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High-Yield Purification of Platelet-Derived Endothelial Cell Growth Factor: Structural Characterization and Establishment of a Specific Antiserum

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Received June 9, 1988; Revised Manuscript Received September 15, 1988

ABSTRACT: Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45-kDa protein that stimulates the growth of endothelial cells [Miyazono, K., et al. (1987) *J. Biol. Chem. 262*, 4098-4103]. Here, we describe a method to purify large quantities of PD-ECGF from human platelet lysate at a high yield (14% overall recovery). The purification method involves five steps, using high-performance liquid chromatography grade hydroxylapatite and hydrophobic chromatographies as the two final steps. The purified material contained two major components of apparent molecular weight values of 46 000 and 44 000. These components coeluted in a high-resolving reversed-phase chromatography and were found to give similar peptide maps after treatments with staphylococcal V8 protease, suggesting that the 44-kDa form is related to the 46-kDa molecule. Partial tryptic digestion of native PD-ECGF revealed that the molecule contains a trypsin-resistant domain of 37-39 kDa. A rabbit antiserum was produced against the purified material and was found to specifically recognize PD-ECGF in immunoblotting. When added to the cell culture medium, an immunoglobulin fraction of the antiserum neutralized the activity of purified PD-ECGF. Furthermore, it completely neutralized the endothelial cell mitogenic activity of platelet lysate, indicating that PD-ECGF is the only mitogen in platelet lysate for this cell type.

Factors with the ability to stimulate the formation of blood vessels have attracted interest as causative agents in angiogenesis of normal and malignant tissues [for a review, see Folkman and Klagsbrun (1987)]. The most well-characterized

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factors in this category are fibroblast growth factors (FGFs),[1] which are a family of heparin-binding endothelial cell mitogens originally isolated from neural tissues [for recent reviews, see Gospodarowicz et al. (1987) and Thomas (1987)]. Another endothelial cell mitogen, platelet-derived endothelial cell growth factor (PD-ECGF), was recently purified and partially characterized (Miyazono et al., 1987). It is a single-chain anionic polypeptide with an apparent molecular weight of 45 000. The bioactivity of PD-ECGF is susceptible to heat or acid but resistant to reducing agents. It stimulates the growth of endothelial cells from various origins, including porcine aorta, human umbilical vein (Miyazono et al., 1987), and murine lung capillary vessels (our unpublished data). The biological and biochemical properties of PD-ECGF are different from those of FGFs; PD-ECGF has a higher molecular weight than FGFs and, unlike FGFs, does not stimulate the growth of human fibroblasts. Furthermore, PD-ECGF does not bind to heparin–Sepharose nor is the activity of PD-ECGF potentiated by exogenous heparin (Miyazono et al., 1987).

We recently described a procedure to prepare small quantities of pure PD-ECGF (Miyazono et al., 1987). In this paper, we describe an improved method to purify PD-ECGF, which allows the purification of sufficient amounts for a thorough structural and functional characterization. Furthermore, we report that PD-ECGF occurs in multiple forms and describe an antiserum that specifically recognizes the factor which has been used to show that PD-ECGF is the only mitogen for porcine aortic endothelial cells in human platelet lysate.

Materials and Methods

Assay for Growth-Promoting Activity. Growth-promoting activity was routinely monitored as the incorporation of [^{3}H]thymidine into DNA, using porcine aortic endothelial cells as target cells (Miyazono et al., 1987). The cells were transferred to Ham's F-10 medium containing 0.5% fetal bovine serum and antibiotics in 24-well tissue culture plates (Costar). After 24 h of incubation, the test samples were added. After an additional 18 h of incubation, 0.2 μCi of [^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear) was added and incubation continued for another 4 h. The ^{3}H radioactivity incorporated into macromolecular material in the cells was determined as previously described (Miyazono et al., 1987).

Purification of PD-ECGF. (A) Initial Steps in PD-ECGF Purification. All procedures were performed at 4 °C, unless otherwise described. The initial isolation procedures of PD-ECGF were performed as previously reported (Miyazono et al., 1987). As starting material, the side fraction from the purification of platelet-derived growth factor from human platelet lysate (Heldin et al., 1987) was used. About 15 L of the flow-through fraction of CM-Sephadex chromatography, which is the first step in the purification of platelet-derived growth factor, was processed at a time. Dry QAE-Sephadex A-50 gel (0.7 g/L; Pharmacia) was added to the starting material and mixed by shaking overnight. The gel was poured into a column (60 × 5 cm; Pharmacia), washed with 75 mM NaCl/10 mM phosphate buffer, pH 7.4, and eluted with 250 mM NaCl/10 mM phosphate, pH 7.4. Ammonium sulfate (247 g/L) was added to the eluate of QAE-Sephadex chromatography. After equilibration for 2 h, the sample was centrifuged at 2075g for 15 min. The precipitate was collected and resuspended in 50 mM NaCl/10 mM Hepes, pH 7.0, treated with 5 mM dithiothreitol at room temperature for 2 h, and dialyzed extensively against the same buffer. The material was applied to a 40-mL column of DEAE-Sepharose CL–6B (Pharmacia) and eluted with a 400-mL linear gradient of NaCl from 50 to 200 mM in Hepes, pH 7.0; the column was operated at a flow rate of 120 mL/h, and 10-mL fractions were collected. The active fractions were pooled and subjected to further purification.

