Activin A/Erythroid Differentiation Factor Is Induced during Human Monocyte Activation

By Marja Erämaa,*‡ Mikko Hurme,* Ulf-Håkan Stenman,§ and Olli Ritvos*‡§

From the *Departments of Bacteriology and Immunology, and ‡Pathology, University of Helsinki; and the §Departments of Obstetrics and Gynecology, Helsinki University Central Hospital, 00290 Helsinki, Finland

Summary

Activin A/erythroid differentiation factor (EDF), a dimeric polypeptide hormone composed of two β_A subunits, regulates growth and erythroid differentiation of human hematopoietic progenitor and erythroleukemia cells. We have identified activated human peripheral blood monocytes as a natural source of activin A/EDF. In these cells, lipopolysaccharide (LPS) induced rapidly the expression of the β_A subunit mRNAs through protein kinase C–dependent transcriptional regulation. The β_A subunit mRNA expression was also increased by 1,25-dihydroxyvitamin D_3, an inducer of macrophage maturation of monocytes. Western analysis with an anti-β_A antibody and an erythroid differentiation bioassay confirmed that the conditioned media of LPS-activated monocytes contained the activin A/EDF protein. We suggest that monocyte/macrophage–derived activin A/EDF may not only modulate hematopoiesis but may also act as a mediator molecule in the diverse physiologic and pathogenetic events in which these cells are involved.

Erythroid differentiation factor (EDF) is a polypeptide that exhibits potent differentiation-inducing activity toward cultured erythroleukemia cells (1, 2) and enhances the growth of normal erythroid precursor cells both in vitro (2, 3) and in vivo (4). This protein, composed of two identical covalently linked 14-kD subunits, was originally found in the culture fluid of human leukemia THP-1 cell line (1). Further characterization by cDNA cloning and sequencing revealed that EDF is encoded by the same mRNA as that of the β_A subunit of inhibin A and activin A, gonadal hormones that suppress and enhance, respectively, the secretion of pituitary follicle–stimulating hormone (5, 6). Inhibins consist of an α subunit, and one of the two β subunits (β_A or β_B) and activins are homo- or heterodimers of the β subunits (6).

Although inhibin subunit gene expression occurs in rat bone marrow and spleen (7), the natural cellular source of activin or inhibin in the hematopoietic system has remained obscure. Activin A/EDF has been shown to be expressed in several human myeloid leukemia cell lines (1, 8), but no data on its production by normal myeloid cells have been reported. We now show that activated human peripheral blood monocytes express the β_A subunit mRNA and secrete the immunoreactive and bioactive activin A/EDF protein.

Materials and Methods

Cell Cultures. Human peripheral blood monocytes were isolated from leukocyte-rich buffy coats obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland) by centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) and 1-h adherence to plastic dishes, and were cultured in RPMI 1640 containing 10% heat-inactivated human AB serum (from the same source as buffy coats), 10 mM Hepes, 2 mM glutamine, and antibiotics. About 90% of the adherent cells were monocytes, as demonstrated by standard morphological criteria or monocyte-specific antibodies (data not shown). The cells were stimulated with PMA (Sigma Chemical Co., St. Louis, MO), LPS (*Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI), and VD_3 (Hoffman-La Roche, Inc., Nutley, NJ), or treated with protein kinase inhibitors HA1004 and H7 (Seigaku America, Inc., St. Petersburg, FL) for indicated time periods. All experiments were repeated at least twice.

RNA Analyses. Total RNA was isolated by guanidine isothiocyanate/CsCl, and 30 μg of RNAs was run in 1.2% formaldehyde-agarose gels, and transferred on nylon membranes according to standard methods (9). In vitro transcribed [^{32}P]UTP-labeled antisense cRNA probes from full-length human inhibin α, and β_A and β_B subunit cDNA templates (10) in pGEM-4Z plasmids (Promega-Biotec, Madison, WI) were hybridized to the filters and high-stringency washes performed as previously described (11). As a control for RNA loading, filters were hybridized to human β-actin cDNA probe, which showed no substantial variation of RNA amounts between different lanes (not shown). For nuclear run-off transcription assay, nuclei were isolated from 2 × 10^7 monocytes induced for 4 h with PMA and used for a 20-min transcription reaction according to Greenberg and Ziff (12). Slot blots with 2 μg of each plasmid were hybridized to 2.0 × 10^6 cpm/ml of ^{32}P-labeled nuclear RNAs for 48 h, washed, and RNase A treated as previously described (12). Reverse-transcription (RT)-

