CELL REGULATION, Vol. 1, 577–596, July 1990

Localization of platelet-derived endothelial cell growth factor in human placenta and purification of an alternatively processed form

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Platelet-derived endothelial cell growth factor (PD-ECGF) was purified to homogeneity from human term placenta, an organ characterized by extensive angiogenesis. N-terminal amino acid sequencing revealed that placental PD-ECGF was proteolytically processed at Thr-6, in contrast to PD-ECGF purified from human platelets, which is processed at Ala-11. The purified factor stimulated porcine aortic endothelial cells as well as two choriocarcinoma cell lines. Immunohistochemical staining revealed that PD-ECGF was present in the connective tissue cells of the placenta. The possibility that PD-ECGF is involved in the development of the placenta is discussed.

Introduction

The placenta is a multifunctional organ characterized by a large proportion of invasive cells that participate in the implantation of the blastocyst into the uterine wall and by extensive neovascularization. The latter is important for the transport of soluble substances between the fetus and the mother. In fact, the term "angiogenesis" was coined to describe the formation of new blood vessels in the placenta (Folkman and Klagsbrun, 1987). Another characteristic feature of the placenta is its regulatory function. It is well known that the placenta produces chorionic gonadotropin, placental progesterone, and placental lactogen. In recent studies, the placenta also has been found to contain a number of growth factors (reviewed in Ohlsson, 1989), including insulin-like growth factor–I (Wang *et al.,* 1988); insulin-like growth factor–II (Ohlsson *et al.,* 1989); platelet-derived growth factor (Goustin *et al.,* 1985); interleukin-1 (Flynn *et al.,* 1982); interleukin-2 (Boehm *et al.,* 1989); transforming growth factor-β (Frolik *et al.,* 1983); and fibroblast growth factor (FGF), which is known to be a potent stimulator of angiogenesis (Moscatelli *et al.,* 1986).

Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45-kDa protein that stimulates growth and chemotaxis of endothelial cells in vitro and angiogenesis in vivo (Ishikawa *et al.,* 1989). In contrast to angiogenic factors of the FGF family (Rifkin and Moscatelli, 1989), PD-ECGF does not stimulate the growth of fibroblasts and has no affinity for heparin. PD-ECGF has been purified from human platelet lysate (Miyazono *et al.,* 1987), and its cDNA was recently cloned from a human placenta cDNA library (Ishikawa *et al.,* 1989); the predicted sequence has no striking similarity with other known proteins. Analysis of the production of PD-ECGF by cultured cells revealed that it is synthesized by human foreskin fibroblasts and by certain cancer cell lines. Consistent with the finding that the primary product lacks a signal sequence, PD-ECGF was found to be inefficiently secreted from the producer cells (Usuki *et al.,* 1989).

It is an interesting possibility that the angiogenesis that occurs during the development of the placenta is stimulated by soluble factors. We describe in this article that human term placenta contains large amounts of PD-ECGF. N-terminal amino acid sequencing of PD-ECGF purified to homogeneity from term placenta revealed that it is differently processed compared with the platelet form. Immunohistochemical stainings revealed that PD-ECGF is present in stromal cells of the placenta. The possibility that PD-ECGF has a function in the normal development of the placenta is discussed.

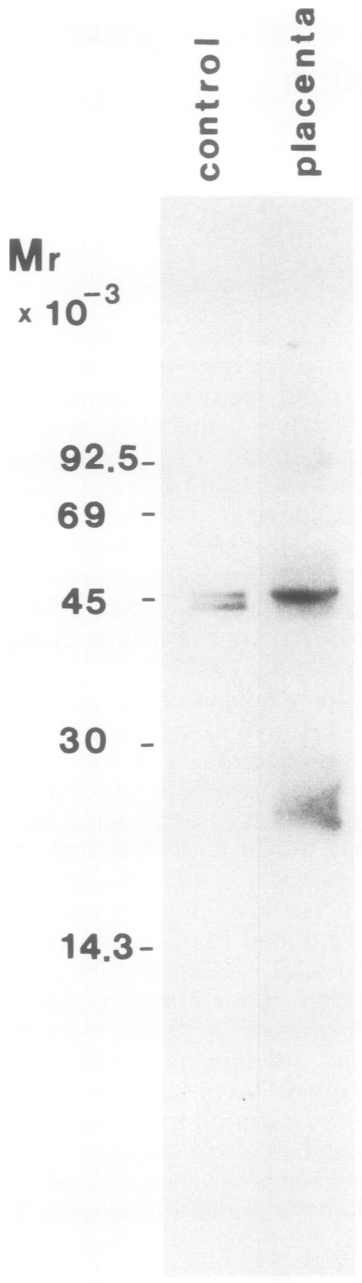


Figure 1. **Immunoblot analysis of human placental extract.** PD-ECGF purified from human platelet lysate was run in parallel as a control; it occurs as a doublet of ~45 kDa, probably because of proteolysis during preparation (Miyazono and Heldin, 1989).

