J. Biochem. 125, 368-374 (1999)

Purification and Characterization of a Novel Vascular Endothelial Cell Growth Inhibitor Secreted by Macrophage-Like U-937 Cells[1]

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Received August 3, 1998; accepted November 9, 1998

A human histiocytic lymphoma cell line, U-937 cells, secretes several vascular endothelial cell growth inhibitors including leukemia inhibitory factor, oncostatin M, tumor necrosis factor-α, and transforming growth factor-β1. Characterization of partially purified fractions from the conditioned media of phorbol ester-treated U-937 cells suggested the existence of unknown endothelial growth inhibitors. Using a combination of copper affinity, heparin affinity, cation exchange, and reversed phase liquid chromatographies, a growth inhibitor for endothelial cells was purified to homogeneity from conditioned media. The purified growth inhibitor migrated as a 65 kDa band on SDS-polyacrylamide gel electrophoresis under both reducing and nonreducing conditions. Microsequencing analyses of the tryptic fragments of the growth inhibitor and a BLAST search analysis revealed a unique sequence showing no homology to known proteins. The purified protein inhibited endothelial cell growth in a dose-dependent manner, but had no effect on smooth muscle cell growth. The factor also blocked endothelial cell growth induced by both fibroblast growth factor-2 and vascular endothelial growth factor, and was additively effective in inhibiting the growth of endothelial cell by U-937 cell-derived endothelial cell growth inhibitors. Thus, this factor appears to be a novel inhibitor with specificity for vascular endothelial cells, and is tentatively called endothelial cell inhibitor (ECI) in this study.

Key words: angiogenesis, endothelial cell, endothelial cell growth inhibitor, macrophage, smooth muscle cell.

Vascular endothelial cell growth is tightly regulated by a balance of growth factors and growth inhibitors (*1, 2*). Angiogenesis, the process by which new blood vessel formation occurs in endothelial cells, participates in numerous physiological events. Under normal conditions, angiogenesis occurs during ovulation, placental development, and wound healing, but also plays roles in various clinically important processes, such as solid tumor growth and metastasis, diabetic retinopathy, atherosclerosis, neovascular glaucoma, and rheumatoid arthritis (*3, 4*). Since the balance normally favors inhibition, endothelial cells in adult tissues are quiescent, despite the wide distribution of angiogenic factors such as fibroblast growth factor-1 (FGF-1, aFGF), fibroblast growth factor-2 (FGF-2, bFGF), and vascular endothelial growth factor (VEGF). In addition, many proteins have been shown to inhibit endothelial cell growth in culture, including transforming growth factor-β1 (TGF-β1) (*5*), tumor necrosis factor-α (TNF-α) (*6*), platelet factor-4 (*7*), interferon-γ (IF-γ) (*8*), cartilage-derived inhibitor (CDI) (*9*), leukemia inhibitory factor (LIF) (*10*), thrombospondin-1 (*11*), oncostatin M (OSM) (*12*), angiostatin (*13*), and endostatin (*14*). Antibodies against integrin αvβ3 also inhibit endothelial cell proliferation (*15*).

Since macrophages usually exist at sites of physiological angiogenesis, such as wounds, and pathological angiogenesis, such as solid tumors and atherosclerotic plaques, macrophages are generally thought to regulate angiogenesis through the production of soluble mediators (*16-18*). Macrophages have been shown to produce and release a number of angiogenic factors such as FGF-2 (*19*), platelet-derived growth factor (PDGF) (*20*), and VEGF (*21*), and, in addition, to influence the angiogenic process by modulating the composition of the extracellular matrix. Based on the hypothesis that macrophages might produce a balance of endothelial cell growth factors and growth inhibitors, we examined the conditioned media of U-937 cells. U-937 cells are a human histiocytic lymphoma line of cells which

[1] This work was supported by Ono Pharmaceutical Co., Ltd.

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Abbreviations: BAE, bovine aortic endothelial cells; ECI, endothelial cell inhibitor; FCS, fetal calf serum; FGF, fibroblast growth factor; IF-γ, interferon-γ; LIF, leukemia inhibitory factor; OSM, oncostatin M; PS, penicillin/streptomycin sulfate; SMC, smooth muscle cells; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

differentiate into macrophage-like cells and which have been shown to produce VEGF (*21*). Previous studies have reported that U-937 cells produce endothelial cell growth inhibitors such as OSM, LIF, TGF-β1 (*12*), and TNF-α (*22*). We subsequently analyzed the conditioned media of U-937 cells, and report here a novel inhibitor for vascular endothelial cells tentatively referred to as ECI in this study. ECI is a 65-kDa protein under both reducing and nonreducing conditions, and has a unique amino acid sequence.