PCR analyses from 1 μg of monocyte total RNAs were performed essentially as described by Rappolee et al. (13). 40 95°C (30 s)–54°C (30 s)–72°C (90 s) PCR cycles were performed in a 50-μl vol with primers 5'-GTTTGCCGAGTCAGGAACAG-3' and 5'-GAGGTT-GGCAAAGGGGCTATGGCCCCGCAT-3' for human β_A subunit (10), and 5'-CCCAGGCACCAGGGCGTGAT-3' and 5'-TCAAA-CATGATCTGGGTCAT-3' for human β-actin (14). The monocyte β_A PCR product was subcloned into pCR1000 vector (InVitrogen, San Diego, CA) and its sequence determined by the dideoxy method.

Western Analysis. For production of anti-β_A Abs, the synthetic peptide Cys-Gly-Gly-(β_A 93–105) was coupled to KLH (custom peptide synthesis and coupling by Multiple Peptide Systems, San Diego, CA) and the conjugate was used to immunize rabbits with 400 μg of the Ag first in CFA and then in IFA at 6-wk intervals. For Western analysis of monocyte-secreted activin-A/EDF, culture media (containing 1% AB serum) conditioned for 48 h with un-stimulated or LPS-stimulated cells (cells cultured at 10⁶/ml) were concentrated with the use of Centricon 10 (Amicon, Beverly, MA). Duplicate 30-μl aliquots equivalent to 500 μl of medium (~180 μg of total proteins) were run on reducing SDS-PAGE and trans-ferred to nitrocellulose. Blots were incubated overnight at 4°C with a 1:2,000 dilution of the rabbit no. 560 antiβ_A(93–105) antipep-tide antiserum. The immunoreactive bands were visualized with ^{125}I-protein A.

Activin A/EDF Bioassay. 1-ml test samples of monocyte-con-ditioned media were concentrated to 60 μl as above and divided into 30-μl heat-treated (5 min at 95°C) and nontreated aliquots. 10 μl of test samples and their threefold serial dilutions was added in duplicate to 10⁴ target K562 cells in 90 μl RPMI 1640-10% FCS. Dilutions of recombinant human (rh)-activin A/EDF (0.1–100 ng/ml) were used as positive controls. Cells were cultured for 4 d at 37°C, and thereafter, hemoglobin-positive cells were stained with benzidine (15) and scored under microscope.

Results and Discussion

Human peripheral blood monocytes did not spontaneously express any of the inhibin subunit mRNAs in our culture conditions, which are strictly free of LPS. By contrast, in RNA from monocytes activated 4 h with LPS, our β_A subunit cRNA probe detected transcripts of molecular size similar to those previously described in other human tissues (8, 16). PMA, a regulator of protein kinase C (17), mim-icked the effect of LPS on β_A mRNA expression (Fig. 1 A). VD₃, which induces macrophage maturation of monocytes (18), also induced β_A subunit mRNA expression at 24 h after stimulation but not yet at 4 h (Fig. 1 A). The induction of β_A mRNA with LPS was rapid and transient, as the ex-pression level declined in 24-h samples as compared with those at 4 h (Fig. 1 A). At 24 h, PMA-induced β_A mRNA ex-pression was about the same as at 4 h (Fig. 1 A). The effect of LPS on β_A mRNAs was inhibited by H7, a potent in-hibitor of both protein kinases A and C, but not by HA1004, which preferentially inhibits A-kinase with only a weak effect on C-kinase (19). This suggests that LPS mediates its effect on monocyte β_A mRNA expression through the protein ki-nase C–dependent signaling pathway. Nuclear run-off assay with human monocytes showed that PMA activates directly β_A subunit gene transcription within 4 h (Fig. 1 B). RT-PCR analysis of RNA of monocytes stimulated for 4 h with
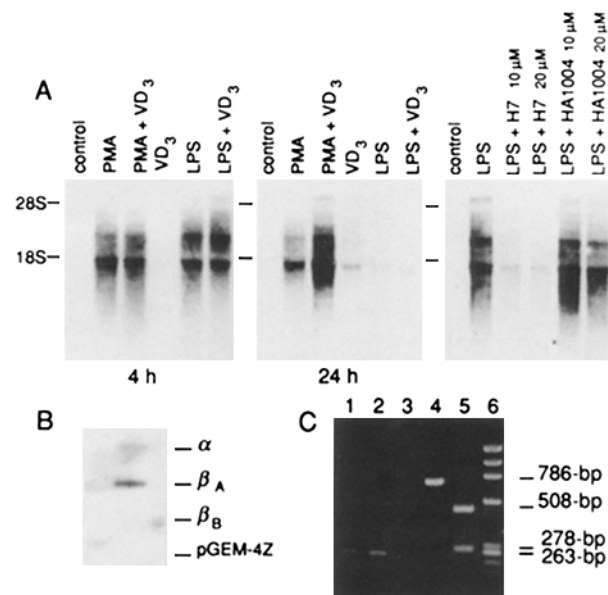

