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EDF-1, a Novel Gene Product Down-regulated in Human Endothelial Cell Differentiation*

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Endothelial cell differentiation is a crucial step in angiogenesis. Here we report the identification of EDF-1, a novel gene product that is down-regulated when endothelial cells are induced to differentiate *in vitro*. The cDNA encoding *EDF-1* was isolated by RNA fingerprinting from human endothelial cells exposed to human immunodeficiency virus type 1 Tat, a viral protein known to be angiogenic. The deduced amino acid sequence of EDF-1 encodes a basic intracellular protein of 148 amino acids that is homologous to MBF1 (multiprotein-bridging factor 1) of the silkworm *Bombyx mori* and to H7, which is implicated in the early developmental events of *Dictyostelium discoideum*. Interestingly, human immunodeficiency virus type 1 Tat, which affects endothelial functions, and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate and culture on fibrin gels, which promote endothelial differentiation *in vitro*, all down-regulate *EDF-1* expression both at the RNA and protein levels. In addition, the inhibition of EDF-1 translation by an antisense anti-EDF-1 construct results in the inhibition of endothelial cell growth and in the transition from a nonpolar cobblestone phenotype to a polar fibroblast-like phenotype. These data suggest that EDF-1 may play a role in the regulation of human endothelial cell differentiation.

Angiogenesis, the formation of new blood vessels, is essential during development, in wound healing, and for the growth of tumors (1). Angiogenesis is a complex process requiring migration of endothelial cells, their proliferation, and their differentiation into tube-like structures (2). Although considerable attention has been given to the mechanisms involved in the regulation of endothelial cell growth, little is known about the molecular events associated with the non-proliferative aspects of angiogenesis, *i.e.* the organization/differentiation of endothelial cells into capillaries (3). The endothelial cell is capable of activating a unique genetic program in response to environmental signals, such as cytokines and extracellular matrix components, that direct and sustain the formation of a differentiated phenotype *in vitro* (4). Among others, interleukin-1, γ-interferon, and the phorbol ester TPA[1] inhibit endothelial growth and promote a morphological change that resembles the polar elongated phenotype assumed by endothelial cells during the early stage of differentiation (5); culture on three-dimensional gels also induces endothelial differentiation and tube formation (6).

It is noteworthy that HIV-1 Tat, which enhances HIV-1 transcription and also affects strategic host genes, can function as a cytokine in the activation of endothelial cells (7). Moreover, HIV-1 Tat plays a role in the pathogenesis of Kaposi's sarcoma (KS), a highly vascularized skin lesion characterized by marked endothelial proliferation and migration, resulting in the formation of new capillaries. Indeed, HIV-1 Tat is angiogenic *in vivo* (8, 9).

Since HIV-1 Tat affects endothelial cell function, we assumed that the isolation of differentially expressed genes in Tat-treated endothelial cells would yield insights into the molecular mechanisms contributing to endothelial dysfunction in AIDS-associated Kaposi's sarcoma and, more generally, in angiogenesis. Here we report the cloning, sequencing, and characterization of a novel and abundant gene product designated EDF-1 (endothelial differentiation-related factor 1) that is down-regulated by Tat in human endothelial cells. We also provide evidence that EDF-1 is down-regulated when endothelial cells are induced to differentiate.

EXPERIMENTAL PROCEDURES

RNA Fingerprinting—1 μg of total RNA from endothelial cells treated with recombinant Tat (Intracel, Cambridge, MA) was reverse-transcribed using an oligo(dT) primer. The cDNAs were then amplified by PCR in the presence of [^{32}P]dCTP using the following primers: 3′-TCT GGG AAC CGG-5′ and 3′-GGG TCG CGA ACA-5′. PCR products were separated on a denaturing polyacrylamide gel and autoradiographed. Several differentially expressed genes were identified. The bands corresponding to differentially expressed genes were excised from the polyacrylamide gel, electroeluted, reamplified by PCR, cloned in Bluescript as described (10), and sequenced.

DNA Sequence Analysis—Plasmid DNA for *EDF-1* was obtained by screening a human umbilical vein endothelial cell (HUVEC) cDNA library (CLONTECH) with the 550-bp fragment obtained by DNA fingerprinting and by RACE-PCR using the Marathon kit (CLONTECH). Double-stranded sequence analysis was performed using the T7 sequencing kit (Amersham Pharmacia Biotech) following the manufacturer's instruction as well as by automatic sequencing (Primm, Milano, Italy). The DNA sequence was analyzed by the Analyze and Interpret programs of the Mac Molly Suite (Berlin). Deduced protein sequences were compared and aligned using BLASTX with ClustalW 1.7 programs made available by Baylor College of Medicine.