EXPERIMENTAL PROCEDURES

Materials—Bovine aortic endothelial cells (BAE) and smooth muscle cells (SMC) were isolated from the thoracic aortae of bovines using methods described previously (*23, 24*). Recombinant human FGF-2, VEGF, TGF-β1, and TNF-α were purchased from Sigma-Aldrich Japan (Tokyo). Recombinant human LIF, OSM, and antibodies to human LIF and OSM were obtained from PeproTech EC (London, England). Antibodies to human TGF-β1 and TNF-α were from R&D Systems (Minneapolis, MN).

Preparation of U-937 Cell Conditioned Media—The human histiocytic lymphoma cell line U-937 was purchased from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (RPMI/10% FCS/PS). U-937 cells were seeded at a density of 1×10^5 cells/50 ml/T-150 flask and incubated for 24 h with RPMI/10% FCS/PS in the presence of 60 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA, phorbol ester) (Calbiochem, CA). After a 24 h incubation with TPA, the medium was replaced with 50 ml of serum-free RPMI/PS, and the conditioned medium was collected and replaced with fresh RPMI/PS at 48 h intervals for 5-7 days. The collected medium was centrifuged at 10,000 rpm for 10 min to remove cell debris. Benzamidine hydrochloride (Sigma-Aldrich Japan) was added to the supernatant to a final concentration of 1 mM in order to protect against protein degradation. The supernatant was maintained at -20°C until used for purification.

Growth Inhibition Assays—BAE and SMC were maintained in Dulbecco's modified Eagle's medium (D-MEM), 10% FCS, and PS. For the assay of cell number, cells were resuspended in D-MEM/10% FCS/PS and seeded onto 6-well microplates (2×10^4 cells/2 ml/well). The plates were incubated for 24 h at 37°C, and re-fed with D-MEM/5% FCS/PS. After 3 h, the samples to be tested for inhibitory activity were added. After a 72 h incubation, the cells were harvested and counted using a Coulter Counter (Coulter Electronics, Luton, England). For the assay of DNA synthesis, BAE were resuspended in D-MEM/10% FCS/PS and seeded onto 96-well microplates (2×10^3 cells/200 μl/well). The plates were then incubated for 24 h at 37°C, and re-fed with D-MEM/5% FCS/PS. After 3 h, the samples and/or growth factors (4 ng/ml FGF-2 or 10 ng/ml VEGF) to be tested for inhibitory activity were added. After a 24 h incubation, 10 μl (0.2 μCi) of [^{3}H]thymidine was added for 6 h, and the incorporation of [^{3}H]thymidine into DNA was determined by liquid scintillation counting (1450 MicroBeta TRILUX, Amersham Pharmacia Biotech, Sweden). To determine BAE growth, BAE were plated at a density of 2×10^4 cells/2 ml/well in 6-well microplates. On the next day, the plates were re-fed with D-MEM/5% FCS/PS. After 3 h, 25 μl/ml of ECI (pooled phenyl fractions) was added (Day 0). The plates were incubated for 3 days, and re-fed with fresh D-MEM/5% FCS/PS.

Purification of Endothelial Cell Growth Inhibitor—Fifty liters of serum-free U-937 conditioned medium was concentrated to 1,800 ml by ultrafiltration (S1Y10 spiral-wound membrane, molecular weight cut off 10,000; Amicon, Beverly, MA). The retentate was applied directly to a copper-chelating Sepharose column (50×150 mm; Amersham Pharmacia Biotech) which had been saturated with cupric sulfate and equilibrated with 20 mM Tris-HCl, pH 8.0/0.5 M NaCl. After extensive washing with the equilibration buffer, the bound proteins were eluted batchwise with 20 mM Tris-HCl, pH 8.0/0.15 M NaCl/60 mM imidazole. The eluate was applied to an AF-Heparin Toyopearl 650 M column (50×100 mm; Tosoh, Tokyo) equilibrated with 20 mM Tris-HCl, pH 7.2/0.15 M NaCl. After extensive washing with the equilibration buffer and subsequently with 20 mM MES, pH 6.0/0.15 M NaCl, the bound proteins were eluted batchwise with 20 mM MES, pH 6.0/1 M NaCl. The eluate was diluted 1:5 with 20 mM MES, pH 6.0, and then applied to an S-Sepharose HP column ($16\times$ 100 mm; Amersham Pharmacia Biotech) which had been equilibrated with 20 mM MES, pH 6.0, using an FPLC system (Amersham Pharmacia Biotech). The column was washed with the equilibration buffer and the bound proteins were eluted with a 120-ml linear gradient of 0–1.5 M NaCl in 20 mM MES, pH 6.0, at a flow rate of 2 ml/min. Fractions of 1.5 ml were collected and 2 μl of each fraction was tested for inhibitory activity. Biologically active fractions were pooled and applied to a phenyl reversed phase column (10×250 mm; nacalai tesque, Kyoto) equilibrated with 5% acetonitrile in 0.05% trifluoroacetic acid (TFA), using a Shimadzu LC-10A HPLC system (Shimadzu, Kyoto). The column was washed extensively with the equilibration solution and the bound proteins were then eluted with a 75-ml linear gradient of 15–50% acetonitrile in 0.05% TFA at a flow rate of 2 ml/min. The eluate was monitored by UV absorption at 280 and 214 nm. Fractions of 1.5 ml were collected and 2 μl of each fraction was tested for inhibitory activity. The fractions corresponding to the endothelial cell growth inhibitory activity were pooled, diluted with 1 volume of doubly distilled, filtered water containing 0.05% TFA, and applied onto a C_4 reversed phase column (4.6×250 mm; Vydac, CA) which had been equilibrated with 5% acetonitrile in 0.05% TFA. The column was washed extensively with the equilibration solution and the bound proteins were then eluted with a 60-ml linear gradient of 20–40% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min. Fractions of 1 ml were collected and 2 μl of each fraction was tested for inhibitory activity. Biologically active fractions were collected, diluted, and rechromatographed on a second C_4 column. The bound protein fractions were eluted with a 90-ml linear gradient of 25–40% acetonitrile in 0.05% TFA. Fractions of 1 ml were collected and 2 μl of each fraction was tested for inhibitory activity.

