Inhibition of Endothelial Cell Growth by Macrophage-like U-937 Cell-derived Oncostatin M, Leukemia Inhibitory Factor, and Transforming Growth Factor β1*

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Conditioned media were collected from phorbol ester-treated human macrophage-like U-937 cells and analyzed for the presence of inhibitors of endothelial cell (EC) proliferation. By a combination of ion exchange and reverse-phase liquid chromatography, three inhibitors were purified to homogeneity as ascertained by microsequencing of 14–17 N-terminal amino acids. These inhibitors were identified as oncostatin M (OSM), leukemia inhibitory factor (LIF), and transforming growth factor β1 (TGF-β1). The identities of the three EC growth inhibitors were confirmed by demonstrating that recombinant human OSM, LIF, and TGF-β1 were inhibitory in the same concentration range. Inhibition of EC proliferation by OSM was a newly described property of this cytokine. OSM was the most potent inhibitor with a half-maximal inhibition by recombinant material of 0.15–2 ng/ml compared with 0.9–1.0 ng/ml for LIF and TGF-β1, respectively. The three factors inhibited basal, vascular endothelial cell growth factor-stimulated, and fibroblast growth factor 2-stimulated EC proliferation. Interleukin-6 and ciliary neurotrophic factor, two cytokines related structurally to OSM and LIF, were not active as EC growth inhibitors. It was concluded that macrophage-like cells secrete a variety of potent EC growth inhibitors and that one of these, OSM, is among the most potent EC growth inhibitors yet reported.

An understanding of the mechanisms that control the proliferation and differentiation of vascular endothelial cells (EC)1 should provide important insights into a wide variety of physiological and pathological events such as embryonic development, wound healing, tumor growth, diabetes, and atherosclerosis (1, 2). ECs in adult tissue are normally quiescent despite the wide distribution of angiogenic factors such as acidic fibroblast growth factor (FGF-1), basic FGF (FGF-2), and vascular endothelial growth factor (VEGF) (2). One possible mechanism that might prevent abnormal EC proliferation might be the expression of EC growth inhibitory factors. A number of polypeptides have been shown to inhibit EC growth in culture. These include transforming growth factor β1 (TGF-β1) (3), tumor necrosis factor-α (TNF-α) (4), platelet factor 4 (5), interferon-γ (6), cartilage-derived inhibitor (7), leukemia inhibitory factor (LIF) (8), thrombospondin (9), and angiostatin (10). Antibodies directed against the integrin αvβ3 also inhibit EC proliferation (11). EC inhibitors in vitro are usually also angiogenesis inhibitors in vivo, but this is not always the case. For example, TGF-β1 and TNF-α, which are EC inhibitors in vitro, are actually stimulators of angiogenesis in vivo. It has been postulated that TGF-β1 and TNF-α act indirectly to stimulate angiogenesis by induction of angiogenesis factors from inflammatory cells, for example, macrophages and T lymphocytes (12). Another possibility is that these EC inhibitors block proliferation but stimulate tube formation, an important component of angiogenesis (13).

Since macrophages commonly reside at sites of physiological angiogenesis such as wounds and of pathological angiogenesis, such as tumors and atherosclerotic plaques, it is possible that this cell type plays an important role in the angiogenic response (14–16). Macrophages have been shown to produce and release a number of angiogenic stimulators such as VEGF (17), FGF-2 (18), and platelet-derived growth factor (19). Based on the idea that macrophages might produce a balance of EC stimulators and inhibitors, we examined the conditioned media (CM) of U-937 cells, a human histiocytic lymphoma cell line that differentiates into macrophage-like cells upon phorbol ester treatment and that has been demonstrated to produce VEGF (17). In a previous report, we demonstrated that macrophages released an EC growth inhibitor that was neither TGF-β1 nor TNF-α (20). This EC inhibitory activity was characterized as being basic and mildly heparin-binding but was not purified to any degree. We subsequently analyzed CM of U-937 cells and found a similar activity. We now report the complete purification, N-terminal microsequencing, and identification of three EC growth inhibitors released by U-937 cells. These are oncostatin M (OSM), LIF, and TGF-β1. LIF and OSM belong to a family of IL-6-related cytokines that exhibit a similar helical structure and share receptor components, while TGF-β1 is a member of the TGF-β superfamily. Unlike TGF-β1 and LIF, OSM has not been reported previously to be an EC growth inhibitor and is particularly potent with half-maximal inhibition at 150–200 pg/ml. We have also found that U-937 cells release VEGF, lending support to the idea that macrophage-like cells produce both EC stimulators and inhibitors.