Plasmid Construction—Antisense pMEXNeo-αEDF was constructed by inserting *Bam*HI- and *Kpn*I-cut EDF-1 into the eukaryotic expression vector pMEXNeo (11). The correct sequence and orientation of the construct were confirmed by sequencing.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ005259.

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[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HIV-1, human immunodeficiency virus type 1; KS, Kaposi's sarcoma; PCR, polymerase chain reaction; HUVEC, human umbilical vein endothelial cell; bp, base pair(s); RACE, rapid amplification of cDNA ends.

A

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                                                        M   A   E   S   D   W   D
          AAT TCC CCG GAC GGA CGC TCG TCT TCG CCC GCC ATG GCC GAG AGC GAC TGG GAC
                   10                  19                  28          37          46                  55

T   V   T   V   L   R   K   K   G   P   T   A   A   Q   A   K   S   K   Q   A
ACG GTG ACG GTG CTG CGC AAG AAG GGC CCT ACG GCC GCC CAG GCC AAA TCC AAG CAG GCT
         64                  73                  82                  91                  100                 109

I   L   A   A   Q   R   R   G   E   D   V   E   T   S   K   K   W   A   A   G
ATC TTA GCG GCA CAG AGA CGA GGA GAA GAT GTG GAG ACT TCC AAG AAA TGG GCT GCT GGC
         124                 133                 142                 151                 160                 169

Q   N   K   Q   H   S   I   T   K   N   T   A   K   L   D   R   E   T   E   E
CAG AAC AAA CAA CAT TCT ATT ACC AAG AAC ACG GCC AAG CTG GAC CGG GAG ACA GAG GAG
         184                 193                 202                 211                 220                 229

L   H   H   D   R   V   T   L   E   V   G   K   V   I   Q   Q   G   R   Q   S
CTG CAC CAT GAC AGG GTG ACC CTG GAG GTG GGC AAG GTG ATC CAG CAA GGT CGG CAG AGC
         244                 253                 262                 271                 280                 289

K   G   L   T   Q   K   D   L   A   T   K   I   N   E   K   P   Q   V   I   A
AAG GGG CTT ACG CAG AAG GAC CTG GCC ACG AAA ATC AAT GAG AAG CCA CAG GTG ATC GCG
         304                 313                 322                 331                 340                 349

D   Y   E   S   G   R   A   I   P   N   N   Q   V   L   G   K   I   E   R   A
GAC TAT GAG AGC GGA CGG GCC ATA CCC AAT AAC CAG GTG CTT GGC AAA ATC GAG CGG GCC
         364                 373                 382                 391                 400                 409

I   G   L   K   L   R   G   K   D   I   G   K   P   I   E   K   G   P   R   A
ATT GGC CTC AAG CTC CGG GGA AAG GAC ATT GGA AAG CCC ATC GAG AAG GGG CCT AGG GCG
         424                 433                 442                 451                 460                 469

K
AAA TGA ACA CAA AGC CTC GAA ATC AGT GCG CTC CAG CTG ATC TCG TTC CGC CGG TTC CCC
         484                 493                 502                 511                 520                 529

TTG GCC GCC AGT TCC GTT CTC CTC ACG GGC CGA ACG GAA CAA GGG GTC CAG CTT GCG GGG
         544                 553                 562                 571                 580                 589

GAC CCT CCC CAG CCC ATT CCT GCT GTC AAA CAA ACA AAA CCT TGC AAA GCG AAA AAA AGG
         604                 613                 622                 631                 640                 649

AAT TCC GGT AAA CTG AAG ACT TTT ATT TGG GAA TGA AAA ACC TTA AAA AAA TCT CTT CAT
         664                 673                 682                 691                 700                 709

CGT TGA ACT GTG CAT TTT CCC TGC ATT TTT TCC CAA CAA AAT TTT GTT GGG GGT TAT GTT
         724                 733                 742                 751                 760                 769

ACT GAA GAA TGA ACA GAT GAG TAA GTG GAG GTG TTA TGT AAA GGC ATA TTG TAC TCG AAA
         784                 793                 802                 811                 820                 829

TCT GAA GAC CTG CAG CAG ATT TAA ATT ACA ACT CTT GTT ATA ACT TTT TAA AAG ATT GTG
         844                 853                 862                 871                 880                 889

AAA ATA TCA AAA TAT AAA TGA ATC AAG TTT TAA TAT ACT GTA TGA TGG GTG GAT GAG GCT
         904                 913                 922                 931                 940                 949