Results

Presence of PD-ECGF in human term placenta

Immunoblotting using a specific rabbit antiserum against PD-ECGF (Miyazono and Heldin, 1989) was used to explore the possibility that PD-ECGF is present in human placenta. A placental extract was first subjected to a crude fractionation using ammonium sulfate precipitation; material precipitating between 28% and 42% saturation of ammonium sulfate, where PD-ECGF should be found (Miyazono and Heldin, 1989), was analyzed. An immunoblotted component of 45 kDa was seen, which was of approximately the same size as PD-ECGF purified from human platelets run in parallel (Figure 1). Based on the stainability of the immunoblotted material in comparison with known amounts of PD-ECGF purified from platelets, it was estimated that placental extract contained several milligrams of PD-ECGF per placenta (data not shown).

Purification of PD-ECGF

To allow a structural and functional characterization of placental PD-ECGF we purified it using an extract from a term human placenta as starting material. The purification was performed essentially as described for the purification of PD-ECGF from human platelet lysate, using QAE-Sephadex chromatography and ammonium sulfate precipitation, followed by chromatographies by anion exchange, high-performance hydroxylapatite, and alkyl-Superose (Miyazono and Heldin, 1989). However, a Q-Sepharose column was used rather than DEAE-Sepharose in the anion exchange step because it was found to give a higher resolution and recovery. In addition, two steps had to be added to obtain a homogeneous product: gel chromatography on a Superose 12 column and anion exchange chromatography on an FPLC Mono Q column. In the last step, two protein peaks, both containing growth-promoting activity, eluted at ~150 mM NaCl (Figure 2A). The protein composition of individual fractions of the Mono Q chromatogram was analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis and silver staining (Figure 2B). The active fractions each contained (a) single component(s) of 47 kDa; no difference in size between the compounds of the two peaks eluting at fractions 48 and 50, respectively, was seen (Figure 2B). We conclude that the two fractions most likely contain variants of the same protein.

A summary of the purification procedure starting from one term placenta is shown in Table I. About 40 μg of pure PD-ECGF was obtained from each preparation. The material was purified 23 000-fold at an overall yield of ~3.6%.

Structural and functional characterization of PD-ECGF purified from human placenta

N-terminal amino acid sequencing of pure placental PD-ECGF gave the sequence TPGTGA-