Figure 1. Northern analysis, nuclear run-off transcription assay, and RT-PCR analysis of activin A/EDF β_A subunit mRNA expression in human monocytes. (A) *Left,* Northern analysis of β_A subunit mRNA in cultured monocytes stimulated 4 h with PMA (10 ng/ml), LPS (100 ng/ml), VD₃ (10⁻⁸ M), and their different combinations: *middle,* same stimulations as in the *left,* but for a 24-h incubation period; *right,* Northern analysis of β_A subunit mRNA in cultured monocytes stimulated 4 h with LPS alone or in combination with H7 or HA1004 (at 10 and 20 μM). Migration of the 28S and 18S rRNAs is indicated. (B) Autoradiogram of ³²P-labeled nuclear run-off transcripts from nuclei of monocytes stimulated for 4 h with PMA (10 ng/ml) hybridized to α, β_A, and β_B subunit cDNAs in pGEM-4Z plasmid or plasmid alone bound to nitrocellulose filter. (C) RT-PCR analyses of human β-actin and β_A subunit mRNAs in monocytes. The figure shows the 263-bp β-actin PCR product used as positive con-trol for RNA integrity in unstimulated (lane 1) and PMA-stimulated (10 ng/ml; 4 h; lane 2) monocytes; absence (lane 3) and presence (lane 4) of the 786-bp β_A PCR product in unstimulated and PMA-stimulated mono-cytes, respectively; lane 5, PstI digest of DNA in lane 4 yielding the ex-pected 508- and 278-bp fragments; lane 6, HaeIII digest of ϕX174RF DNA used as molecular weight marker.

PMA yielded the expected 786-bp human β_A PCR product (Fig. 1 C), which was identified as identical to previously described human β_A sequences (5, 10) by restriction anal-ysis (Fig. 1 C) and DNA sequencing (not shown).

By Western analysis we found that LPS-activated mono-cytes cultured for 48 h secrete a protein with a molecular mass ~14 kD that upon reduction reacts specifically with our rabbit no. 560 anti-β_A (93–105) antiserum (Fig. 2). Its comigration with a rh-activin A/EDF standard further indi-cates that it represents monocyte-secreted activin A/EDF pro-tein (Fig. 2). From these Western analyses, we estimate that LPS-stimulated monocytes secrete ~20–40 ng/ml/10⁶ cells of activin A/EDF during a 48-h culture. We also measured the activin A/EDF bioactivity of monocyte-conditioned media with the human erythroleukemia K562 cell differentiation assay (2, 20). The same concentrated conditioned media that were positive in Western analysis induced effectively hemo-globin synthesis in K562 cells (Table 1). Heat treatment
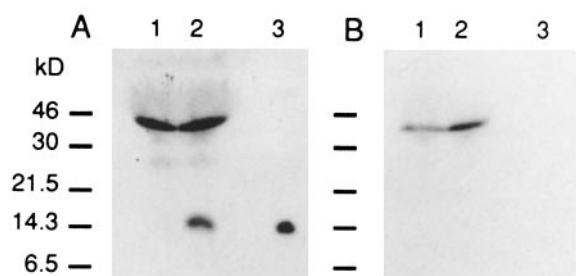
Figure 2. Western analysis of activin A/EDF β_A subunit protein in human monocyte-conditioned media. (A) Concentrated media conditioned for 48 h with unstimulated (lane 1), LPS-stimulated monocytes (lane 2), and rh-activin A/EDF (10 ng) (lane 3) were run on reducing 15% SDS-PAGE, and transferred to nitrocellulose filter probed with the no. 560 antiβ_A(93–105) antipeptide antiserum and ^{125}I-protein A. One lane between lanes 2 and 3 has been left empty to avoid contamination during gel loading. (B) A filter identical to that in A probed with the same antiβ_A(93–105) antipeptide antiserum preabsorbed with the peptide antigen. The nonspecific binding of the antiserum at the 45–50-kD range is probably due to protein overloading of lanes 1 and 2 (human AB serum proteins). The migration of molecular mass markers is indicated.