GTC CAT TGT ACC ATT TGT TTG AAT TC
         964                 973
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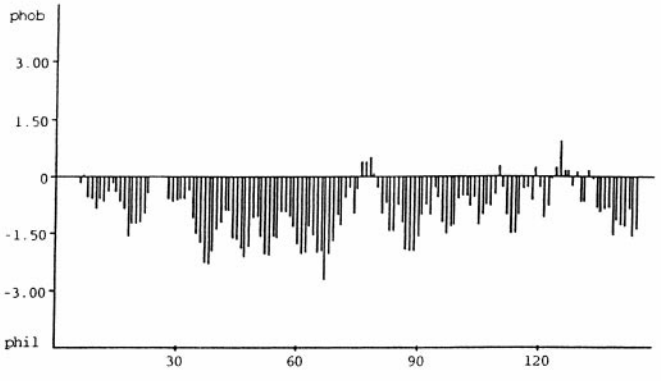
B



C

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EDF-1     MAESDWDTVT VLRKKG------PTA AQAKSKQAILAAQRR GEDVETSKKWAAGQN KQHSITKNTAKLD RETEELHHDRVTLEV     77
MBF-1     --MSDWDTVT ILRKKP------PKA SALKTEQAVNAARRQ GIPVDTQQKYGAGTN KQHVTTKNTAKLD RETEELRHEKIPLDL     75
yeast     --MSDWDTNT IIGSRARAGGSGPRA NVARSQGQINAARRQ GLVVSVDKKYGSTNT RGDNEGQRLTKVD RETDIVKPKKLDPNV     81
Dictyost. ---------- --------------- --------------- ---MDVQTKYGAGQN KVLGGANQKKIAE SEEDIALP-ELNPSV     39

EDF-1     GKVIQQGRQSKGLTQ KDLATKINEKPQVIA DYESGRAIPNNQVLG KIERAIGLKLRGKDI GKPIEKGPR-AK    148
MBF-1     GKLIMQGRQAKGMSQ KDLATKICEKPQIVN DYEAGRGIPNNIVLG KIERAIGIKLRGKER GQPLQP-PGGQK    146
yeast     GRAISRARTDKKMSQ KDLATKINEKPTVVN DYEAARAIPNQQVLS KLERALGVKLRGNNI GSPLGAPKKK--    151
Dictyost. PQAIQRARNALKMTQ KELAFKINERPGVIN EYESGSAIPSQAVLS KLEKALNVKLRGKEI GKPLK-------    104
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FIG. 1. **Nucleotide and deduced amino acid sequences of human EDF-1.** *A,* the nucleotide (positions 1–980) and deduced amino acid (positions 1–148) sequences are shown for the human *EDF-1* cDNA. The Kozak consensus translation initiation sequence is *underlined*. *B,* the hydropathic profile of EDF-1 is shown. *phob*, hydrophobic; *phil*, hydrophilic. *C,* the amino acid sequence of EDF-1 is aligned with those of *B. mori* MBF1 (GenBank™ accession number AB001078), *D. discoideum* H7 (*Dictyost.*; GenBank™ accession number X15385), and *S. cerevisiae* deduced from chromosome XV region residues 877231–877678 (from the *S. cerevisiae* genome data base).

Protein Expression and Purification—To produce EDF-1 bearing six consecutive histidine residues (His_6-EDF-1) in *Escherichia coli*, a *Bam*HI-*Kpn*I *EDF-1* fragment was obtained by PCR and cloned into pQE30 (QIAGEN Inc.). The oligomers used were as follows: 5′-CTA GGA TCC GCC ATG GCC GAG AGC GAC-3′ (sense) and 5′-CTA GGT ACC CAA GGG GAA CCG GCG GAA C-3′ (antisense). The correct sequence was confirmed by sequencing. Recombinant His_6-EDF-1 was purified using nickel-immobilized resin (QIAGEN Inc.).

Immunological Methods—Antiserum against EDF-1 was prepared using His_6-EDF-1 to immunize rabbits by standard procedures. IgGs against EDF-1 were purified on a protein A-Sepharose column. For Western blotting, cell extracts (75 μg/lane) were resolved by SDS-

polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets at 150 mA for 16 h, and probed with anti-EDF-1 IgGs (10 μg/ml). Secondary antibodies were labeled with horseradish peroxidase (Pierce). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins. For immunoprecipitation, HUVECs were incubated with 150 μCi/ml [^{35}S]methionine in methionine-free medium for 6 h. Cells were then washed, lysed in ice-cold radioimmune precipitation assay buffer, and centrifuged. After pre-clearing with preimmune serum, lysates were immunoprecipitated with anti-EDF-1 IgGs (10 μg/ml). The immunocomplexes were bound to protein G-Sepharose, extensively washed, and eluted in Laemmli buffer at 95 °C for 5 min.