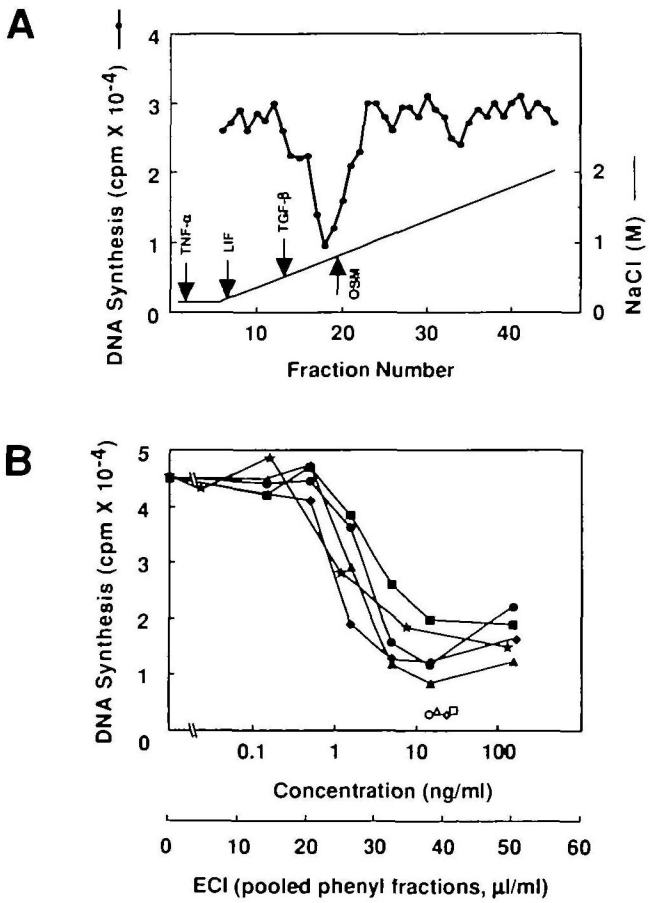
For all these procedures, the reaction tubes were siliconized with Sigmacote (Sigma-Aldrich Japan) to avoid activity loss as a result of non-specific absorption.

SDS-Polyacrylamide Gel Electrophoresis—Samples were analyzed by electrophoresis on 15% polyacrylamide

gels (25) and stained with a silver staining kit, Sil-Best Stain (nacalai tesque).

Western Blotting Analysis—The active fraction concentrated from 3 ml of pooled phenyl fractions, TNF-α (100 ng), TGF-β1 (30 ng), LIF (50 ng), and OSM (50 ng) were fractionated by electrophoresis on a 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skimmed milk in PBS overnight at 4°C and incubated with a mixture of rabbit anti-TNF-α, anti-TGF-β1, anti-LIF, and anti-OSM antibodies in 5% skimmed milk/PBS for 4 h at room temperature. The membrane was washed three times at 10 min intervals with 0.05% Tween 20 in PBS, and then incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The membrane was then washed again three times at 10 min intervals with 0.05% Tween 20 in PBS, and incubated with avidin-HRP (Vectastatin, Vector Laboratories) for 30 min at room temperature. The membrane was washed five additional times with 0.05% Tween 20 in PBS, and HRP was detected by an ECL kit (Amersham Pharmacia Biotech) and autoradiography.

