. Second, these species may represent alternative proteolytic processing. Third, the doublet may reflect differences in the primary translation product arising from alternatively spliced A-chain transcripts; the presence of multiple transcripts in endothelial-cell RNA from an apparently single copy gene[7] suggests that the EC transcripts are, in fact, differentially spliced. Interestingly, Sejersen et al.[25] recently demonstrated that rat smooth and skeletal muscle cells express only the smaller two A-chain transcripts. Thus, tissue and/or species-specific processing of A-chain transcripts apparently occurs. It is conceivable that various human vascular cells also exhibit tissue-specific splicing of A chain and that these events may have important functional consequences. Evidence for species-specific splicing of the PDGF B chain has previously been suggested.[26]

The suggestion that HUVEs secrete A-chain homodimer raises the interesting possibility that there may be functional differences among the PDGF-like growth factors produced by the cells within the vascular wall in both normal and pathologic settings. In addition to endothelial cells, platelets,[17–20] macrophages,[27,28] and smooth muscle cells[29,30] produce PDGF species that stimulate the proliferation,[1,2,31] migration,[32] and contraction[33] of vascular smooth muscle cells. PDGF purified from normal human platelets contains approximately equal quantities of PDGF A and B chains, which suggests the possibility of heterodimers[17–21]; the subunit composition of the smooth muscle-derived mitogen and that of the activated macrophage-derived growth factor are not known. Although both A- and B-chain homodimers are biologically active, the affinities of these mitogens for PDGF receptors may differ from each other and from a heterodimer. The differences in chain composition between these mitogens may also be important in intracellular processing events, targeting of the protein, or stability of the mitogen within the vessel wall.

Endothelial cell gene expression may be modulated by nondenuding injury or by activation.[1,2,34] For example, levels of PDGF B-chain transcripts in cultured human ECs have been shown to undergo dynamic changes in an *in vitro* model of vascular morphogenesis.[35] The genes for PDGF A and B chains have been localized to different chromosomes (7 and 22, respectively[7,36]), and preliminary data suggest that A- and B-chain expression can be regulated independently.[7] Furthermore, evidence has been presented that the product of the *v-sis* gene (a B-chain homodimer) remains associated with, or rapidly associates with the cell membrane.[37,38] If PDGF B chains are constitutively translated by the endothelial cell, they may not be properly dimerized, processed, or secreted; alternatively, B-chain homodimers or A- and B-chains heterodimers may be formed and secreted by the endothelial cell but remain associated with the cell surface, or rapidly degraded.

Two additional implications can be drawn from our data: First, EC biosynthesis of the PDGF-like mitogen may be independent of B-chain expression. This limits the validity of measuring B-chain transcript levels as a means of quantifying endothelial growth factor production. And second, in various pathophysiologic settings,[39,40] activated or dysfunctional ECs may independently alter the quantity of A-chain and B-chain biosynthesis. This could result in more efficient PDGF secretion or even alter the biologic spectrum of PDGF activity. In conclusion, synthesis and secretion of PDGF-like molecules by ECs may be far more complex than previously thought, and further analysis with A- and B-chain–specific reagents will be necessary to elucidate the role of these mitogens in vessel wall pathophysiology.

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