Cell Culture, Proliferation Assays, and Transfection—ECV cells (12) were cloned to obtain a monoclonal population (13) and maintained by serial passage in medium 199 supplemented with 10% fetal calf serum. HUVEC-C cells were from American Type Culture Collection and cultured in Ham's F-12 medium containing 10% fetal calf serum, endothelial cell growth supplement (150 μg/ml), and heparin (5 units/ml) on gelatin-coated dishes. TTB and LE cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Fibrin matrices were prepared by rapidly mixing human fibrinogen (20 mg/ml) with bovine thrombin (25 units/ml) in a cell culture dish and allowing the mixture to solidify for 30 min at room temperature (14).

pMEXNeo and antisense pMEXNeo-αEDF were transfected into 2 × 10^5 HUVEC-C cells via the calcium phosphate coprecipitation technique (13). Transfectants were selected in growth medium containing G418 (500 μg/ml).

To perform proliferation assays, HUVECs were seeded at low density (7500/cm^2) in growth medium. At various time intervals, cells were trypsinized and counted using a Burker chamber.

Purification of RNA and Northern Blotting—Cells were treated with Tat (10 ng/ml) or TPA (10 nM) or were grown on fibrin gels for different times, rinsed with phosphate-buffered saline, and lysed in 4 M guanidinium isothiocyanate. RNA was purified as described (15). RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, capillary-blotted onto nylon membranes, and UV-cross-linked. EDF-1 and glyceraldehyde-3-phosphate dehydrogenase or β-actin were labeled with a random primer labeling kit (Ambion Inc.). Filters were hybridized in 0.5 M sodium phosphate (pH 7.2) containing 7% SDS, 1 mM EDTA, and 20% formamide at 65 °C for 20 h and extensively washed at high stringency. mRNAs were visualized by autoradiography.

Zoo Blot—A Southern blot containing 4 μg of genomic DNA/lane from nine eukaryotic species was purchased from CLONTECH and hybridized as described above.

RESULTS

Cloning and cDNA Sequencing of EDF-1—To obtain cDNAs representing mRNAs regulated by Tat in human endothelial cells, we utilized a modified PCR-based differential screening approach commonly referred to as RNA fingerprinting (10). We obtained four differentially expressed clones. The characterization of one of these clones, termed *EDF-1*, whose expression is down-regulated by Tat, is the subject of this report. The *EDF-1* insert (550 bp) hybridized to a mRNA species of 1.0 kilobases (see below). To obtain a full-length clone, the initial 550-bp *EDF-1* fragment was used as a probe to screen an endothelial cell cDNA library; a 700-bp clone was obtained, which did not contain the 5′-end region. By RACE-PCR, we obtained the full-length cDNA coding for *EDF-1*. The complete nucleotide sequence of the *EDF-1* cDNA is 980 bp long and contains, at the 5′-end, a consensus translation initiation sequence (Fig. 1*A*) (16). This is followed by an open reading frame encoding 148 amino acids. The predicted polypeptide is basic, with a pI of 8.2. No known protein motifs were found in the EDF-1 protein. The deduced amino acid sequence of EDF-1 is highly hydrophilic, with no hydrophobic NH_2-terminal region (Fig. 1*B*).

Sequence comparison of EDF-1 by the BLASTX program revealed 66% identity at the amino acid level to MBF1 (multiprotein-bridging factor 1) of the silkworm *Bombyx mori*, a transcriptional coactivator that may play a role in differentiation (17), and 46% identity to H7, a developmentally regulated gene of *Dictyostelium discoideum* (18, 19). In Fig. 1*C*, these sequences and the homologous gene product of *Saccharomyces cerevisiae* have been aligned using the ClustalW program.

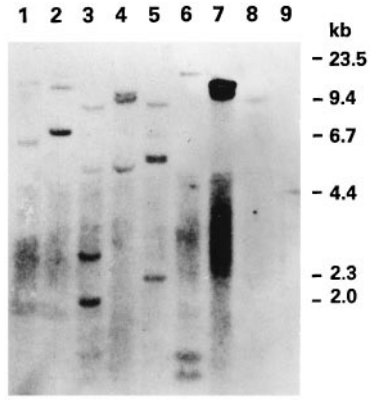

FIG. 2. **Southern blot analysis of *EDF-1*.** The zoo blot was hybridized at high stringency to *EDF-1* cDNA and visualized by autoradiography. *Lane 1*, human; *lane 2*, monkey; *lane 3*, rat; *lane 4*, mouse; *lane 5*, dog; *lane 6*, cow; *lane 7*, rabbit; *lane 8*, chicken; *lane 9*, yeast. *kb*, kilobases.