(B) Chromatography on a High-Performance Hydroxylapatite Column. The material obtained from DEAE-Sepharose chromatography was filtered through a 0.22-μm filter (Millipore) and loaded at room temperature onto a high-performance hydroxylapatite column (100 × 7.8 mm; Bio-Rad) equipped with a guard column (50 × 4.0 mm; Bio-Rad). The column was preequilibrated with 1 mM phosphate buffer, pH 6.8, 50 mM NaCl, and 0.01 mM $CaCl_2$ and eluted at a flow-rate of 0.5 mL/min with a gradient of 1–100 mM phosphate, pH 6.8, 50 mM NaCl, and 0.01 mM $CaCl_2$. The column was then washed with 1 M phosphate buffer, pH 6.8, and 0.01 mM $CaCl_2$. Fractions of 1 mL were collected and tested for growth-promoting activity on porcine endothelial cells.

(C) Chromatography on an Alkyl-Superose Column. The active fractions from the high-performance hydroxylapatite chromatography were pooled and mixed with an equal volume of 2.8 M ammonium sulfate (HPLC-grade, Bio-Rad) and 100 mM phosphate buffer, pH 6.8. The material was applied to an alkyl-Superose column (HR5/5, Pharmacia), preequilibrated with 1.4 M ammonium sulfate/100 mM phosphate buffer, pH 6.8, and eluted with a gradient of ammonium sulfate from 1.4 to 0 M in 100 mM phosphate buffer, pH 6.8. The flow-rate was 0.5 mL/min, and the column was operated at room temperature. The absorbance of the column effluent was monitored at 280 nm. Fractions of 500 μL were collected and assayed for growth-promoting activity.

Radioiodination. Pure PD-ECGF was labeled with ^{125}I using the method of Hunter and Greenwood (1962). Briefly, 5 μg of PD-ECGF (200 μL of volume) obtained from alkyl-Superose chromatography, containing 0.5 mCi of Na^{125}I (Amersham), was given 10 μL of chloramine-T (2 mg/mL) and incubated for 1 min at room temperature. The reaction was stopped by adding 25 μL of sodium metabisulfite (2 mg/mL) and desalted by using a PD-10 column (Pharmacia), equilibrated with 150 mM NaCl, 50 mM phosphate buffer, pH, 7.4, and 1 mg/mL bovine serum albumin. Protein A (Pharmacia) was labeled with ^{125}I using Iodobeads (Pierce). Briefly, 0.2 mg of protein A was incubated with 0.5 mCi of Na^{125}I and Iodobeads for 30 min at room temperature and desalted on a PD-10 column equilibrated with 150 mM NaCl/10 mM phosphate buffer, pH 7.4.

SDS Gel Electrophoresis. SDS gel electrophoresis was performed according to the method of Blobel and Dobberstein (1975), using gradient gels of 10–18% polyacrylamide. After electrophoresis, gels were analyzed by silver staining (Morrissey, 1981) or used for immunoblotting or peptide mapping. The following were used as molecular weight markers (Pharmacia): phosphorylase b (94K); bovine serum albumin (67K); ovalbumin (43K); carbonic anhydrase (30K); soybean trypsin inhibitor (20.1K); and α-lactalbumin (14.4K).

Peptide Mapping. ^{125}I-PD-ECGF was analyzed by SDS gel electrophoresis and autoradiography. The principal bands of molecular weight values of 46 000, 44 000, and 41 000 were cut out from the gel and subjected to peptide mapping ac-

[1] Abbreviations: FGFs, fibroblast growth factors; FPLC, fast protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; PD-ECGF, platelet-derived endothelial cell growth factor; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor β.

cording to the method of Cleveland et al. (1977), using various concentrations of staphylococcal V8 protease (Sigma). Samples were analyzed by one-dimensional SDS gel electrophoresis on 15% polyacrylamide gels, followed by autoradiography.

Preparation of PD-ECGF Antiserum. Pure PD-ECGF obtained by alkyl-Superose chromatography was diluted to 20 μg/mL by 150 mM NaCl/10 mM phosphate buffer, pH 7.4. Ten micrograms of pure PD-ECGF was then mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into a rabbit. The rabbit was boosted 2 weeks later with 10 μg of PD-ECGF in Freund's incomplete adjuvant. After that, the rabbit was boosted every 2–3 weeks with 5 μg of PD-ECGF in Freund's incomplete adjuvant. The immune serum used in this paper was obtained after more than four injections. The immunoglobulin fraction was purified by applying 4 mL of immune serum to a 2-mL column of protein A–Sepharose (Pharmacia) equilibrated with 100 mM phosphate buffer, pH 7.4. The column was washed with the same buffer and then eluted with 50 mM citrate buffer, pH 3.0. The eluate from the column was rapidly neutralized with 1 M Tris-HCl, pH 7.4.

