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 (multipeptid-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 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 [α-32P]dCTP using the following primers: 5′-TCT GGG AAC CGG-3′ and 5′-GGG TCG CGA ACA-3′. 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 CLUSTALW 1.7 programs made available by Baylor College of Medicine.

**Plasmid Construction—Antisense pMEXNeo-αEDF** was constructed by inserting BamHI- and KpnI-cut EDF-1 into the eukaryotic expression vector pMEXNeo (11). The correct sequence and orientation of the construct were confirmed by sequencing.
To produce EDF-1 bearing six consecutive histidine residues (His<sub>6</sub>-EDF-1) in Escherichia coli, a BamHI-KpnI EDF-1 fragment was obtained by PCR and cloned into pQE30 (QIAGEN Inc.). The oligomers used were as follows: 5'-CTAGATCCGCCATGGCCAGAGAC-3' (sense) and 5'-CTAGTACCGCACTATTCGAGGAAAAGC-3' (antisense). The correct sequence was confirmed by sequencing. Recombinant His<sub>6</sub>-EDF-1 was purified using nickel-immobilized resin (QIAGEN Inc.).

**Immunological Methods**—Antiserum against EDF-1 was prepared using His<sub>6</sub>-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-PAGE.
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 [35S]methionine in methionine-free medium for 6 h. Cells were then washed, lysed in ice-cold radiommune 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 μM) 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/cm²) in growth medium. At various times, 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 mM guanidinium isothiocyanate. RNA was purified as described (15). RNA was electrophoresed on a 1% agarose gel containing 2.2M formaldehyde, diarganated 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 x 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. 1A) (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 NH2-terminal region (Fig. 1B).

Sequence comparison of EDF-1 by the BLASTX program revealed 66% identity at the amino acid level to MBF1 (multiple-protein-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. 1C, these sequences and the homologous gene product of Saccharomyces cerevisiae have been aligned using the ClustalW program.

Evolutionary Conservation and Tissue Distribution of EDF-1—EDF-1 cDNA was used as a probe to hybridize a Southern blot containing EcoRI-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-
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. 4A, 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. 4B).

EDF-1 Expression in Differentiating Endothelial Cells—The possibility that EDF-1 is regulated in endothelial differentiation is suggested by the similarity between EDF-1 and H7, a developmentally regulated gene isolated in D. discoideum (18, 19) (Fig. 1C). 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. 5A). 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. 5B), thus suggesting that EDF-1 is down-regulated in the early phases of endothelial differentiation in vitro. Fig. 5C 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. 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.
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 [35S]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.
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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REFERENCES

1. Folkman, J. (1995) Nat. Med. 1, 27–31
2. Risau, W. (1997) Nature 386, 671–674
3. Maciag, T., Kadish, J., Wilkins, L., Stemerman, M. B., and Weinstein, R. (1982) J. Cell Biol. 94, 511–520
4. Risau, W. (1995) FASEB J. 9, 926–933
5. Maciag, T. (1990) in Advances in Oncology (DeVita, V., and Rosenberg, S., eds) pp. 83–96, J. B. Lippincott Co., Philadelphia
6. Ingber, D. E., and Folkman J. (1989) J. Cell Biol. 109, 317–330
7. Hofman, F. M., Wright, A. D., Dohadwala, M. M., Wong-Staal, F., and Walker, S. M. (1993) Blood 82, 2774–2780
8. Albini, A., Barillari, G., Benelli, R., Gallo, R. C., and Ensoli, B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4838–4842
9. Albini, A., Soldi, R., Giuncaglini, D., Giraudou, K., Benelli, R., Primo, L., Noonan, D., Salin, M., Camussi, G., Rockl, W., and Busaido, F. (1996) Nat. Med. 2, 1371–1375
10. Consalez, G. G., Corradi, A., Ciarmatorti, S., Bosso, S., M. Aligaretti, N., and Stattau, C. L. (1996) Trends Genet. 12, 455–456
11. Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T., and Barbacid, M. (1989) Mol. Cell. Biol. 9, 24–33
12. Takahashi, K., Sawasaki, Y., Hata, J., Mukai, K., and Goto, T. (1990) In Vitro Cell. Dev. Biol. 26, 265–274
13. Maier, J. A. M., Statuto, M., and Ragnotti, G. (1994) Mol. Cell. Biol. 14, 1845–1851
14. Zimrin, A., Villeponteau, B., and Maciag, T. (1995) Biochem. Biophys. Res. Commun. 213, 630–635
15. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Kozak, M. (1989) J. Cell Biol. 108, 229–241
17. Takemaru, K.-I., Li, F.-Q., Ueda, H., and Hirose, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7251–7256
18. Singleton, C. K., Manning, S. S., and Ken, R. (1989) Nucelic Acids Res. 17, 9679–9692
19. Singleton, C. K., Delube, R. L., Ken, R., Manning, S. S., and McPherson, C. E. (1991) Dev. Genet. 12, 88–97
20. Montesano, R., and Orii, L. (1985) Cell 42, 469–477
21. Dvorak, H. F., Harvey, V. S., Estrella, P., Brown, L. F., McDonagh, J., and Dvorak A. M. (1987) Lab. Invest. 57, 675–686
22. Scanlon, K. J., Ohta, Y., Ishida, H., Kijima, H., Ohkawa, T., Kaminaski, A., Tsui, J., Horng, G., and Kashani-Sabet, M. (1995) FASEB J. 9, 1288–1296
23. Zhang, Y. M., Bachman, S., Hemmer, C., Van Luunzen, J., Von Stemm, A., Kern, P., Dietrich, M., Ziegler, R., Waldherr, R., and Nawroth, P. P. (1994) Am. J. Pathol. 144, 51–59
24. Cavallaro, U., Gasparini, G., Seria, M., and Maier, J. A. M. (1996) AIDS 10, 1211–1219
25. Jaye, M., McMahon, E., Drohan, W., Tong, B., Deuel, T., and Maciag, T. (1985) Science 228, 882–885
26. Hla, T., and Maciag, T. (1990) J. Biol. Chem. 265, 9308–9313
27. Lee, M.-J., Van Brocklyn, J. R., Thangada, S., Liu, C. H., Hand, A. R., Menzel, S., Spiegel, S., and Hla, T. (1998) Science 279, 1552–1555
28. Zimrin, A., Pepper, M. S., McMahon, G. A., Nguyen, F., Montesano, R., and Maciag, T. (1996) J. Biol. Chem. 271, 32499–32502
29. Vogel, J., Hinrichs, S. H., Reynolds, R. K., Luciw, P. A., and Jay, G. (1988) Nature 335, 506–511
30. Sturzl, M., Brandtstetter, H., and Roth W. K. (1992) AIDS Res. Hum. Retroviruses 8, 1753–1763
31. Kim, U., Qin, X.-F., Gong, S., Stevens, S., Luo, Y., Mussenzweig, M., and Roeder, R. G. (1996) Nature 383, 542–547
32. Brownell, J. E., Zhou, J., Hanali, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996) Cell 84, 843–851
33. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakataki, Y. (1996) Cell 87, 953–959
34. Devreotes, P. (1989) Science 245, 1054–1058

Endothelial Differentiation-related Factor