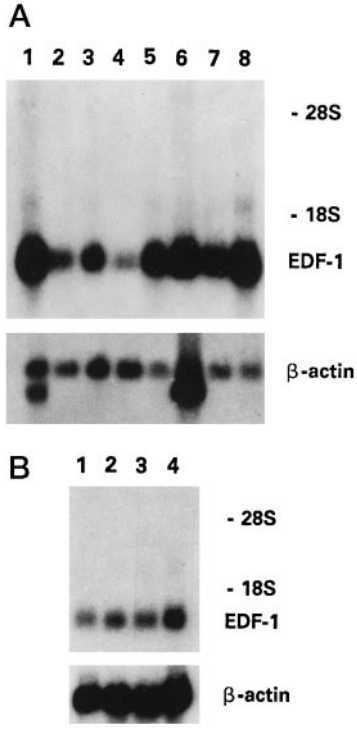

FIG. 3. **Tissue distribution of *EDF-1*.** *A,* a human adult multiple-tissue Northern blot was purchased from CLONTECH. Northern blotting was performed at high stringency using the human *EDF-1* cDNA probe. Hybridization to β-actin indicates that similar amounts of RNA were used per lane; as indicated by the manufacturer, heart and skeletal muscle contain two forms of β-actin, 2.0 and 1.8 kilobases. *Lane 1*, heart; *lane 2*, brain; *lane 3*, placenta; *lane 4*, lung; *lane 5*, liver; *lane 6*, muscle; *lane 7*, kidney; *lane 8*, pancreas. *B,* a human fetal multiple-tissue Northern blot was purchased from CLONTECH. Northern blotting was performed as described above. *Lane 1*, brain; *lane 2*, lung; *lane 3*, liver; *lane 4*, kidney.

Evolutionary Conservation and Tissue Distribution of EDF-1—*EDF-1* cDNA was used as a probe to hybridize a Southern blot containing *Eco*RI-cut genomic DNA from human and other species (CLONTECH). Under conditions of high stringency hybridization, we observed strong specific signals in all the species examined, including yeast, reflecting the existence of orthologues of *EDF-1* in each of these species (Fig. 2). We also determined the distribution of *EDF-1* transcript in human tissues. *EDF-1* was present in every human adult tissue exam-

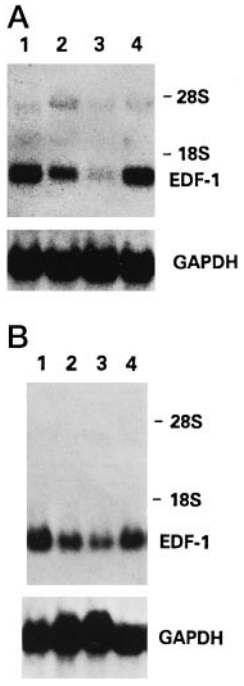

FIG. 4. **Modulation of *EDF-1* expression by Tat in human endothelial cells.** Confluent cultures of ECV cells (*A*) and cytokine-treated HUVECs (*B*) were exposed to 10 ng/ml Tat for 1, 4, and 24 h. Total RNA (10 μg/lane) was analyzed by Northern blotting as described under "Experimental Procedures." The blot was rehybridized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to verify equal amounts of RNA loading among the lanes. *Lane 1*, untreated cells; *lane 2*, cells exposed to Tat for 1 h; *lane 3*, cells exposed to Tat for 4 h; *lane 4*, cells exposed to Tat for 24 h.

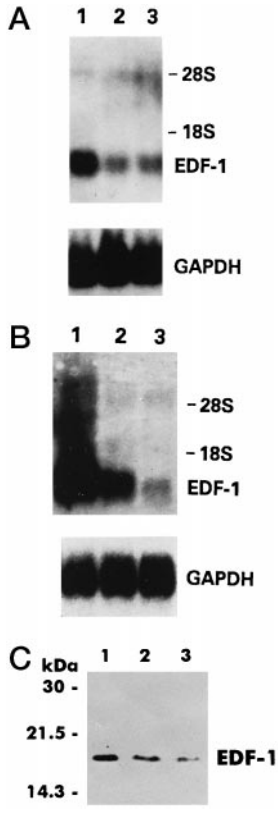

FIG. 5. **Modulation of EDF-1 expression in differentiating human endothelial cells.** *A,* confluent cultures of HUVECs were exposed to 10 nM TPA for 45 min (*lane 2*) and 4 h (*lane 3*) or left untreated (*lane 1*). Total RNA (10 μg/lane) was analyzed by Northern blotting as described under "Experimental Procedures." *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. *B,* Northern blotting was performed on RNA obtained at 0, 4, and 24 h (*lanes 1–3*, respectively) from human endothelial cells seeded on fibrin as described. *C,* confluent HUVECs were exposed to 10 nM TPA or cultured on fibrin for 15 h. Cell extracts (75 μg/lane) were loaded on a 15% SDS-polyacrylamide gel, blotted onto nitrocellulose, incubated with anti-EDF-1 IgGs, and visualized by chemiluminescence as described under "Experimental Procedures." *Lane 1*, untreated cells; *lane 2*, TPA-treated HUVECs; *lane 3*, HUVECs seeded on fibrin.