Immunoblotting. SDS gel electrophoresis was performed as described above; samples on the gel were then electrophoretically transferred to nitrocellulose sheets in a buffer containing 20% ethanol, 150 mM glycine, and 20 mM Tris-HCl, pH 8.4, at 200 mA for 5 h at room temperature (Burnette, 1981). Nonspecific protein binding was blocked by incubating for 30 min at 40 °C in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 5% bovine serum albumin. The nitrocellulose sheets were then incubated with a 1:50 dilution of antiserum in the same buffer. Blots were washed once with 150 mM NaCl/10 mM Tris-HCl, pH 7.4, for 10 min, washed twice with the same buffer containing 0.05% Triton X-100 for 20 min per wash, and finally for 10 min in 150 mM NaCl/10 mM Tris-HCl, pH 7.4, only. Then, the blots were incubated with ^{125}I-labeled protein A (3×10^5 cpm/mL) in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 5% bovine serum albumin, washed according to the same procedure as above, and analyzed by autoradiography.

Protein Determination. Protein concentration was determined by the dye fixation assay of Bradford (1976), with bovine serum albumin as a standard. The amount of pure PD-ECGF was determined by amino acid analysis.

Results

High-Yield Purification of PD-ECGF. For the purification of PD-ECGF, a side fraction from the purification of platelet-derived growth factor, i.e., the flow-through fraction of CM-Sephadex chromatography (Heldin et al., 1987), was used. The initial steps of the purification, i.e., QAE-Sephadex chromatography, ammonium sulfate precipitation, and DEAE-Sepharose chromatography, were performed as reported (Miyazono et al., 1987). As the next step, chromatography on an HPLC-grade hydroxylapatite column was used. PD-ECGF bound to the gel in 1 mM phosphate buffer, pH 6.8, and was eluted with a linear gradient of phosphate (Figure 1A). The recovery of activity was about 32% in this step. Individual fractions from the chromatogram were analyzed by SDS gel electrophoresis and silver staining; the growth-promoting activity coeluted with two components of 46 and 44 kDa and some other components (Figure 1B).

Final purification was achieved by hydrophobic chromatography using FPLC. The pooled fractions from the high-performance hydroxylapatite chromatography were applied to an alkyl-Superose column, equilibrated with 1.4 M ammonium sulfate/100 mM phosphate buffer, pH 6.8, and eluted