Table 1. *Effects of Monocyte-conditioned Media on the Differentiation of K562 Cells*

| | EDF titer | |
Sample	No treatment	Heat treatment
	U/ml	
rh-activin A/EDF (0.3 ng/ml)	1	ND
LPS (0.001–10 μg/ml)	0	ND
Unstimulated monocyte medium	0	6
LPS-stimulated monocyte medium	60	180

The 48-h-conditioned media were tested with and without heat treatment (5 min at 95°C). The EDF titer (expressed as K562 EDF U/ml) is defined as the reciprocal of the highest dilution in 1 ml at which differentiation of the K562 cells is still visible compared with complete RPMI 1640 (20). In our bioassay one K562 EDF U/ml is equal to 0.3 ng/ml of rh-activin A/EDF, being consistent with previously reported activity of activins in this assay (2, 20). We obtained reproducibly ∿40–45% differentiation of the target cells with the maximal effective concentration of rh-activin A/EDF (100 ng/ml). LPS did not affect K562 hemoglobin synthesis at any concentrations used, nor did the nonconditioned control media. Spontaneous differentiation accounted for only 4–7% in this assay and is defined as zero EDF U/ml. The data are from three experiments all giving similar results.

markedly increased the bioactivity of the samples, possibly due to the conversion of latent proactivin A to the mature active hormone. The EDF titers of LPS-stimulated monocyte-conditioned nontreated and heat-treated media were ∿60 and ∿180 EDF U/ml/10[6] cells, respectively, corresponding to ∿20 and ∿60 ng/ml/10[6] cells of activin A/EDF when compared with the rh-activin A/EDF standard (Table 1). The conditioned media of nonactivated monocytes also contained minute amounts of EDF activity (EDF titer, 6 U/ml/10[6] cells) but only after heat treatment. Although activated monocytes may secrete other factors able to induce erythrodifferentiation in K562 cells, our findings suggest that activin A/EDF accounts for most of this activity as the results of the Western and bioassay analyses correlated fairly well.

The ability of human monocyte/macrophages to express the β_A mRNA and secrete the immunoreactive and bioactive activin A/EDF protein provides insight into the possible cellular origin of activin A/EDF during human hema-

topoiesis. This report and the recent finding that murine bone marrow stromal cell lines produce activin A/EDF after PMA, TNF, and IL-1 stimulation (21) suggest that both these cell types may play an important role in the physiology of activin A/EDF-regulated hematopoiesis. These two cell types are likely to contribute to the activin A/EDF bioactivity recently observed in crude murine bone marrow cultures (22). As peripheral blood monocytes mature to tissue macrophages and scatter throughout most organs of the body, activin A/EDF may have a wider range of biological effects than we presently know this versatile differentiation/growth factor has.

References

1. Eto, Y., T. Tsuji, M. Takezawa, S. Takano, Y. Yokogawa, and H. Shibai. 1987. Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. *Biochem. Biophys. Res. Commun.* 142:1095.

2. Yu, J., L. Shao, V. Lemas, A. Yu, J. Vaughan, J. Rivier, and W. Vale. 1987. Importance of FSH-releasing protein and inhibin in erythrodifferentiation. *Nature (Lond.).* 330:765.