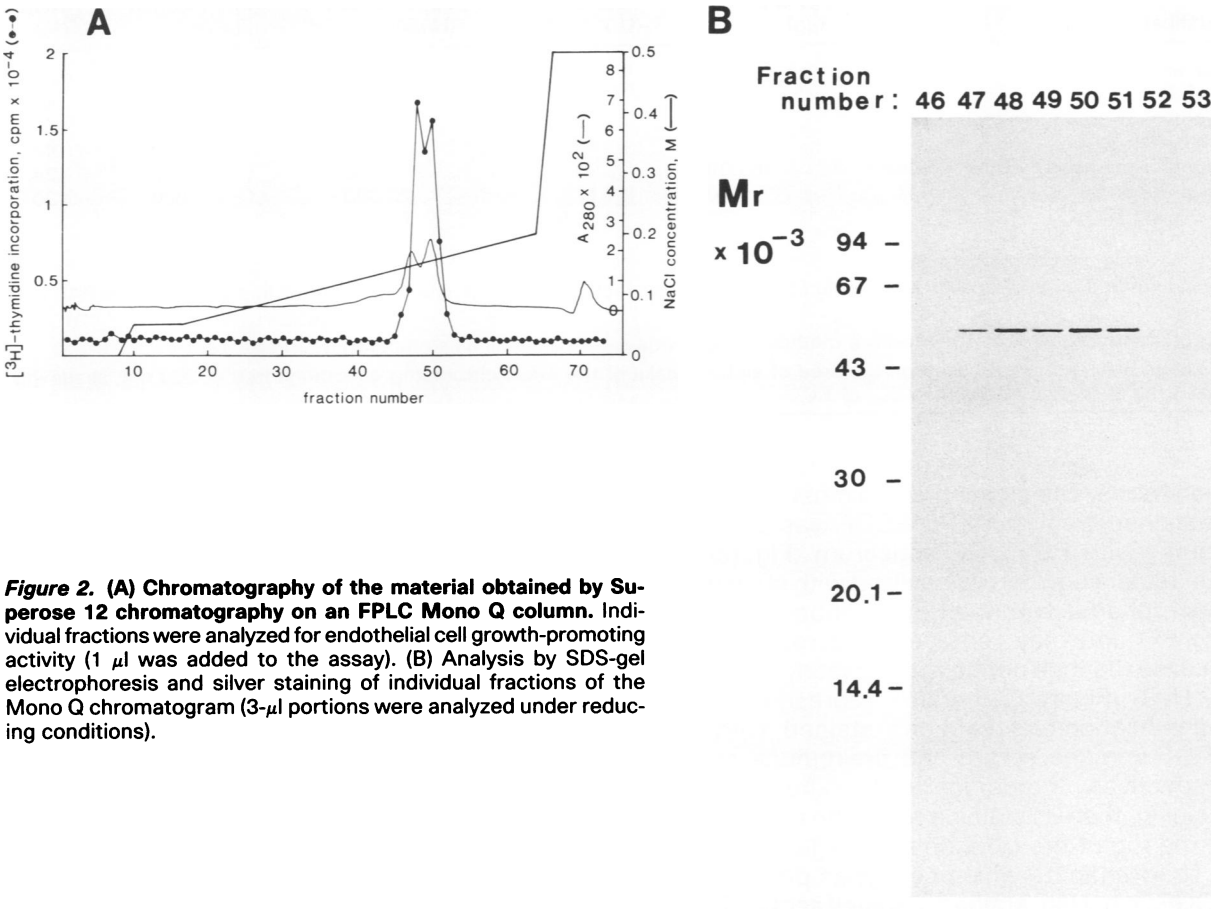


***Figure 2.* (A) Chromatography of the material obtained by Superose 12 chromatography on an FPLC Mono Q column.** Individual fractions were analyzed for endothelial cell growth-promoting activity (1 μl was added to the assay). (B) Analysis by SDS-gel electrophoresis and silver staining of individual fractions of the Mono Q chromatogram (3-μl portions were analyzed under reducing conditions).

PPA. . . . Thus, compared with the form purified from platelets, placental PD-ECGF contains 5 additional amino acids in its N-terminus (Figure 3). Comparison with the sequence predicted from the cDNA (Ishikawa *et al.,* 1989) reveals that the primary translational product is processed 5 and 10 amino acids from the translation-initiating methionine residue in the placental and platelet forms of PD-ECGF, respectively.

The purified placental PD-ECGF stimulated [^{3}H]thymidine incorporation into porcine aortic endothelial (PAE) cells in a dose-dependent manner (Figure 4A). Maximal stimulation of the purified sample occurred at ~35 ng/ml, i.e. at a similar concentration as for platelet PD-ECGF (30 ng/ml) (Usuki *et al.,* 1989). As previously observed for PD-ECGF purified from human platelets, the activity decreased at higher concentrations of placental PD-ECGF. The endothelial growth-promoting activity given by 35 ng/ml of pure material was completely inhibited by an antiserum raised against platelet PD-ECGF but not by nonimmune serum (Figure 4B).

PD-ECGF also stimulated DNA synthesis in two choriocarcinoma cell lines, JAR and JEG-3 (Figure 5). Maximal activity was observed at ~30 ng/ml of PD-ECGF. The decrease in activity on porcine aortic endothelial cells seen at high concentration was also observed in both of the two choriocarcinoma cell lines.

Immunohistochemical localization of PD-ECGF in sections of human placenta

A rabbit antiserum made against PD-ECGF purified from platelets was used to localize PD-ECGF in sections of human term placenta by an immunoperoxidase staining technique. As seen in Figure 6D, strong positive staining was seen in the stromal parts of the villi, mainly in the multipotent stromal cells and also diffuse in

Table 1. Purification of PD-ECGF from human placenta

Purification step	Total protein (μg)	Total activity (U × 10^{-6})[a]	Specific activity (U/μg)	Purification (-fold)	Yield (%)
Placental extract	26 000 000[b]	83	3.2	1	100
QAE-Sephadex	7 600 000[b]	37	4.9	1.5	45
Ammonium sulfate precipitation	390 000[b]	57	150	47	69
Q-Sepharose	26 000[c]	40	1500	470	48
High-performance hydroxyl apatite	10 000[c]	20	2000	630	24
Alkyl-Superose	730[c]	17	23 000	7200	20
Superose 12	130[c]	3.5	26 000	8100	4.2
Mono Q	40[c]	3	75 000	23 000	3.6

[a] Five hundred units of activity is the quantity of the growth factor required to give half-maximal stimulation in the PAE cell assay.