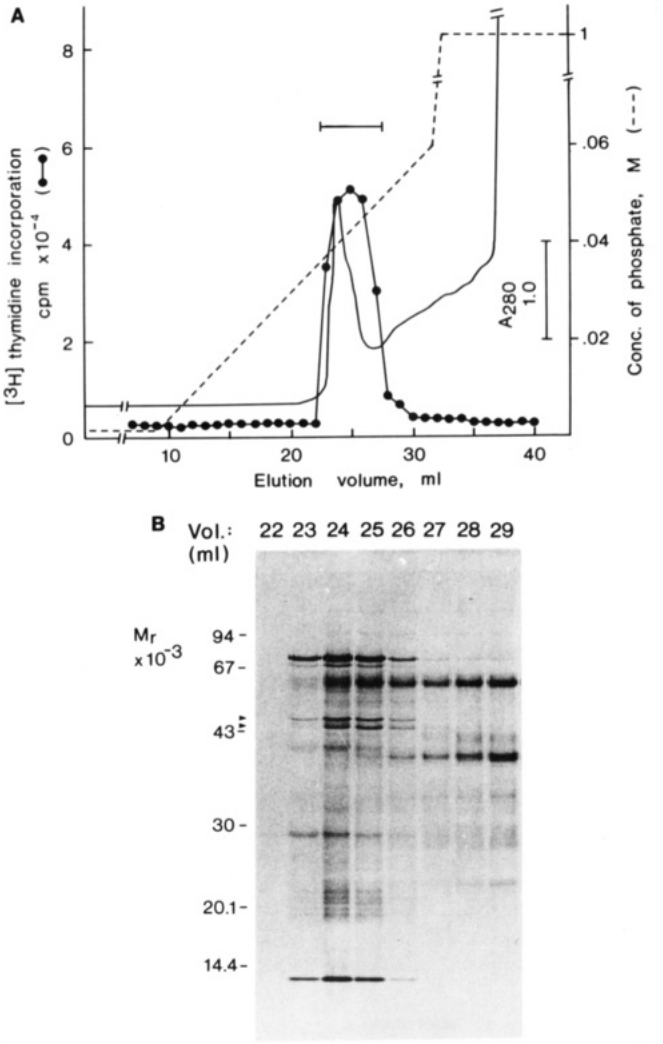

FIGURE 1: Chromatography on a high-performance hydroxylapatite column. (A) Chromatography of the material obtained by DEAE-Sepharose chromatography on a high-performance hydroxylapatite column. For experimental details, see Materials and Methods. Two-microliter aliquots of each fraction were added to endothelial cells for bioassay. Fractions under the horizontal bar were collected for the next step. (B) Analysis by SDS gel electrophoresis and silver staining of individual fractions of the hydroxylapatite chromatogram. Five-microliter aliquots of each fraction were analyzed individually under reducing conditions. The 46- and 44-kDa bands, which coeluted with the growth-promoting activity, are shown by arrowheads.

with a decreasing gradient of ammonium sulfate in 100 mM phosphate buffer, pH 6.8. A single peak of growth-promoting activity was obtained at about 0.8 M ammonium sulfate (Figure 2A). The increase in specific activity was 50-fold in this step at a recovery of 82%. The protein compositions of individual fractions of the alkyl-Superose chromatogram were analyzed by SDS gel electrophoresis and silver staining (Figure 2B); again, the active fractions contained the principal two components of 46 and 44 kDa, indicating that PD-ECGF was essentially pure. When the purified material was analyzed under nonreducing conditions, bands of the same sizes were observed (Figure 2C). Furthermore, a very faint band of M_r 42 000 was reproducibly found. The proportion of these proteins in the silver-stained gels differed from preparation to preparation, but the 46- and 44-kDa components were always predominant.

A summary of the purification procedure starting from 300 g of platelet protein, corresponding to approximately 800–1000 L of human blood, is shown in Table I. About 34 μg of pure PD-ECGF was obtained from each preparation. The material

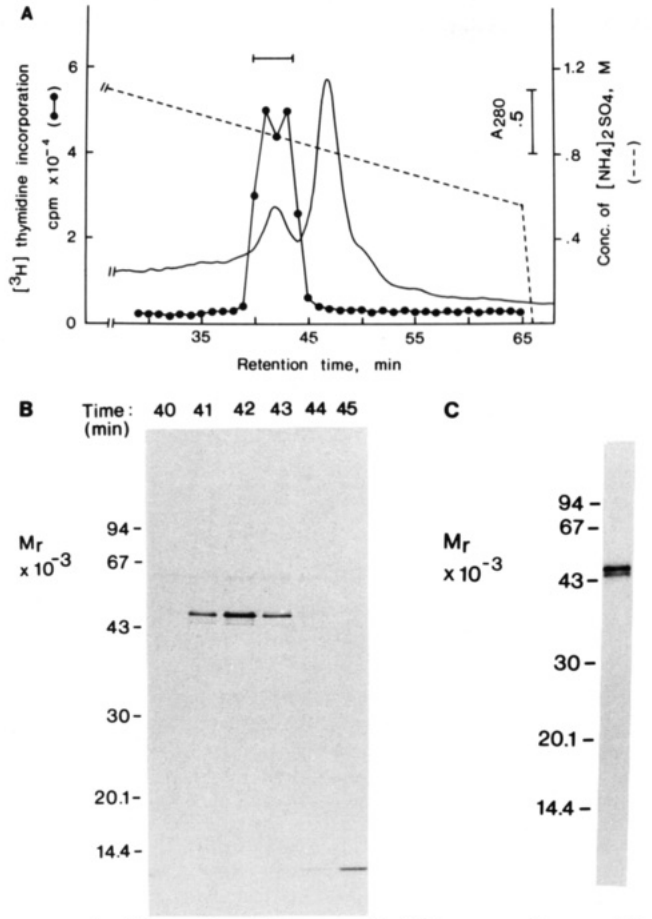

FIGURE 2: Chromatography on an alkyl-Superose column. (A) Alkyl-Superose chromatography of the material obtained by high-performance hydroxylapatite chromatography. Aliquots of the fractions were diluted 10-fold with 100 mM phosphate buffer, pH 6.8, and 10-μL aliquots were added to endothelial cells for bioassay. (B) Analysis by SDS gel electrophoresis and silver staining of the fractions from alkyl-Superose chromatography. Two-microliter aliquots of each fraction were analyzed individually under reducing conditions. (C) Analysis of the purified material (from 40 to 43 min) by SDS gel electrophoresis and silver staining under nonreducing conditions.