Tryptic Digestion and Microsequencing—Fractions corresponding to growth inhibitor activities from the second C_4 column chromatography were pooled, dried, and resuspended in 150 μl of 4 M guanidine HCl, 0.1 M Tris-HCl, pH 8.0. Dithiothreitol was added to a final concentration of 20 mM, the reaction mixture was incubated at 37°C for 60 min, and then alkylated by the addition of solid iodoacetamide to a final concentration of 25 mM. The reaction mixture was then incubated for 30 min at room temperature, desalted on a C_{18} reversed phase HPLC column (4.6× 150 mm; Vydac, gradient of 10 to 40% acetonitrile in 0.1% TFA), and the protein was dried and resuspended in 150 μl of 0.1 M ammonium bicarbonate. Trypsin (0.4 μg, Boehringer Mannheim, Mannheim, Germany) was added, and the mixture was then incubated at 27°C for 2 h. A second aliquot of trypsin (0.3 μg) was added, and the mixture was incubated for a further 2 h at 27°C. The digestion products were then fractionated on a C_{18} HPLC column using a



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	ED50
ECI (pooled phenyl fractions)	25 μl/ml
LIF	3 ng/ml
OSM	2 ng/ml
TNF-α	4 ng/ml
TGF-β	1 ng/ml

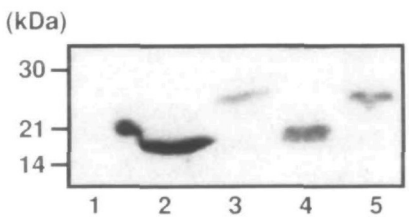

Fig. 1. **Characterization of U-937 cell-derived endothelial cell inhibitors.** A: The samples prepared from concentrated U-937 cell conditioned media using copper affinity, heparin affinity, cation exchange, and phenyl reversed phase liquid chromatographies were applied to a heparin Sepharose column (7×25 mm). Fractions were eluted with a gradient of 0.15–2 M NaCl, and 2 μl of each fraction was tested for its ability to inhibit the incorporation of [^{3}H]thymidine into BAE. Arrows show the elution positions of recombinant LIF, OSM, TNF-α, and TGF-β1. B: Increasing amounts of ECI (pooled phenyl fractions, closed stars), LIF (closed circles), OSM (closed triangles), TNF-α (closed squares), and TGF-β1 (closed diamonds) were added to BAE. At 10 ng/ml LIF (open circles), OSM (open triangles), TNF-α (open squares), and TGF-β1 (open diamonds), 25 μl/ml of ECI (pooled phenyl fractions) was added to the BAE cells. The cells were incubated and tested for [^{3}H]thymidine incorporation. Each point is the mean of duplicate measurements. C: ED_{50}s were estimated from Fig. 1B. ECI concentrated from 3 ml of pooled phenyl fractions (lane 1), TNF-α (100 ng, lane 2), TGF-β1 (30 ng, lane 3), LIF (50 ng, lane 4), and OSM (50 ng, lane 5) were analyzed by Western blotting using a mixture of antibodies against LIF, OSM, TNF-α, and TGF-β1.

gradient of 3% to 63% acetonitrile in 0.1% TFA. Two major peaks were collected and applied to a Hewlett Packard protein sequencing system (HP G1005A).

RESULTS AND DISCUSSION

Characterization of TPA-Treated U-937 Cell-Derived Endothelial Cell Inhibitors—Previous studies demonstrated that a human histiocytic lymphoma cell line, U-937 cells, secretes several vascular endothelial cell growth inhibitors, including OSM, LIF, TGF-β1 (*12*), and TNF-α (*22*). In order to identify novel endothelial cell growth inhibitors, we further analyzed the conditioned media using copper affinity, heparin affinity, cation exchange, and phenyl reversed phase liquid chromatographies.

In order to examine whether the active fraction which was eluted from the phenyl column contained any known endothelial growth inhibitors such as LIF, OSM, TNF-α, or TGF-β1, we examined the retention of these factors on a heparin Sepharose column. Recombinant LIF, OSM, and TGF-β1 were eluted at 0.2, 0.8, and 0.5 M NaCl, respectively, while TNF-α did not bind (Fig. 1A). The active fraction was eluted at 0.75 M NaCl. The active fraction showed dose-dependent growth inhibitory activity (Fig. 1B) with an ED_{50} of approximately 25 μl/ml. LIF, OSM, TNF-α, and TGF-β1 inhibited the DNA synthesis of BAE dose-dependently with ED_{50}s of 3, 2, 4, and 1 ng/ml, respectively. Three milliliters of the active fraction, which was 120-fold in excess of the ED_{50} value of the active fraction, was analysed by Western blotting using antibodies against the four proteins, but no band was observed (Fig. 1C). The minimum detectable levels of LIF, OSM, and TNF-α were 50 ng/lane, and that of TGF-β1 was 30 ng/lane by Western blotting analysis. Accordingly, the levels of LIF, OSM, TNF-α, and TGF-β1 in the active fraction were estimated to be less than 14, 21, 10, and 25%, respectively. Furthermore, we examined the additional effect of the active fraction on LIF, OSM, TNF-α, and TGF-β1 at their activity-saturating concentrations. At 10 ng/ml of LIF, OSM, TNF-α, or TGF-β1, 25 μl/ml of the active fraction inhibited the proliferation of endothelial cells in an additive manner (Fig. 1B). These data suggest that the active fraction contains growth inhibitors other than LIF, OSM, TNF-α, and TGF-β1.