[b] Protein was assayed by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

[c] Protein was estimated based on the area of elution peaks of the FPLC chromatograms monitored at 280 nm, assuming that 1 mg of protein/ml gives an E_{280} of 1.0.

the stroma. The staining was almost completely quenched when pure PD-ECGF was added together with PD-ECGF antiserum (Figure 6C). The remaining stain colocalized with staining for macrophages with the RFD7 antibody (see Figure 6F) and may represent macrophage peroxidase that had not been completely exhausted by H_2O_2. Figure 6, B and A, represents higher magnifications of sections stained with PD-ECGF immune serum and preimmune serum, respectively. In addition to the diffuse stromal staining, there is staining along the sinusoid and at the top of the trophoblasts (Figure 6B).

To investigate which cell types produce PD-ECGF, we also stained parallel sections with antibodies against Factor VIII to visualize endothelial cells (Figure 6E) and with antibodies against human chorionic gonadotropin (HCG) to demonstrate trophoblasts (Figure 6H). Figure 6G is van Gieson staining of corresponding areas, showing that some villi contain an abundance of collagen.

Discussion

We show in the present study that PD-ECGF, an angiogenic factor originally purified from human platelets, is present at high quantities in human term placenta. Based on quantitative immunoblotting and the total amount of mitogenic activity for PAE cells, it was estimated that one placenta contained several milligrams of PD-ECGF, which is considerably more than can be accounted for by the PD-ECGF in the platelets of the placental blood. Structural analysis of PD-ECGF purified to homogeneity from term placenta showed that the placental factor was differently processed in the N-terminus compared with the platelet protein and contained five extra amino acids (Figure 3). This is consistent with the observation that the placental factor migrated in SDS-gel electrophoresis as a component of 47 kDa (Figure 1), whereas the platelet factor migrates slightly faster, corresponding to a size of 45–46 kDa (Miyazono *et al.*, 1987; Miyazono and Heldin, 1989). The placental and platelet forms were approximately equipotent in their mitogenic activities on PAE cells; whether their structural difference is associated with other functional differences is currently being investigated.

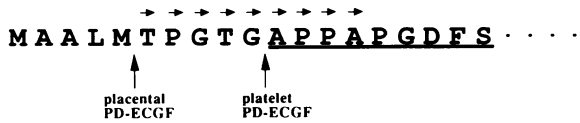


***Figure 3.* N-terminal amino acid sequences of PD-ECGF purified from human term placenta and human platelets.** The N-terminal sequence of PD-ECGF deduced from the cDNA (Ishikawa *et al.*, 1989) is shown; the amino acid sequence obtained by sequencing of placental PD-ECGF is indicated by arrows and the N-terminal sequence of platelet PD-ECGF underlined. The vertical arrows denote cleavage sites for PD-ECGF purified from placenta and platelets.

The purification of PD-ECGF to homogeneity from human placenta involved a 23 000-fold purification at a yield of 3.6%. The corresponding figures for the purification of PD-ECGF from human platelets are a 1 250 000-fold purification at a 14% yield (Miyazono and Heldin, 1989). The differences most likely result partly from the fact that placenta also contains other endothelial cell mitogens, e.g., FGFs (Moscatelli *et al.*, 1986), whereas PD-ECGF seems to be the only major mitogen in platelets for PAE cells (Miyazono and Heldin, 1989). The placenta also contains growth inhibitors for endothelial cells, such as transforming growth factor–β (Frolik *et al.*, 1983), which may explain the increase in