Table I: Summary of the Purification of PD-ECGF[a]

purification step	protein (μg)	max stimulation (ng/mL)	purification (x-fold)	yield (%)
platelet lysate (flow-through from CM-Sephadex)	300 000 000	*b*	1	*b*
QAE-Sephadex	12 000 000	1 000 000	20[b]	80
ammonium sulfate pptn	800 000	80 000	250	58
DEAE-Sepharose	80 000	10 000	2 000	53
high-performance hydroxyapatite	2 000	800	25 000	17
alkyl-Superose	34[c]	16	1 250 000	14

[a] The results represent mean values of four individual preparations. [b] The growth-promoting activity in platelet lysate was variable, maybe due to the presence of growth inhibitors (Miyazono et al., 1987). Therefore, the purification in the QAE-Sephadex chromatography was estimated at 20-fold on the basis of the amount of protein recovered and an assumed recovery of 80% of activity (Miyazono et al., 1987). [c] Protein concentration was determined by amino acid analysis.

was purified 1 250 000-fold at an overall yield of about 14%.

Multiple Forms of PD-ECGF. In order to investigate whether the 46- and 44-kDa components (Figure 2B,C) were

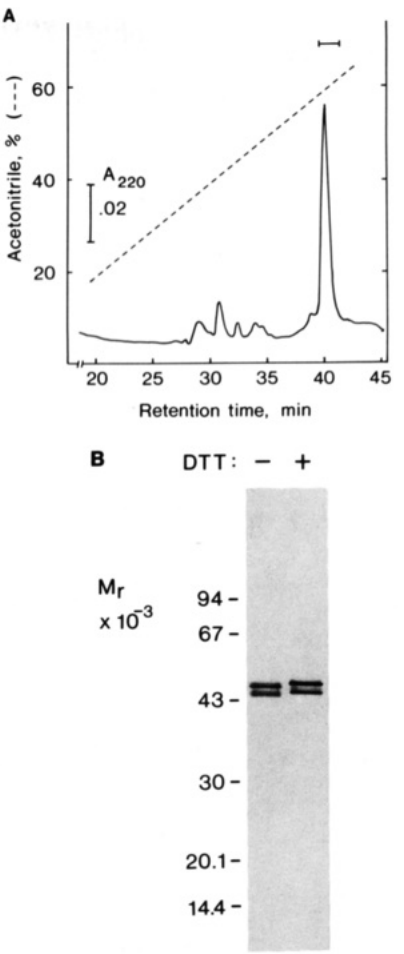

FIGURE 3: Reversed-phase HPLC of PD-ECGF. (A) Chromatography of PD-ECGF obtained from alkyl-Superose on a narrow-bore C4 column eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 0.2 mL/min, and eluted protein peaks were collected manually. (B) The eluate under the horizontal bar (panel A) was subjected to SDS gel electrophoresis in the presence or absence of dithiothreitol (DTT) and stained with silver. PD-ECGF migrated slightly faster in the absence of dithiothreitol as observed before (Miyazono et al., 1987).

related to each other, the material obtained by alkyl-Superose chromatography was subjected to reversed-phase chromatography on a narrow-bore C4 column (7 μm, 2.1 × 30 mm; Brownlee Labs) eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid. A homogeneous protein peak that accounted for >90% of the UV-adsorbing material was obtained, eluting at about 60% acetonitrile (Figure 3A). Since the mitogenic activity of PD-ECGF is destroyed at low pH, no mitogenic activity was recovered from the chromatogram. Analysis by SDS gel electrophoresis in the presence or absence of dithiothreitol revealed that the protein peak contained both the 46- and 44-kDa components (Figure 3B). Furthermore, a very faint band of 42 kDa could also be observed in the absence of dithiothreitol. Thus, these components could not be separated in this high-resolving chromatographic system.

To further evaluate the relationship between the 46- and 44-kDa bands, PD-ECGF was radiolabeled by the chloramine-T method and subjected to peptide mapping according to Cleveland et al. (1977). When the iodinated material was analyzed by SDS gel electrophoresis under reducing conditions, followed by autoradiography, three bands were seen of 46, 44, and 41 kDa (Figure 4A). Only a low amount of ^{125}I radioactivity was incorporated into the 46- and 44-kDa PD-ECGF components. This may be related to the fact that PD-ECGF by amino acid composition analysis was found to contain very

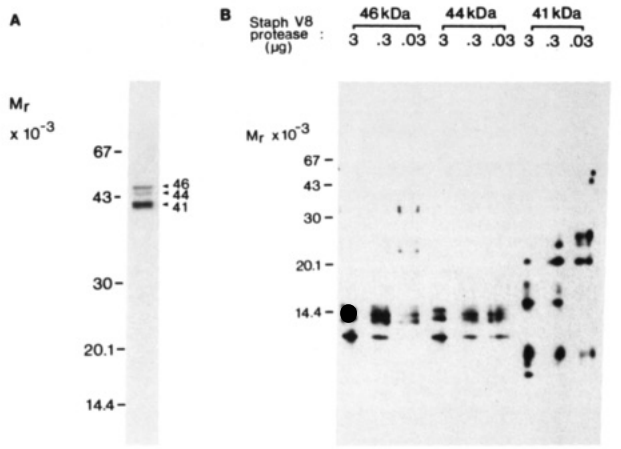

FIGURE 4: Comparison by peptide mapping of the components of purified PD-ECGF. (A) Purified PD-ECGF was radiolabeled by the chloramine-T method and analyzed under reducing conditions by SDS gel electrophoresis and autoradiography. Three bands of 46, 44, and 41 kDa, indicated by arrowheads, were cut out from the gel and subjected to peptide mapping. (B) Peptide maps after digestion with various concentrations of staphylococcal V8 protease. Samples were analyzed by SDS gel electrophoresis and autoradiography.