3. Broxmeyer, H.E., L. Lu, S. Cooper, R.H. Schwall, A.J. Mason, and K. Nikolics. 1988. Selective and indirect modulation of human multipotential and erythroid hematopoietic progenitor cell proliferation by recombinant human activin and inhibin. *Proc. Natl. Acad. Sci. USA.* 85:9052.

4. Shiozaki, M., R. Sakai, M. Tabuchi, Y. Eto, M. Kosaka, and H. Shibai. 1989. In vivo treatment with erythroid differentiation factor (EDF/activin A) increases erythroid precursors (CFU-E and BFU-E) in mice. *Biochem. Biophys. Res. Commun.* 165:1155.

5. Murata, M., Y. Eto, H. Shibai, M. Sakai, and M. Muramatsu. 1988. Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin β_A chain. *Proc. Natl. Acad. Sci. USA.* 85:2434.

6. Vale, W., A. Hsueh, C. Rivier, and J. Yu. 1990. The inhibin/activin family of hormones and growth factors. *Handb. Exp. Pharmacol.* 95:211.

7. Meunier, H., C. Rivier, R.M. Evans, and W. Vale. 1988. Gonadal and extragonadal expression of inhibin α, β_A, and β_B subunits in various tissues predicts diverse functions. *Proc. Natl. Acad. Sci. USA.* 85:247.

8. Takahashi, S., T. Yamashita, Y. Eto, H. Shibai, K. Miyamoto, and E. Ogata. 1990. Inducible expression of activin A/erythroid differentiation factor in HL-60 cells. *Biochem. Biophys. Res. Commun.* 167:654.

9. Sambrook, J., E.F. Fritch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, vol. 1-3. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

10. Mason, A.J., H.D. Niall, and P.H. Seeburg. 1986. Structure of two human ovarian inhibins. *Biochem. Biophys. Res. Commun.* 135:957.

11. Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 12:7035.

12. Greenberg, M.E., and E.B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature (Lond.).* 311:433.

13. Rappolee, D.A., A. Wang, D. Mark, and Z. Werb. 1989. Novel method for studying mRNA phenotypes in single or small numbers of cells. *J. Cell. Biochem.* 39:1.

14. Ponte, P., S.-Y. Ng, J. Engel, P. Gunning, and L. Kedes. 1984. Evolutionary conservation in the untranslated region of actin mRNAs: DNA sequence of a beta-actin cDNA. *Nucleic Acids Res.* 12:1687.

15. Orkin, S.H., F.I. Harosi, and P. Leder. 1975. Differentiation in erythroleukemic cells and their somatic hybrids. *Proc. Natl. Acad. Sci. USA.* 72:98.

16. Davis, S.R., Z. Krosowski, R.I. McLachlan, and H.G. Burger. 1987. Inhibin gene expression in the human corpus luteum. *J. Endocrinol.* 115:R21.

17. Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular signalling. *Nature (Lond.).* 334:661.

18. Kreutz, M., and R. Andreesen. 1990. Induction of human monocyte to macrophage maturation in vitro by 1,25-dihydroxyvitamin D_3. *Blood.* 76:2457.

19. Hidaka, H., M. Inagaki, S. Kawamota, and Y. Sasaki. 1984. Isoquinoline sulfonamides, novel and potent inhibitors of cyclic nucleotide-dependent protein kinase and protein kinase C. *Biochemistry.* 23:5036.

20. van den Eijnden-Van Raaij, A.J.M., E.J.J. van Zoelent, K. van Nimmen, C.H. Koster, G.T. Snoek, A.J. Durston, and D. Huylebroeck. 1990. Activin-like factor from a Xenopus laevis cell line responsible for mesoderm induction. *Nature (Lond.).* 345:732.

21. Yamashita, T., S. Takahashi, and E. Ogata. 1992. Expression of activin A/erythroid differentiation factor in murine bone marrow stromal cells. *Blood.* 79:304.

22. Shiozaki, M., R. Sakai, M. Tabuchi, T. Nakamura, K. Sugino, H. Sugino, and Y. Eto. 1992. Evidence for the participation of endogenous activin A/erythroid differentiation factor in the regulation of erythropoiesis. *Proc. Natl. Acad. Sci. USA.* 89:1553.