Endothelial cells which were treated with 25 μl/ml of the active fraction became spindle shaped on Day 1 and showed a suppressed cell growth. On Day 3, the cells remained in the same state as on Day 1. After the cells were changed to fresh medium without the active fraction on Day 3, the cells regrew normally (Fig. 2). Endothelial cells which were treated with the active fraction did not show apoptotic nor necrotic cell death, suggesting that the growth inhibition of BAE is not due to a decrease in cell viability.

Purification and Microsequencing of TPA-Treated U-937 Cell-Derived Endothelial Cell Inhibitors—In order to purify and characterize the novel macrophage-like cell derived factor, we attempted to purify it to homogeneity. Fifty liters of TPA-treated U-937 cell conditioned medium (approximate 10 g of protein) was concentrated to 1,800 ml and purified batchwise on a copper-chelate column, followed by a heparin affinity column. The bound proteins were applied to an S-Sepharose column and eluted with a gradient on an FPLC system. Fractions corresponding to inhibitory activities were eluted with 0.6–0.75 M NaCl

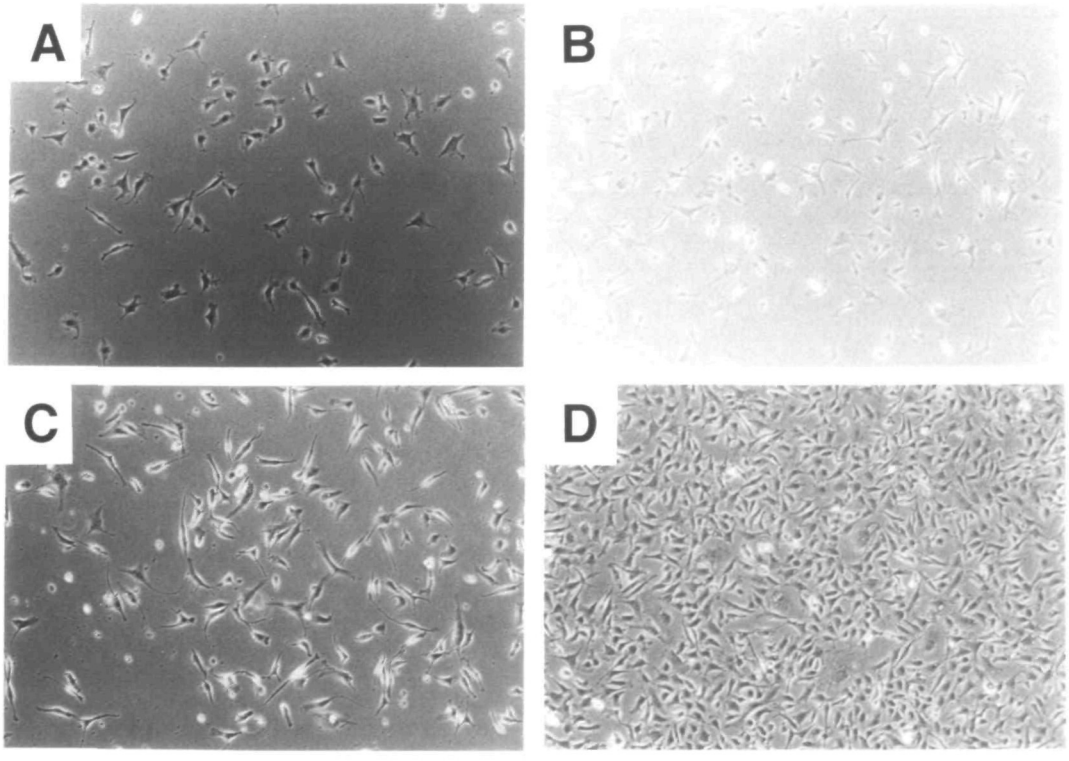

Fig. 2. **Effect of partially purified ECI on BAE.** BAE were seeded onto 6-well microplates (2×10^4 cells/2 ml/well). After 24 h, the plates were re-fed with D-MEM/5% FCS/PS. After 3 h, 25 μl/ml of ECI (pooled phenyl fraction) was added (Day 0, A). The plates were incubated for 3 days, and re-fed with fresh media. B, C and D show cells on Day 1, 3, and 5, respectively. Magnifications are 10×10.

(Fig. 3A). The active fractions were further chromatographed by HPLC on a phenyl column. Two fractions which showed inhibitory activities were eluted at approximately 38 and 45% acetonitrile (Fig. 3B).