few tyrosine residues, probably only one in the whole molecule (our unpublished observation). The 41-kDa component represents a contaminant, not visible on silver-stained SDS gels, which incorporates ^{125}I radioactivity much more efficiently. These bands were cut out from the gel, subjected to staphylococcal V8 protease digestion, and analyzed by SDS gel electrophoresis and autoradiography. Figure 4B shows that the sizes of the peptides derived from the 46- and 44-kDa components were very similar, whereas the 41-kDa component gave a completely different peptide map. These results indicate that the 46- and 44-kDa peptides are microheterogeneous forms of the same protein. The two components could be partially separated by alkyl-Superose using a very shallow gradient of ammonium sulfate. Analysis of the growth-promoting activity of the various fractions indicated that the M_r 46 000 component had biological activity and suggested that the 44-kDa component was less active; whether it has any activity at all remains to be determined (not shown).

Tryptic Digestion of PD-ECGF. In order to further characterize the structure–function relationship of PD-ECGF, native PD-ECGF was subjected to trypsin digestion for various time periods. The samples were then analyzed by SDS gel electrophoresis and silver staining, and for mitogenic activity. Native PD-ECGF was found to be relatively resistant to trypsin cleavage; incubation with 10% (w/w) trypsin had very little effect on the molecule (not shown). Incubation of PD-ECGF with a higher concentration of trypsin (200%, w/w) destroyed the bioactivity of PD-ECGF (Figure 5A). Loss of the bioactivity after 10–20 min of incubation was accompanied by degradation of the 44–46-kDa doublet to components of sizes between 37 and 39 kDa (Figure 5B). The 37–39-kDa components were rather resistant to further degradation, indicating that PD-ECGF contains a trypsin-resistant domain of this size. Only after a long incubation time were small amounts seen of degradation products of lower molecular weights (about 23K without reduction and about 13K after reduction) (Figure 5B).

Characterization of a Rabbit Antiserum against PD-ECGF. A rabbit antiserum was raised against purified native PD-ECGF and was characterized by immunoblotting. Components of 46 and 44 kDa were recognized by the antiserum when samples were run under reducing conditions, and an additional 42-kDa component was found under nonreducing conditions

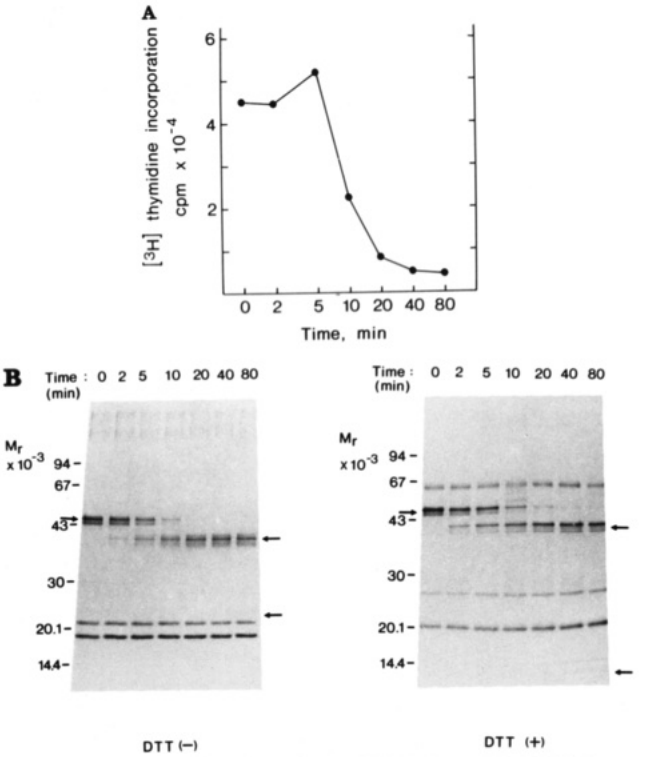

FIGURE 5: Tryptic digestion of PD-ECGF. Pure PD-ECGF was incubated with trypsin (200%, w/w; Sigma) at 37 °C for 0–80 min. After indicated periods of incubation, the reaction was terminated by the addition of a 2-fold (w/w) excess of soybean trypsin inhibitor (Sigma). Aliquots of the incubation mixture were tested for growth-promoting activity at a concentration of 40 ng/mL (panel A) or subjected to SDS gel electrophoresis in the presence or absence of dithiothreitol (DTT) (panel B). Intact and proteolytic derivatives of PD-ECGF are indicated by arrows to the left and right, respectively. The bands at 22 and 19 kDa (nonreducing conditions) and at 26 and 20 kDa (reducing conditions) represent trypsin and soybean trypsin inhibitor, respectively. The M_r 60 000 bands observed in the presence of dithiothreitol are contaminants in the reagents used.