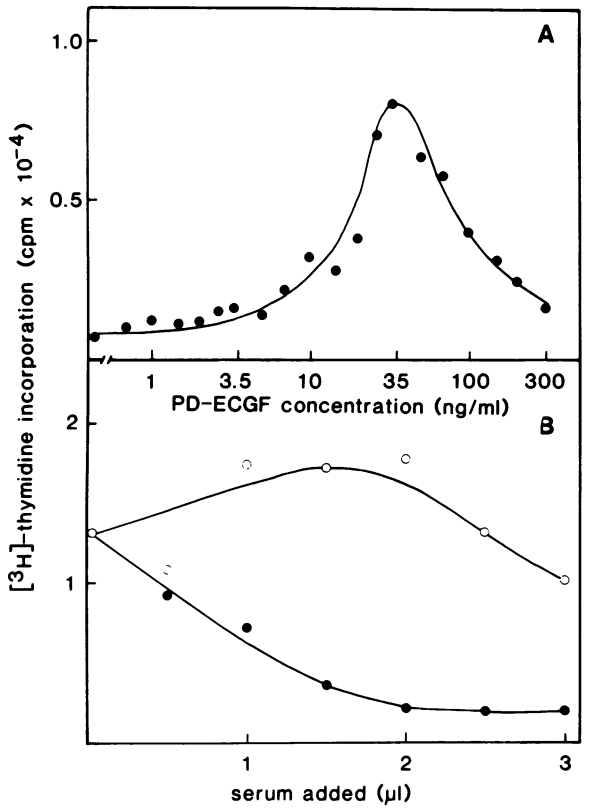


Figure 4. **(A) Dose-response curve of PD-ECGF purified from human placenta on porcine aortic endothelial cells.** (B) Growth-promoting activity of 35 ng/ml PD-ECGF in the presence of increasing volumes of a rabbit PD-ECGF antiserum (●) or nonimmune serum (○). Values represent [^{3}H]thymidine incorporation into DNA (means of duplicates).

growth–promoting activity noticed after the ammonium sulfate precipitation step in the preparation procedure (Table 1).

Immunohistochemical staining of sections from term placenta revealed that PD-ECGF was present in stromal cells, whereas trophoblasts and endothelial cells were negative (Figure 6). This is consistent with our previous finding that human foreskin fibroblasts in culture produced PD-ECGF (Usuki *et al.*, 1989). A staining along the sinusoids was also seen. This most likely represents adsorbed PD-ECGF derived from platelets of the maternal blood, because partial coagulation and activation of platelets would occur at some sites in the placental tissue during labor, as well as after delivery before freezing of the tissue. In addition to endothelial cells, trophoblasts are also possible targets for PD-ECGF secreted from stromal cells in the placenta, because PD-ECGF stimulates the growth not only of endothelial cells, but also of choriocarcinoma cell lines, which are derived from trophoblasts (Figure 5). It remains to be investigated, however, whether PD-ECGF promotes the growth of nontransformed trophoblasts. It is thus possible that, at a later stage of placental development, stromal PD-ECGF of the placenta may play a key role in angiogenesis through the promotion of growth of trophoblasts and endothelial cells, which build up the vascular network in direct contact with blood in sinuses and in vessels, respectively.

Apart from mitogenicity, PD-ECGF stimulates chemotaxis of endothelial cells (Ishikawa *et al.*, 1989). Furthermore, with the use of immunoblot analysis with anti-PD-ECGF specific serum, the factor has been found in porcine uterus and in cultured human vascular smooth muscle cells (Usuki *et al.*, unpublished data). It is thus possible that PD-ECGF also has a role at the early stages of placental development; PD-ECGF from uterus smooth muscle cells and stromal fibroblasts might promote the implantation of the blastocyste into the uterine wall by stimulating chemotaxis of trophoblasts.

Materials and methods

Assay of growth promoting activity

Porcine aortic endothelial cells were cultured in Ham's F-10 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and antibiotics (Miyazono *et al.*, 1987). Human choriocarcinoma cell lines JAR and JEG-3, obtained from American Type Culture Collection (Rockville, MD), were grown in RPMI 1640 (GIBCO) and minimum essential medium (GIBCO), respectively, in the presence of 10% FCS. Cells were trypsinized and replated sparsely (~1 × 10^4 cells/well) in 500 μl of Ham's F-10 medium containing 0.5% FCS in 24-well tissue culture plates. After 24 h of incubation, samples were added to the wells. Eighteen hours later, [^{3}H]thymidine (7.4 kBq [0.2 μCi]/well, 6.7 Ci/mmol; New England Nuclear, Boston, MA) was added. After an additional 6 h, the cells were fixed with ice-cold 5% (w/v) trichloroacetic acid for 20 min. The resulting precipitates were washed extensively with water and solubilized with 200 μl of 1 M NaOH. After incubation at room temperature for 20 min, 250 μl of 1 M HCl was given to the wells. ^{3}H-radioactivity was then determined in a liquid scintillation counter with the use of 10 ml of Ready Safe (Beckman, Fullerton, CA) per sample.