The major active fractions which eluted with approximately 45% acetonitrile were further purified by repeated HPLC on a C_4 reversed phase column (Fig. 3, C and D). Active fractions corresponding to fraction numbers 83 to 87 from the second C_4 column chromatography were analyzed by SDS-PAGE under both reducing and nonreducing conditions (Fig. 4). A single band corresponding to 65 kDa was detected under both conditions, suggesting that ECI consists of a single polypeptide chain. Approximately 2 μg of purified ECI protein as estimated by silver staining was obtained. In order to analyze internal amino acid sequences, the remainder of the purified ECI was digested with trypsin and fractionated by reversed phase HPLC on a C_{18} column (Fig. 5). Two major peaks, Peak-I and Peak-II, were isolated and subjected to microsequencing analyses. No sequence for a peptide Peak-I was detected, suggesting the possibility of a blocked amino-terminal fragment. However, the sequence of peptide Peak-II was determined to be QSRVQISW, which shows an 87% sequence homology with *C. elegance* cosmid F32B5 (*26*), but no homology with any protein described to date in the protein-database, as evidenced by a FASTA3 Search. Based on these results, ECI appears to be a novel and unique factor.

The second active fraction (No. 32-35), shown in Fig. 3B, was also subjected to further purification. However, an insufficient amount was isolated to permit amino acid sequence determination.

Characterization of Purified Endothelial Cell Inhibitor— In order to examine the functional specificity and character of purified ECI, we first examined ECI-mediated cell growth regulation on BAE and SMC. ECI inhibited the proliferation of BAE in a dose-dependent manner, but