ined and was most abundant in the heart, pancreas, and liver (Fig. 3*A*). *EDF-1* was highly expressed also in the fetal tissues examined and was most abundant in the kidney (Fig. 3*B*). We also detected EDF-1 in several human adult tissues (brain, liver, lung, kidney, and heart) by Western blotting using anti-EDF-1 IgGs (data not shown).

EDF-1 mRNA Expression in Human Endothelial Cells Exposed to Tat—The down-regulation of *EDF-1* mRNA by Tat was confirmed by Northern blot analysis of ECV cells exposed to Tat for 1, 4, and 24 h. As shown in Fig. 4*A*, an early and transient down-regulation of *EDF-1* mRNA was observed, starting at 60 min and becoming maximal after 4 h. Similar results were obtained using synthetic Tat (data not shown). ECV cells were isolated by spontaneous transformation of HUVECs (12). We therefore extended our studies to HUVECs, which represent a widely accepted experimental model for the study of endothelial biology and pathophysiology. To be sensitive to Tat, HUVECs must be pre-activated by exposure to pro-inflammatory cytokines (8). HUVECs were exposed to a mixture of interleukin-1 (1 ng/ml) and tumor necrosis factor (0.5 unit/ml) for 24 h before the addition of Tat (10 ng/ml). Under these experimental conditions, Tat decreased *EDF-1* mRNA, with a maximal down-regulation observed after 4 h of exposure to Tat and a return to the base line within 24 h (Fig. 4*B*).

EDF-1 Expression in Differentiating Endothelial Cells—The possibility that EDF-1 is modulated in endothelial differentiation is suggested by the similarity between *EDF-1* and H7, a developmentally regulated gene isolated in *D. discoideum* (18, 19) (Fig. 1*C*). A number of *in vitro* endothelial cells systems have been suggested as models for the study of endothelial differentiation. Among others, the phorbol ester TPA, which inhibits endothelial growth, promotes endothelial differentiation into capillary-like, tubular structures (20). TPA (10 nM) decreased the steady-state level of *EDF-1* mRNA in HUVECs as early as 45 min after addition to the medium (Fig. 5*A*). Moreover, three-dimensional fibrin gels have been used to mimic differentiation as an *in vitro* approximation of the *in vivo* phenomenon since endothelial cells invade blood clots in the process of wound repair, and fibrin provides a provisional stroma to newly formed capillaries in tumor angiogenesis (14, 21). In an attempt to confirm a role of EDF-1 in differentiation, we plated HUVECs on fibrin, and we observed a rapid decrease in *EDF-1* mRNA over the course of 24 h (Fig. 5*B*), thus suggesting that EDF-1 is down-regulated in the early phases of endothelial differentiation *in vitro*. Fig. 5*C* shows that TPA and culture on fibrin markedly reduced the levels of EDF-1, as detected by Western blotting using anti-EDF-1 IgGs.

To delineate a functional role for EDF-1, we utilized an antisense approach. Similar strategies have previously proved useful as a means of repressing the translational efficiency of a wide variety of transcripts *in vitro* (22). *EDF-1* cDNA was inserted in the antisense orientation into the eukaryotic expression vector pMEXNeo (11) to attain high level constitutive expression of antisense mRNA. pMEXNeo-αEDF was then transfected into HUVECs (13). Several single clones were isolated, propagated, and characterized. The results of some representative clones are shown in Fig. 6. The expression of the antisense anti-EDF-1 mRNA resulted in a significant reduction