Purification of PD-ECGF from human placenta

A term human placenta was obtained from Department of Obstetrics, University Hospital, Uppsala, within 2 h after delivery and stored at −20°C until use. All procedures were performed at 4°C, unless otherwise described. The placenta was thawed at 4°C overnight, dissected free from amnion membrane and the umbilical cord, and then ground in a meat grinder. The minced placenta (~450 g) was homogenized (Food Processor, Electrolux, Alingsås, Sweden, maximal speed for 5 min in N_2 atmosphere) in four times its weight of 0.15 M NaCl, 10 mM imidazol buffer, pH 7.4, 5 mM dithiothreitol. The homogenate was cleared from debris by centrifugation for 10 min at 1300 × *g* and then filtered through two layers of cheesecloth. The filtrate was clarified by recentrifugation at 13 700 × *g* for 1 h and then diluted

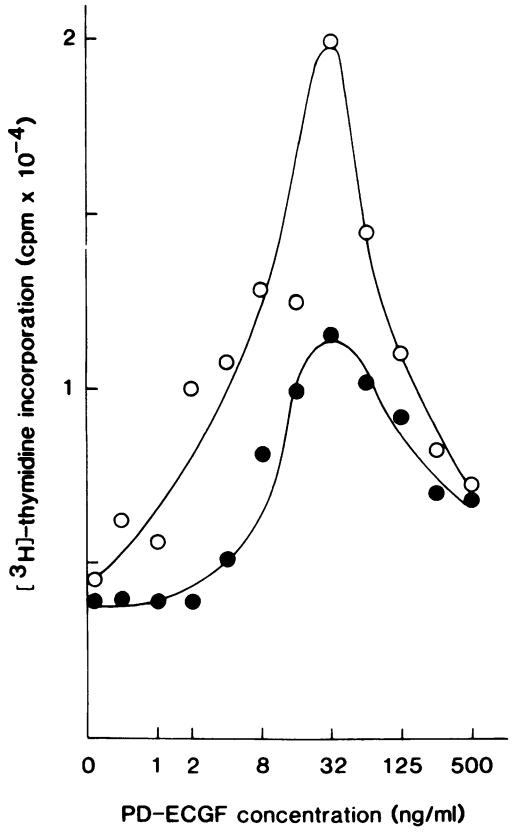


Figure 5. **Dose-response curve of pure PD-ECGF on two human choriocarcinoma cell lines, JAR (○) and JEG-3 (●).** Values represent [^{3}H]thymidine incorporation into DNA (means of duplicates).

to 75 mM NaCl with distilled water; 30 g of dry QAE-Sephadex A-50 gel (Pharmacia-LKB, Uppsala, Sweden) was then added, and the slurry was mixed by shaking overnight. The gel was poured into a column (60 × 5 cm; Pharmacia-LKB) and washed with 75 mM NaCl, 10 mM imidazol buffer, pH 7.4; the sample was then eluted with 250 mM NaCl, 10 mM imidazol buffer, pH 7.4. Ammonium sulfate (247 g/l) in 50 mM sodium phosphate buffer, pH 7.4, was added to the eluate of the QAE-Sephadex column. After equilibration for 2 h, the precipitate was pelleted by centrifugation at 3000 × *g* for 30 min, then resuspended in phosphate-buffered saline (PBS); treated with 5 mM dithiothreitol at room temperature for 2 h; and dialyzed extensively against 50 mM NaCl, 10 mM Bis-tris(hydroxymethyl)aminomethane/HCl (Bis-Tris/HCl), pH 6.8. After filtration through a 0.22-μm filter (Millipore, Bedford, MA), the sample was applied to a 30-ml column (1.5 × 15 cm) of Q-Sepharose (Pharmacia-LKB) and eluted with a 400-ml linear gradient of NaCl from 100 mM to 200 mM in Bis-Tris, pH 6.8; the column was operated at a flow rate of 5 ml/min; and 10-ml fractions were collected. The fractions containing endothelial cell growth-promoting activity were pooled and subjected to a high-performance hydroxylapatite column (7.8 × 100 mm; Bio-Rad, South Richmond, CA) equipped with a guard column (4.0 × 50 mm; Bio-Rad). After washing with 1 mM phosphate buffer, pH 6.8, 50 mM NaCl, 0.01 mM $CaCl_2$, the bound material was eluted with a gradient of 20–80 mM phosphate buffer pH 6.8 (24 ml), in 50 mM NaCl, 0.01 mM $CaCl_2$, at a flow rate of 0.4 ml/min; and 1-ml fractions were collected. The active fractions from the high-performance hydroxylapatite chromatography were adjusted to 1.4 M ammonium sulfate, 100 mM phosphate buffer, pH 6.8, and loaded onto an alkyl-Superose column (HR 5/5, Pharmacia-LKB) preequilibrated with the same buffer. The material was eluted with a gradient of ammonium sulfate from 1.4–0 M in 100 mM phosphate buffer, pH 6.8 (20 ml), at a flow rate of 0.5 ml/min; fractions of 500 μl were collected. The fractions containing PD-ECGF activity were pooled, concentrated to 100 μl with the use of Centricon 10 microconcentrator (Amicon, Danvers, MA), and applied to a Superose 12 column (HR 10/30, Pharmacia-LKB). The column was equilibrated with 50 mM NaCl, 10 mM phosphate buffer, pH 7.4, and eluted at a flow rate of 0.5 ml/min. The active fractions were pooled and directly loaded onto a Mono Q column (HR 5/5, Pharmacia-LKB), and eluted with a gradient of 50–200 mM NaCl in 10 mM phosphate buffer, pH 7.4, at a flow rate of 1.0 ml/min. Fractions of 1 ml were collected and tested for endothelial cell growth-promoting activity.