(Figure 6A). This pattern is thus similar to that observed after SDS gel electrophoresis and silver staining of purified PD-ECGF (Figure 2) and indicates that the antiserum recognized PD-ECGF. The specificity of the antiserum was investigated by subjecting samples from the various stages in the purification of PD-ECGF to immunoblotting. Except for the most crude material, only components of 46 and 44 kDa were recognized by the antiserum, suggesting that the antiserum is highly specific for PD-ECGF (Figure 6B). In the starting material, additional components of 24–30 kDa were recognized by the antiserum (Figure 6B, lane a). At present, it is not known whether these components represent degradation products of PD-ECGF or cross-reactivity with other components in the platelet lysate. The antiserum was found not to cross-react in immunoblotting with FGFs (not shown).

When added to the cell culture medium, a purified immunoglobulin fraction of the PD-ECGF antiserum efficiently neutralized the mitogenic activity of pure PD-ECGF (Figure 7A). This ability was used to investigate whether platelet lysate contains other endothelial cell mitogens. As shown in Figure 7B, the PD-ECGF antiserum completely inhibited the mitogenic activity of platelet lysate on porcine aortic endothelial cells, indicating that PD-ECGF is the only mitogen for these cells in the platelet lysate.

Discussion

The present report describes a procedure to purify PD-ECGF at a high yield. The purification of PD-ECGF has been

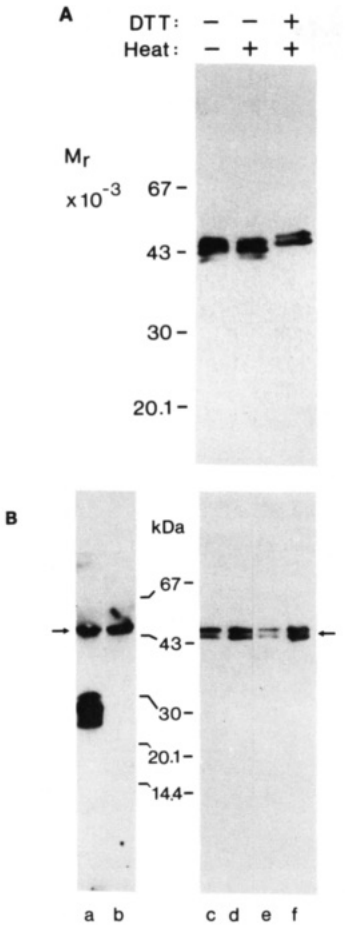

FIGURE 6: Immunoblotting using anti-PD-ECGF antiserum. (A) Comparison of native and denatured PD-ECGF by immunoblotting. For denaturation, pure PD-ECGF was heated at 95 °C for 3 min in the absence or presence of dithiothreitol (DTT). (B) Immunoblotting of PD-ECGF at different stages of purification. Lane a, flow-through of CM-Sephadex chromatography; lane b, material from ammonium sulfate precipitation; lane c, from DEAE-Sepharose chromatography; lane d, from high-performance hydroxylapatite chromatography; lane e, pure PD-ECGF from alkyl-Superose chromatography; lane f, denatured pure PD-ECGF from narrow-bore C4 reversed-phase HPLC. Samples were reduced and alkylated prior to SDS gel electrophoresis. Bands corresponding to PD-ECGF are indicated by arrows.

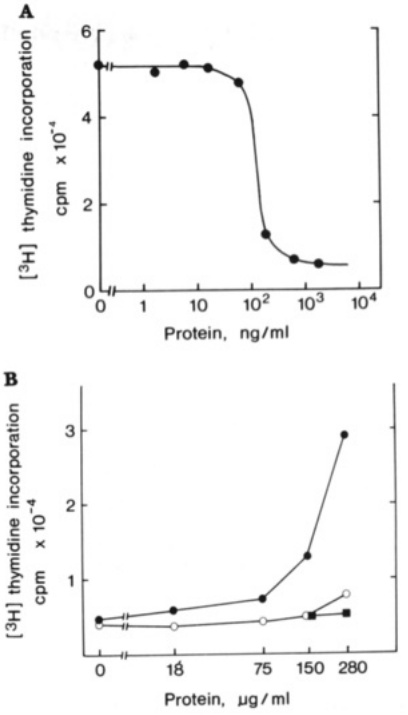

FIGURE 7: Immunoneutralization of the PD-ECGF activity. (A) Various amounts of PD-ECGF antibodies, purified by protein A–Sepharose, were added to the endothelial cell culture medium in the presence of 800 ng/mL semipure PD-ECGF obtained from high-performance hydroxylapatite chromatography. (B) Immunoneutralization of the endothelial cell growth-promoting activity of crude platelet lysate. Fresh human platelet lysate was obtained from normal volunteers. Crude platelet lysate was prepared as described before (Miyazono et al., 1985). Cells were incubated in various amount of crude platelet lysate in the absence (●) or presence [(○) 2.5 µg of protein/mL; (■) 5.0 µg of protein/mL] of PD-ECGF antibodies purified by protein A–Sepharose.