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1 The abbreviations used are: EC, endothelial cells; BAE, bovine aortic EC; BCE, bovine capillary EC; CM, conditioned media; CNTF, ciliary neurotrophic factor; DMEM, Dulbecco’s Modified Eagle’s medium; EC1, endothelial cell inhibitor; FCS, fetal calf serum; FGF, fibroblast growth factor; GPS, glutamine/penicillin/streptomycin; IL-6, interleukin-6; LIF, leukemia inhibitory factor; OSM, oncostatin M; RPLC, reverse phase liquid chromatography; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor α; TPA, 12-O-tetradecanoylphorbol-13-acetate; VEGF, vascular endothelial growth factor; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; rh, recombinant human.
EXPERIMENTAL PROCEDURES

Materials

The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), and glutamine/penicillin/streptomycin (GSP) were purchased from Sigma. Bovine calf serum was purchased from Colorado Serum Co. (Denver, CO). Further cell media components were purchased from Life Technologies, Inc. Recombinant human FGF-2 was obtained from Scios Nova (Mountain View, CA). Recombinant human TGF-β1, LIF, and OSM were purchased 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 was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and GSP. Cells were seeded in roller bottles at a density of 5 x 10⁶ cells/ml in fresh RPMI 1640 culture medium containing 5% (v/v) FCS and TPA. Two days later, cells were washed with serum-free media and were incubated in serum-free RPMI 1640 for 48 h, at which time the supernatants were collected and replaced with fresh serum-free RPMI 1640. CM were collected again at 4 and 6 days after 48 h of serum-free culture incubation. The samples of CM were pooled and used as the starting material for the purification of EC inhibitors.

Endothelial Cell Growth Inhibition Assays

Bovine capillary EC (BCE) and bovine aortic EC (BAE) were used to assay EC growth inhibitors. BCE cells were maintained in 21-day-old bull calf blood co’s modified Eagle’s medium (DMEM), 10% bovine calf serum, and 3 ng/ml recombinant human FGF-2. BAE cells (22) were maintained in DMEM, 10% FCS, and GPS. For EC growth inhibition assays, BCE were resuspended in DMEM, 10% bovine calf serum, and BCE cells were resuspended in DMEM, 1% FCS. Both cell types were seeded onto gelatinized 96-well microtiter plates (2 x 10⁵ cells/well/200 μl). The plates were incubated for 24 h at 37° C, and samples were tested for inhibitory activity. The samples were added after 48 h, 10 μl (0.2 μCi) of [3H]thymidine (specific activity, 27 mCi/mg; DuPont NEN) was added for 6 h, and [3H]thymidine incorporation into DNA was determined by liquid scintillation counting (20).

Purification of Endothelial Cell Growth Inhibitors

Three liters of serum-free U-937 CM were concentrated to 125 ml by ultrafiltration (S1Y10 spiral-wound membrane, molecular weight cut off 10,000; Amicon, Inc., Beverly, MA). The retentate was dialyzed against 0.02 M acetic acid, pH 4.0, and after centrifugation (50,000 x g, 30 min) the supernatant was applied to a TSK gel SP-5PW column (7.5 x 75 mm, Tosoh Haas, Montgomeryville, PA). The column was equilibrated with 0.02 M acetic acid, pH 4.0, and bound material was eluted with a linear gradient of NaCl (0–1.5 M) at a flow rate of 1 ml/min. Fractions (1 ml) were collected and tested for bioactivity. Three active peaks of EC growth inhibitory activity (ECI) were pooled and designated as endothelial cell inhibitors 1, 2, and 3 (ECI 1, ECI 2, ECI 3).

ECI 1—Fractions collected from the first peak of inhibitory activity eluted from the TSK gel SP-5PW column (0.1–0.3 M NaCl) were pooled and injected onto a Vydac C₄ RPLC column (4.6 x 250 mm) equilibrated with 10% acetonitrile, 0.1% trifluoroacetic acid. Fractions (1 ml) were eluted with a linear gradient of 20–60% acetonitrile at a flow rate of 1 ml/min. Next, the inhibitory fractions from the C₄ RPLC column were diluted 2-fold with 0.1% trifluoroacetic acid and injected onto a TSK gel SP-5PW column (4.6 x 50 mm) equilibrated with 2-propanol. Fractions (1 ml) were eluted with a linear gradient of 25–35% 2-propanol at a flow rate 0.5 ml/min. All the fractions collected during the various purification steps were tested for EC growth inhibitory activity at a final dilution of 1:200 (1 μl of fraction/200 μl of medium/well).