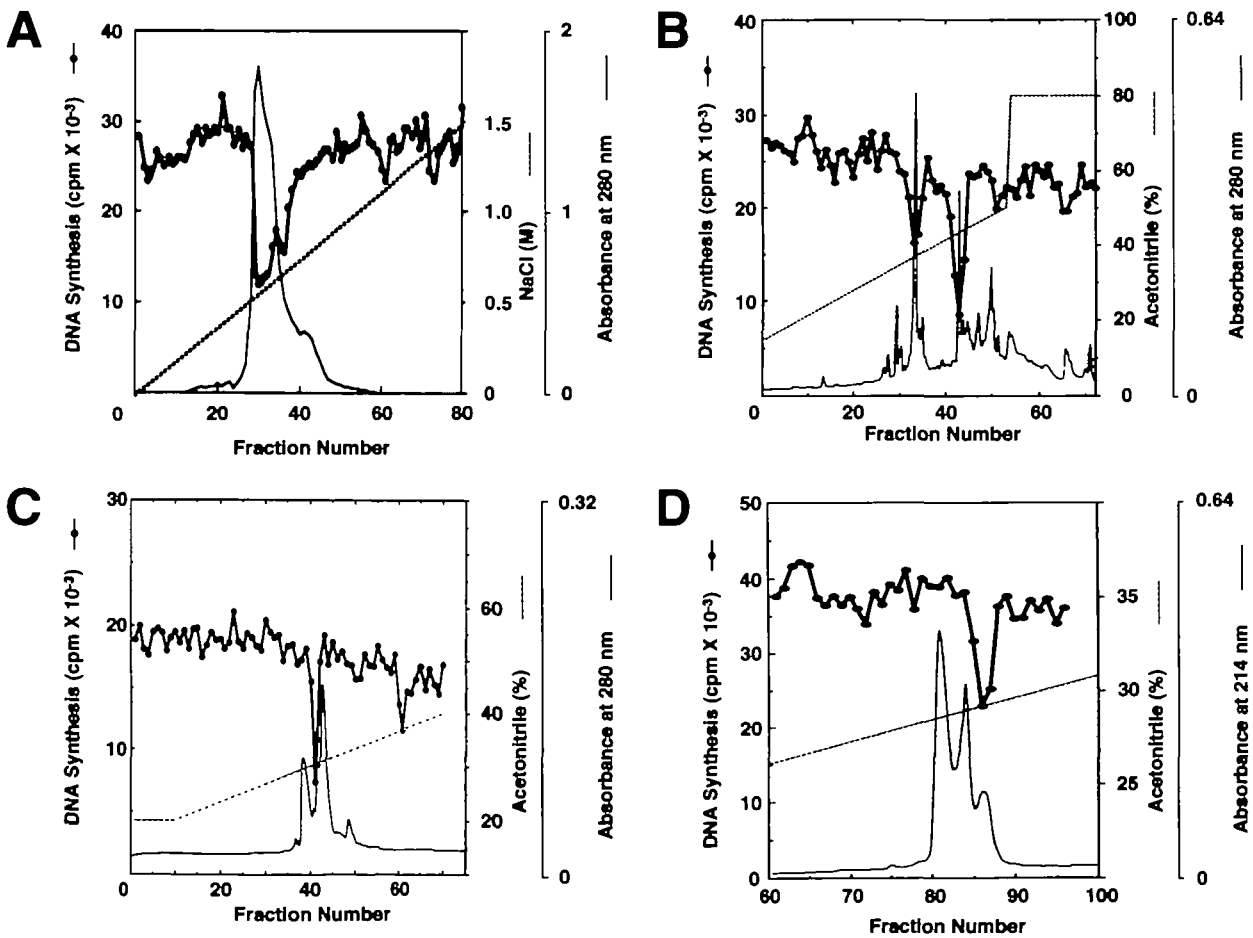

Fig. 3. **Purification of ECI from U-937 cell conditioned media.** A: S-Sepharose FPLC. Samples prepared from concentrated phorbol ester-treated U-937 cell conditioned media using copper affinity and heparin affinity columns were applied to an S-Sepharose HP column (16×100 mm). Fractions (1.5 ml) were eluted with a gradient of 0-1.5 M NaCl, and 2 μl of each fraction was tested for its ability to inhibit [^{3}H]thymidine incorporation into BAE. B: Phenyl reversed phase HPLC. Fractions 29-36 obtained from S-Sepharose HP column chromatography were pooled and applied to a phenyl column on HPLC. Then, bound fractions were eluted with a gradient of 5-60% acetonitrile, and 2 μl of each fraction was tested for inhibitory activity. C: C_4 reversed phase HPLC. Fractions 41-44 obtained from phenyl reversed phase HPLC were applied to a C_4 column. The bound fractions were eluted with a gradient of 20-40% acetonitrile, and 2 μl of each fraction was tested for inhibitory activity. D: Second C_4 reversed phase HPLC. Fractions 40-41 obtained from the first C_4 column chromatography were applied to a second C_4 column. The bound fractions were eluted with a gradient of 25-40% acetonitrile, and 2 μl of each fraction was tested for inhibitory activity.

neither inhibited nor stimulated the proliferation of SMC (Fig. 6A).

The growth of endothelial cells is induced by certain growth factors such as FGF-2 and VEGF. To investigate whether ECI is capable of functioning as an antagonist of the activities of these factors, we examined the effect of ECI on BAE, which had been stimulated by growth factors. FGF-2 and VEGF upregulated [^{3}H]thymidine incorporation into endothelial cell DNA as had been shown previously (*12*). The addition of ECI inhibited both FGF-2- and VEGF-induced [^{3}H]thymidine incorporation relative to the basal level in a dose-dependent manner (Fig. 6B). Based on these results, ECI appears to antagonize the growth factor activities of both FGF-2 and VEGF. In terms of the molecular mechanism that underlies this effect, two possibilities exist: (i) ECI could bind directly to FGF-2 and VEGF, thus blocking access to their receptors; or (ii) ECI

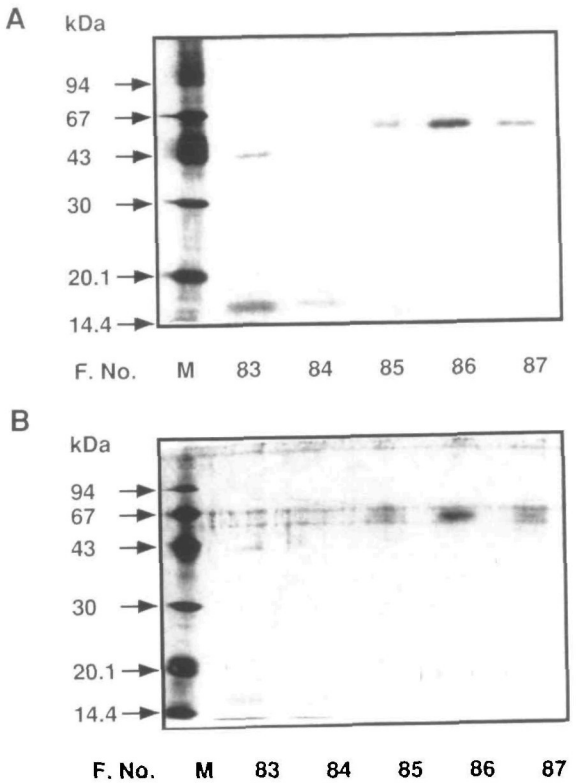

Fig. 4. **SDS-PAGE of ECI.** The active fractions 83-87 obtained from the second C_4 reversed phase HPLC shown in Fig. 3D were analyzed by 15% SDS-PAGE under nonreducing (A) and reducing (B) conditions, and visualized by silver staining.

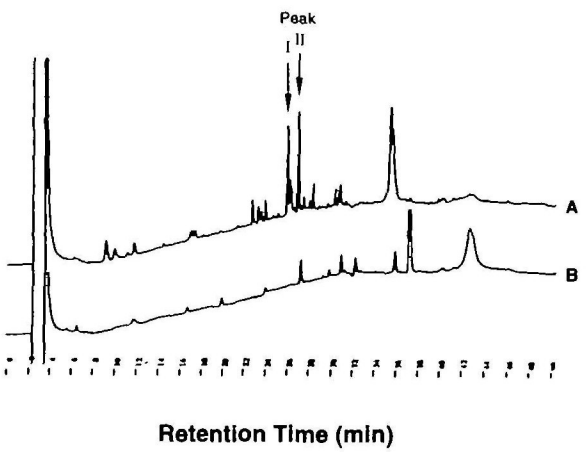

Fig. 5. **Peptide mapping of the ECI tryptic digest.** Fractions 85-87 from the second C_4 reversed phase HPLC were carboxymethylated and digested with trypsin as described under "EXPERIMENTAL PROCEDURES." The digestion products (A) and control (B) were fractionated on a C_{18} HPLC column using a gradient of 3 to 63% acetonitrile in 0.05% TFA. Two major peaks, referred to as peak I and peak II, were subjected to microsequence analyses.