complicated, on one hand, by the fact that PD-ECGF occurs at fairly low quantities in platelets, making necessary more than a millionfold purification, and on the other, by the fact that its biological activity is labile and is lost, e.g., at low pH. The success of the present purification procedure was due to the use of two high-resolving HPLC-grade columns operated at neutral pH, i.e., a hydroxylapatite column and an alkyl-Superose column. The method allows an 1 250 000-fold purification of 30–40 µg close to homogeneous PD-ECGF per batch, at a 14% yield.

The purified PD-ECGF appeared as two close bands of molecular weight values of 46 000 and 44 000. Peptide mapping indicated that these components are microheterogeneous forms of the same protein. Since PD-ECGF does not have affinity for concanavalin A or wheat germ agglutinin immobilized to Sepharose CL–6B gels (our published observation), the microheterogeneity is probably not due to a difference in the degree of glycosylation; it seems likely that it is due to partial proteolysis of PD-ECGF.

The relationship between the M_r 42 000 component, which was observed under nonreducing conditions in silver-stained gels (Figure 2C) and in immunoblotting (Figure 7A), remains to be established. Since this component could be detected only in the absence of dithiothreitol, it is possible that it represents a proteolyzed version of PD-ECGF which falls apart upon reduction.

Our results indicate that platelets contain a potent endothelial cell mitogen that is distinct from FGFs. Recently, an endothelial cell mitogen which, like PD-ECGF, does not have affinity for heparin was found to be produced by a poorly differentiated bronchial carcioma cell line, 1PT (Walker et al., 1987). The 1PT cell-derived growth factor has similar biological and biochemical properties as PD-ECGF; whether it is identical with PD-ECGF remains to be elucidated.

Platelets are known to contain a variety of growth regulatory proteins, such as platelet-derived growth factor and transforming growth factor β (TGF-β). However, FGFs, which are synthesized by various cell types (Moscatelli et al., 1986; Lobb et al., 1986), including macrophages (Baird et al., 1985), have not been identified in platelets so far. Our finding, that a specific antiserum against PD-ECGF neutralizes all endothelial cell growth-promoting activity in human platelet lysate, is compatible with this observation and indicates that PD-ECGF is the only mitogen for endothelial cells in platelets.

Platelets contain large quantities of TGF-β which recently was reported to be a potent inhibitor of endothelial cell growth (Fråter-Schröder et al., 1986; Baird & Durkin, 1986; Heimark et al., 1986). TGF-β is, however, stored in platelets as a high molecular weight latent form (Pircher et al., 1986; Miyazono et al., 1988; Wakefield et al., 1988). Activation of TGF-β in vitro occurs after transient acidification, alkalinization, or treatment with chaotropic agents; the mechanism for activation of TGF-β in vivo is not known. Thus, platelets contain both

a potent mitogen for endothelial cells and even larger quantities of a powerful inhibitor of endothelial cell growth. The net effect of platelet releasate on endothelial cell growth will thus be dependent on the extent of activation of TGF-β from its latent complex.

The large-scale purification method of PD-ECGF that is described here allows the purification of sufficient amounts of PD-ECGF for a thorough structural and functional characterization, which is currently ongoing in our laboratory. Amino acid sequencing of tryptic fragments of PD-ECGF has allowed the cloning of cDNA for the factor; the deduced sequence has confirmed that PD-ECGF is distinct from previously known proteins. Furthermore, analysis of material synthesized by cells transfected with constructs of this cDNA verified that the 44–46-kDa PD-ECGF molecule is responsible for the growth-promoting activity on endothelial cells (Ishikawa et al., unpublished results).

The recent finding that PD-ECGF has angiogenic activity (Risau et al., unpublished results) is of potential interest for the in vivo function of PD-ECGF. The availability of a specific antiserum against PD-ECGF will make possible studies on the biosynthesis, tissue distribution, and possible involvement of PD-ECGF in normal and pathological angiogenesis.

Acknowledgments

We thank Linda Baltell for valuable help in the preparation of the manuscript, Ulf Hellman for help in reversed-phase HPLC, and Tetsuro Okabe and Fumimaro Takaku for helpful discussions. We also thank the Finnish Red Cross, Helsinki, and the Department of Virology, Uppsala, for the supply of platelets.

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