Amino acid sequence analysis

Before the sequence analysis, the PD-ECGF preparation was desalted on a narrow-bore reversed-phase column (2.1 × 30 mm) with a gradient of acetonitrile in 0.1% trifluoroacetic acid.

The N-terminal sequence analysis was performed in an Applied Biosystems Model 477A Protein Sequencer, equipped with an online PTH-Analyzer Model 120A (Foster City, CA). The instruments were operated according to the manufacturer's instructions.

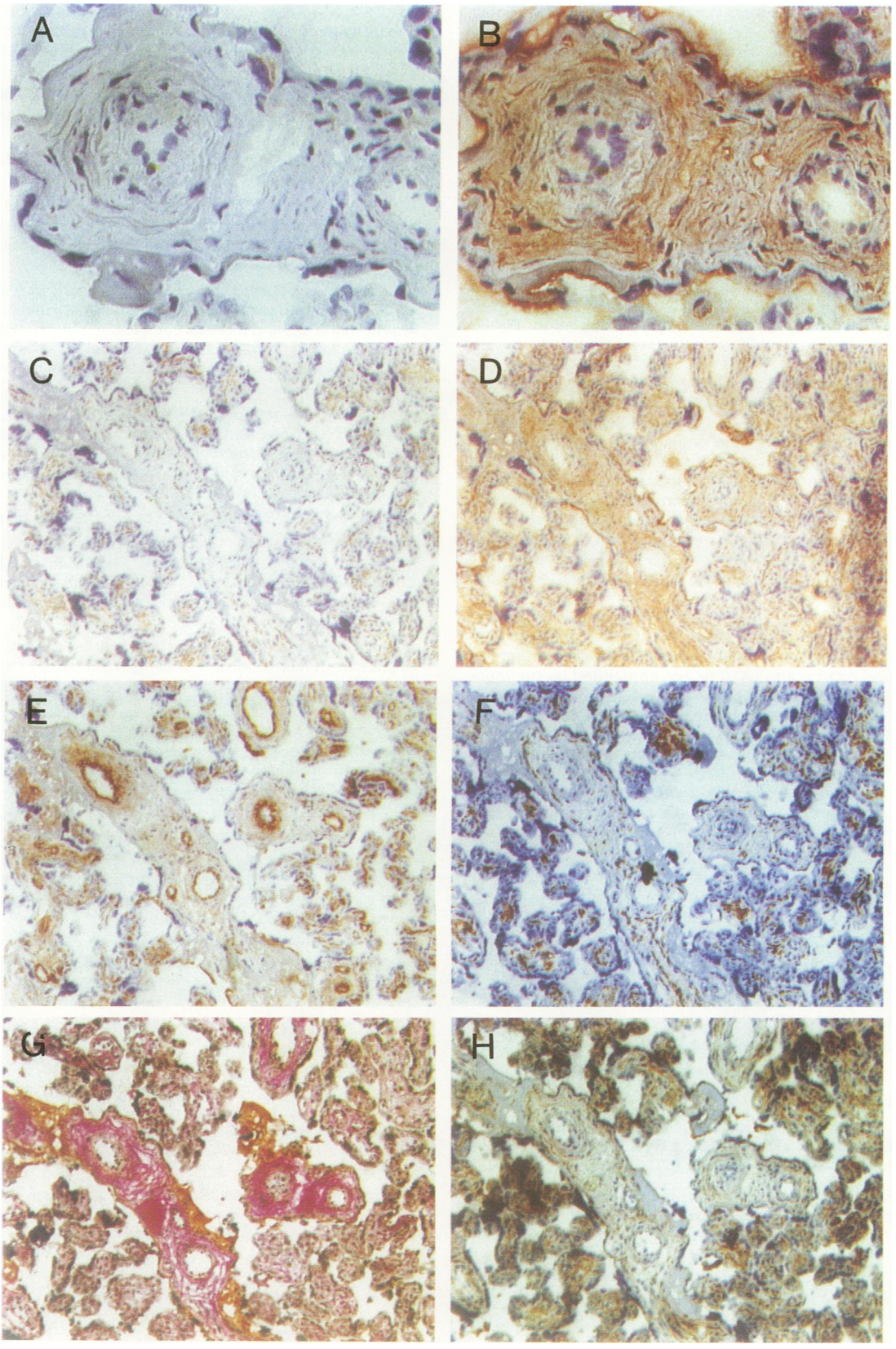
Immunoblotting

SDS-gel electrophoresis was performed in 10%–18% gradient polyacrylamide gels under reducing conditions (Blobel and Dobberstein, 1975). Samples on the gel were transferred to nitrocellulose membranes in a buffer containing 10% ethanol, 150 mM glycine, and 20 mM Tris-base, pH 8.4. The nitrocellulose membranes were incubated in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10% bovine serum albumin (BSA; Fraction V, Boehringer Mannheim, Mannheim, FRG) to block non-specific binding, then incubated in a 1:50 dilution of a specific rabbit PD-ECGF antiserum (Miyazono and Heldin, 1989), and washed twice with 150 mM NaCl, 10 mM Tris-HCl, pH 7.4 and twice with 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Triton X-100. The nitrocellulose membranes were then incubated with ^{125}I-labeled staphylococcal protein A (5 × 10^5 cpm/ml) and washed as described above. Blots were subjected to autoradiography.

Immunohistochemistry

Biopsies from term placentas—obtained from Department of Obstetrics, University Hospital, Uppsala—were frozen

Figure 6. **Immunohistochemical stainings of serial sections of a human term placenta.** Staining with an anti-PD-ECGF antiserum (B, D) gave the most abundant staining in the stroma. Preimmune serum (A) or blocking of the anti-PD-ECGF antiserum by pure PD-ECGF (C) gave essentially no staining. Trophoblasts were visualized by an antiserum against HCG (H), macrophages by the monoclonal antibody RFD7 (F), and endothelial cells by an antiserum against Factor VIII (E). A van Gieson staining is also shown (G). Magnifications: A and B, ×400; C–H, ×100.