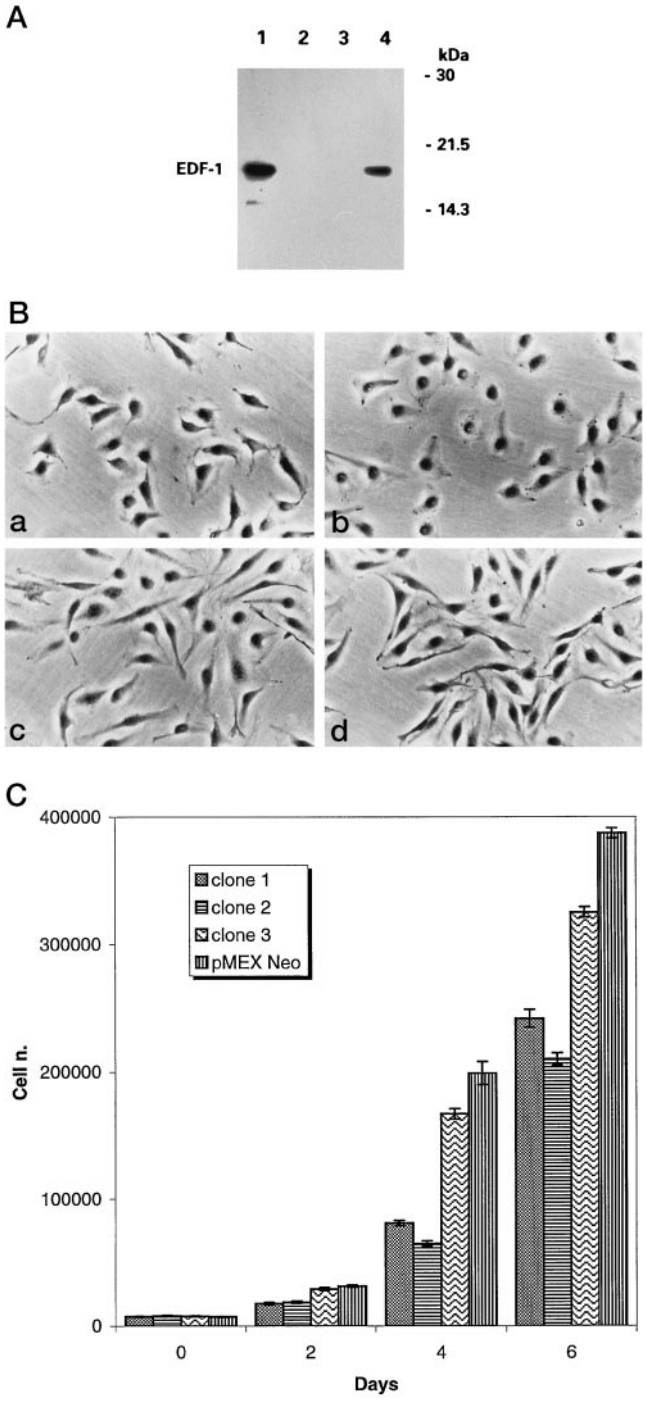

FIG. 6. **Effects of inhibition of EDF-1 translation in human endothelial cells.** *A,* shown are the results from immunoprecipitation analysis of antisense-transfected and mock-transfected cells. Transfected HUVECs were labeled with [^{35}S]methionine, lysed, and immunoprecipitated with anti-EDF-1 IgGs. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, and proteins were visualized by autoradiography. *Lane 1*, pMEXNeo-transfected cells; *lane 2*, antisense pMEXNeo-αEDF-transfected clone 1; *lane 3*, antisense pMEXNeo-αEDF-transfected clone 2; *lane 4*, antisense pMEXNeo-αEDF-transfected clone 3. *B,* the morphology of HUVECs transfected with the antisense anti-EDF-1 construct was examined by phase-contrast microscopy (magnification × 40). *Panel a,* pMEXNeo-transfected cells; *panel b,* antisense pMEXNeo-αEDF-transfected clone 3; *panel c,* antisense pMEXNeo-αEDF-transfected clone 1; *panel d,* antisense pMEXNeo-αEDF-transfected clone 2. *C,* the cell proliferation assay in transfected cells was performed as described under "Experimental Procedures." At the indicated time intervals, the cells were harvested by digestion with trypsin, and viable cells were counted using a Burker chamber. Data refer to means ± S.D. of two separate experiments performed in triplicate.

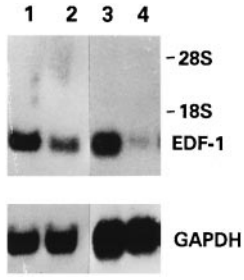

FIG. 7. **Expression of *EDF-1* in human and murine KS spindle cells.** Total RNA (10 μg/lane) from human and murine spindle cells was analyzed by Northern blotting. *Lane 1*, HUVECs; *lane 2*, KS spindle cells; *lane 3*, LE cells; *lane 4*, TTB cells. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

of EDF-1 in clone 3 (Fig. 6*A*, *lane 4*) and in the undetectability of EDF-1 in clones 1 and 2 (*lanes 2* and *3*, respectively). Fig. 6 (*A* and *B*) shows the relation between the levels of EDF-1 and cell shape; the inhibition of EDF-1 translation by the antisense mRNA promoted the transition from a cobblestone monolayer phenotype to a spindle-shaped, fibroblast-like phenotype (Fig. 6*B*), which is very similar to the morphology of endothelial cells during the early phases of their differentiation pathway *in vitro* (5). Since agents that promote endothelial differentiation or that modulate endothelial phenotype antagonize cell proliferation (5), we also evaluated the growth rate of pMEXNeo-αEDF-transfected clones. Cells were seeded at low density and counted every other day. As shown in Fig. 6*C*, the lower the levels of EDF-1, the slower the proliferation rate.