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The molecular masses of EC inhibitors were determined by SDS-PAGE under non-reducing conditions and silver stained as described previously (23). In order to extract EC growth inhibitors, non-reduced samples were electrophoresed on a 1-mm thick, 10-cm long 15% SDS-polyacrylamide gel. The polyacrylamide gels were sliced into 1.5–2-mm pieces, which were each crushed and extracted for 6 h at 4 °C with 50 μl of phosphate-buffered saline. The polyacrylamide gel extracts were assayed for EC growth inhibitory activity.

Amino Acid Analysis and Microsequencing

Non-reduced samples were electrophoresed on 15% SDS-PAGE gels, as described above, and transferred to PVDF membranes (ProBlett, Applied Biosystems, Foster City, CA). After staining with 0.1% Coomassie Brilliant Blue, protein bands corresponding to EC inhibitory activity were excised and dried. One-tenth of the sample was used for amino acid analysis to estimate the total protein amount, and the other nine-tenths was analyzed for N-terminal amino acid sequences by microsequencing. Amino acid analysis and microsequencing were performed by Dr. Bill Lane of the Harvard Microchemistry Facility using Pico-Tag amino acid analysis HPLC and a gas-phase sequenator (Applied Biosystems).

RESULTS

Initial Purification of U-937 Cell-derived EC Inhibitors—Human U-937 cells treated with 10 nM TPA for 24 h became adherent and acquired many of the characteristics of macrophages (24). The serum-free CM collected from the TPA-treated U-937 cells, but not from untreated U-937 cells, inhibited EC growth. About 50% inhibition of [3H]thymidine incorporation into both capillary and aortic ECs occurred at a concentration of 1–2% (v/v). In order to purify the EC inhibitory activity initially, 3 liters of U-937 cell CM (150 mg of protein) were concentrated, dialyzed, and applied to a strong cation exchange column, TSK gel SP-5PW. At least three peaks of EC growth inhibitory activities were detected that were eluted with about 0.2, 0.6, and 0.85 M NaCl, respectively (Fig. 1). These three peaks were termed ECI 1, ECI 2, and ECI 3 in the order of their increasing concentrations of NaCl needed for elution. Both BCE and BAE cells were inhibited, but only the BAE cell
Inhibition of endothelial cell proliferation by ECI 1, ECI 2, and ECI 3

TABLE I

| Sample         | Cell number       |
|----------------|-------------------|
| No addition    | 42,326 ± 2245     |
| ECI 1          | 26,458 ± 2349     |
| ECI 2          | 22,432 ± 3249     |
| ECI 3          | 15,426 ± 3249     |

RESULTS

Purification of ECI 1—ECI 1 was purified by a combination of C₄ RPLC (Fig. 2A), TSK gel TMS-250 RPLC (Fig. 2B), and C₄ RPLC (Fig. 2C) as described under “Experimental Procedures.” Active fractions from the last column were pooled, lyophilized, and analyzed by SDS-PAGE (Fig. 3A). A major protein band of about 70 kDa was detected. The SDS-polyacrylamide gels were extracted, and EC inhibitory activity was found to be associated with the 40-kDa band (Fig. 3B). The remainder of the sample was analyzed on a replicate SDS gel, and the bands were transferred to a PVDF membrane. The 40-kDa band was excised and subjected to microsequencing (Table II), and the N-terminal amino acid sequence was found to be SPLPITPV, corresponding to the N-terminal sequence of human LIF (25). The final yield of pure LIF from 3 liters of U-937 CM was estimated to be 20 pmol (0.8 μg).

Purification of ECI 2—ECI 2 was purified by a combination of C₄ RPLC (Fig. 4A) and C₁₈ RPLC (Fig. 4B) as described under “Experimental Procedures.” The EC inhibitory fractions from the C₁₈ column were pooled and analyzed by SDS-PAGE, and a major protein band of 30 kDa and minor bands of 25 and 70 kDa were detected (Fig. 5A). After gel extraction, it was found that the inhibitory activity of ECI 2 was associated with the 30-kDa band (Fig. 5B). The remainder of the sample was analyzed on a replicate SDS-polyacrylamide gel, and the bands were transferred to a PVDF membrane. The 30-kDa band was excised and subjected to microsequencing (Table II), and the N-terminal amino acid sequence was found to be AAIGSKEYRVLL corresponding to the N-terminal sequence of human OSM (26). The final yield in pure protein from 3 liters of U-937 cell CM, as estimated by amino acid analysis, was 8 pmol (0.24 μg).