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within 30 min after delivery in a mixture of dry ice and liquid isopentane. Six-micrometer-thick cryosections were cut and stored at −70°C. The sections were fixed sequentially in 50% cold acetone for 30 s and 100% cold acetone for 5 min and finally air dried. Fixed sections were washed in PBS and exhausted for endogenous peroxidase by incubation in 0.3% H_2O_2 for 15 min. After new washes in PBS, the sections were incubated for 30 min with normal swine serum (Dakopatts, Glostrup, Denmark) diluted 1:10 in PBS containing 4% BSA. Sections were reacted for 30–60 min with 30 μl of rabbit anti–PD-ECGF antiserum (diluted 1:400) (Miyazono and Heldin, 1989), preimmune serum (diluted 1:400), or rabbit anti–Factor VIII antiserum (diluted 1:400) and washed with PBS. In the second step, sections were incubated for 30–60 min with swine anti-rabbit IgG (Dakopatts) diluted 1:500 in PBS containing 4% BSA and then washed with PBS. In the third step, sections were incubated for 30–60 min with rabbit peroxidase/anti-peroxidase (rabbit-PAP; Dakopatts) diluted 1:1000 in PBS containing 4% BSA. The peroxidase reaction was carried out by the use of a diethyl carbazole–containing buffer (Kaplow, 1975), and the sections were counterstained with hematoxylin and mounted in glycerin-gelatin. Both the swine anti-rabbit IgG and the rabbit-PAP reagent had been preabsorbed with normal human serum immobilized on CNBr-activated Sepharose-4B (Pharmacia-LKB) to suppress unspecific binding to the placental tissue. Control stainings included 1) exchange of the primary antiserum with preimmune serum from the same rabbit and 2) stainings with anti–PD-ECGF antiserum that had been preincubated with a threefold molar excess of purified PD-ECGF for 24 h at 4°C.

Human chorionic gonadotropin–producing cells and macrophages were also visualized by immunohistochemistry. An anti-HCG rabbit antiserum was obtained commercially (Dakopatts) and used as recommended by the manufacturer. The tissue macrophage-specific monoclonal antibody RFD7 was kindly provided by Dr. Leonard Poulter (Department of Immunology, Royal Free Hospital, London, UK) (Poulter *et al.*, 1986) and used at a concentration of 1 μg/ml. Sections were pretreated as described above and stained immunohistochemically with each of the antibodies, employing a biotin-avidin-peroxidase complex (ABC)-based system (Vectastain, Vector Laboratories, Burlingame, CA).

Acknowledgments

We thank Ingegärd Schiller for valuable help in the preparation of this manuscript. These studies were in part supported by the Swedish Cancer Society.

Received: April 26, 1990.
Revised and accepted: May 22, 1990.

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