Kaposi's sarcoma spindle cells show a unique phenotype and may originate from an endothelial precursor (23). We therefore compared endothelial and human KS spindle cells for their levels of *EDF-1* mRNA. As shown in Fig. 7, spindle cells expressed lower levels of *EDF-1* transcript compared with endothelial cells. The same result was obtained in a murine transgenic model of KS: TTB cells, which share several characteristics of human KS spindle cells (24), expressed lower amounts of *EDF-1* mRNA compared with murine LE endothelial cells (Fig. 7).

DISCUSSION

Angiogenesis is central to a number of physiologic and pathologic processes, such as wound healing, placental development, rheumatoid arthritis, and tumor growth (1). Although endothelial cell differentiation is recognized as an early and important component of angiogenesis, very little is known about the molecular mechanisms of its regulation. *In vitro*, the endothelial cell is capable of activating a unique genetic program in response to environmental signals that direct and sustain the formation of a differentiated phenotype. Some cytokines, the phorbol ester TPA, and extracellular matrix components including fibrin and collagen induce changes from the traditional nonpolar cobblestone monolayer into a polar elongated fibroblast-like phenotype (4, 5). These polar endothelial cells ultimately organize into three-dimensional capillary-like structures (3). Endothelial cell differentiation has been shown to have a transcriptional basis. Indeed, endothelial organization into a cellular network has been associated with an increase in the transcripts encoding fibronectin (25); the protein G-coupled receptor EDG-1 (26), recently identified as the receptor for sphingosine 1-phosphate (27); and Jagged, the ligand for the Notch receptor (28); and with a decrease in *sis*-mRNA (25).

Here we describe the isolation and characterization of a novel human cDNA designated *EDF-1*, which is down-regulated in endothelial cells induced to differentiate by exposure to TPA or fibrin. Since agents that promote endothelial differentiation or that modulate endothelial phenotypic changes also

antagonize endothelial proliferation (5), it is noteworthy that endothelial cells in which EDF-1 translation is inhibited by an antisense anti-EDF-1 construct assume a spindle-shaped phenotype and proliferate slower than controls. Since we show that Tat inhibits EDF-1 expression, it is tempting to speculate about a potential pro-differentiative action of Tat in endothelial cells. To this purpose, since Tat plays a role in the pathogenesis of KS (29), it is noteworthy that KS spindle cells, which have the characteristic immunohistochemical and ultrastructural features of endothelial cells (30) and which seem to differentiate from a vascular progenitor (23), express lower levels of *EDF-1* when compared with endothelial cells used as controls.

The *EDF-1* cDNA sequence (980 bp in length) contains an open reading frame of 447 nucleotides and predicts a basic polypeptide of 148 amino acids corresponding to a molecular mass of 16 kDa. This was confirmed by immunoprecipitation on radiolabeled HUVECs and by Western blot analysis using anti-EDF-1 IgGs.

EDF-1 is homologous to silkworm MBF1, a transcriptional cofactor that mediates transactivation by stabilizing the protein-DNA interactions (17). Cofactors such as MBF1 are important in the regulation of gene expression in various systems (31–33). On these bases, we postulate that EDF-1 could act as a bridging molecule that interconnects the regulatory proteins and the basal transcriptional machinery, thus modulating the transcription of genes involved in endothelial cell differentiation. Preliminary experiments support the aforementioned hypothesis. It is noteworthy that *EDF-1* is also highly homologous to the H7 gene of *D. discoideum*, which is deactivated upon the onset of development (19, 20). When *D. discoideum* differentiates, multicellular aggregates align, organize, and result in the formation of stalks (34). Interestingly, the human endothelial cell utilizes a similar series of morphological correlates during its differentiation pathway (3, 5); individual cells migrate, align, and organize to form multicellular capillary-like structures. Although we do not know whether the reduced expression of *EDF-1* transcript is necessary for HUVEC differentiation *in vitro*, the analogy with the *D. discoideum* pathway suggests that EDF-1 may play a role in the regulation of human endothelial cell differentiation.

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