An EC mitogenic peak was eluted from the C₄ column with 30% acetonitrile (Fig. 4A). This mitogenic activity was neutralized about 50% by incubation with anti-VEGF neutralizing antibodies (data not shown), consistent with previous reports of VEGF production by U-937 cells (17).

Purification of ECI 3—ECI 3 was purified by a combination of C₂₅ RPLC using a 2-propanol gradient column (Fig. 6A) and C₄ RPLC using a 2-propanol gradient (Fig. 6B) as described under “Experimental Procedures.” EC inhibitory activity from this second C₂₅ column was pooled, concentrated, and analyzed by SDS-PAGE that showed the presence of a single band of about 28 kDa (Fig. 6C). Since this was the only protein band detected, it was transferred directly to a PVDF membrane, followed by microsequencing. The N-terminal sequence was found to be ALDNTYFSSTLKN (Table II), corresponding to the N-terminal sequence of human TGF-β1 (27). The final yield of pure ECI 3 was estimated to be 12 pmol (0.34 μg).

The N-terminal sequences obtained for human U-937 cell-derived ECI 1, 2, and 3 corresponded to human but not bovine LIF, OSM, and TGF-β1 N-terminal sequences, respectively, making it unlikely that the EC inhibitors were artifically derived from bovine serum during the purification process (Table II).
EC Growth Inhibition by Recombinant Human LIF, OSM, and TGF-β1—To confirm that LIF, OSM, and TGF-β1 were responsible for the three EC inhibitory activities purified from U-937 cell CM, recombinant human (rh) LIF (rhLIF), OSM (rhOSM), and TGF-β1 (rhTGF-β1) were tested. All three recombinant proteins induced a dose-dependent inhibition of BAE (Fig. 7A) and BCE (Fig. 7B) cell proliferation. Half-maximal inhibition of BAE and BCE cells, respectively, was obtained with 0.9 and 0.6 ng/ml, 0.15 and 0.2 ng/ml, and 1.0 and 0.9 ng/ml rhLIF, rhOSM, and rhTGF-β1, respectively. Thus, OSM appeared to be the most potent of the U-937 cell-derived ECIs, followed by LIF and TGF-β1. Since LIF and OSM belong to the same Interleukin-6 related cytokine superfamily, two other members of this family, rhIL-6 and rhCNTF, were tested for EC inhibitory activity (Fig. 7). However, these cytokines were inhibitory only in the 100–200 ng/ml range, or log 3 less than the rhOSM.

The inhibition of BAE cell proliferation by the three EC inhibitors was non-toxic and reversible in that upon removal of the inhibitors, EC proliferation resumed (data not shown). rhLIF, rhOSM, and rhTGF-β1 inhibited not only basal EC proliferation but VEGF- and FGF-2-stimulated EC proliferation as well and to about the same extent (Table III).

DISCUSSION

We have demonstrated that in addition to the EC growth stimulator, VEGF, macrophage-like U-937 cells release EC growth inhibitors as well. We have purified three such inhibi-

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**Table II**

N-terminal amino acid analysis of endothelial cell growth inhibitors

| ECI 1 | SPLPITPVXATXAIRHP |
|--------|-------------------|
| LIF    | SPLPITPVXATXAIRHP |
| ECI 2  | AAIGSCSKEYRVLL   |
| OSM    | AAIGSCSKEYRVLL   |
| ECI 3  | ALDTNYXSTKLN     |
| TGF-β1 | ALDTNYXSTKLN     |

*The bovine LIF gene sequence has been deposited in the GenBank data base with accession number D50377.*
Inhibition of basal, VEGF-, and FGF-2-stimulated EC proliferation

| Inhibitor Basal | VEGF | FGF-2 |
|----------------|------|-------|
| LIF (4 ng/ml)  | 90.4 | 91.0  | 85.3 |
| OSM (2 ng/ml)  | 91.3 | 93.1  | 80.2 |
| TGF-β1 (5 ng/ml)| 84.0 | 88.5  | 90.1 |

Notes: The table lists the percentage of inhibition (mean ± S.D., n = 3) for each inhibitory concentration tested. VEGF and FGF-2 were used as mitogens at 5 ng/ml, and OSM was tested at 2 ng/ml.

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