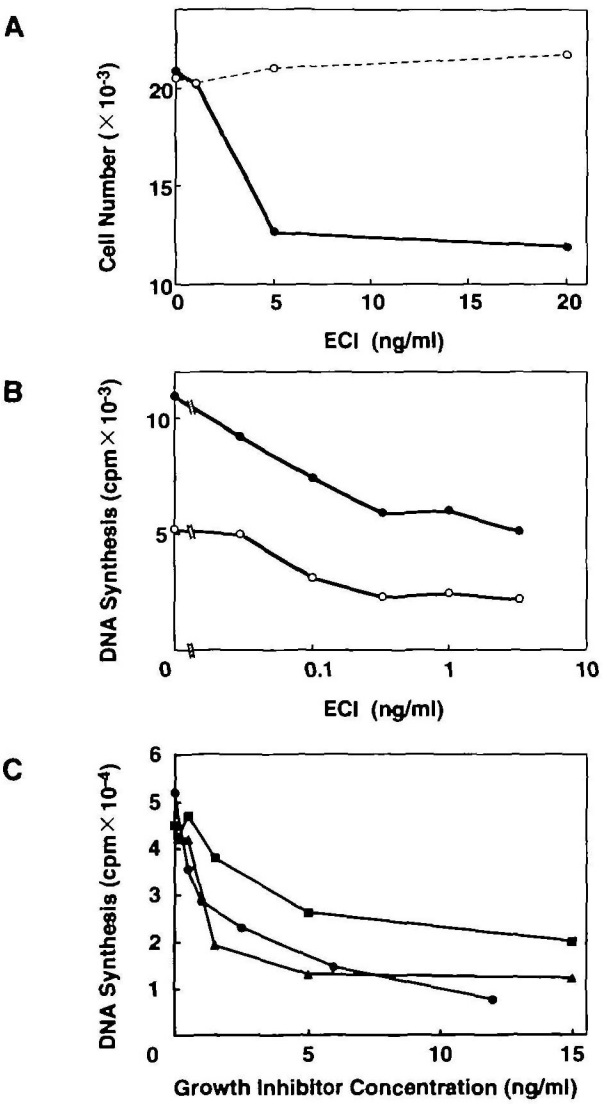

Fig. 6. **Biological characterization of ECI.** A: Increasing amounts of ECI were added to BAE (closed circles) and SMC (open circles). After a 72 h incubation, the cells were harvested and counted by a Coulter Counter. B: ECI was added to BAE stimulated by 4 ng/ml FGF-2 (closed circles) or 10 ng/ml VEGF (open circles). After a 24 h incubation, 0.2 μCi of [^{3}H]thymidine was added, and 6 h later, [^{3}H]-thymidine incorporation into DNA was measured by liquid scintillation counting. C: ECI (closed circles), TNF-α (closed squares), and TGF-β1 (closed triangles) were added to BAE stimulated with 4 ng/ml FGF-2, and [^{3}H]thymidine incorporation into DNA was determined, as above. Each point is the mean of duplicate measurements.

could bind to a specific receptor, thus evoking a signaling cascade that interferes with a growth signaling cascade. ECI is also able to inhibit the basal growth of endothelial cells, and there is little possibility that ECI binds to both FGF-2 and VEGF, interfering with the binding of FGF-2 and VEGF to their receptors on endothelial cells. Thus, the second hypothesis appears to be the most viable.

To evaluate the potency of ECI for endothelial cells, the inhibitory activity of ECI was compared to those of TNF-α and TGF-β1. TNF-α and TGF-β1 showed nearly the same inhibitory patterns, and were half-maximally effective at 4 and 1 ng/ml, respectively. ECI showed a similar inhibition pattern to TNF-α and TGF-β1 and inhibited endothelial cell growth with a half maximal inhibition at approximately 2 ng/ml (Fig. 6C).

In summary, we purified an endothelial cell growth inhibitor from the conditioned media of macrophage-like U-937 cells. Although the biological properties, molecular weight, and internal amino acid sequence of ECI found in this study leads us to conclude that ECI might be a novel factor, further studies including gene cloning and characterization of the activity of the recombinant protein are required to confirm this hypothesis, and to suggest possibilities for future studies of the regulation of angiogenesis by ECI. Based on the fact that macrophages produce several of the growth factors and growth inhibitors of endothelial cells, it can be speculated that the balance of these factors might control endothelial cell proliferation as well as angiogenesis in response to macrophage infiltration.

We gratefully acknowledge Drs. Yoshinao Wada and Koichi Honke, Research Institute, Osaka Medical Center for Maternal and Child Health, for helpful discussion.

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