

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

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### **Summary**

Activin A/erythroid differentiation factor (EDF), a dimeric polypeptide hormone composed of two  $\beta_A$  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  $\beta_A$  subunit mRNAs through protein kinase C-dependent transcriptional regulation. The  $\beta_A$  subunit mRNA expression was also increased by 1,25-dihydroxyvitamin D<sub>3</sub>, an inducer of macrophage maturation of monocytes. Western analysis with an anti- $\beta_A$  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.

**E**rythroid 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  $\beta_A$  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  $\alpha$  subunit, and one of the two  $\beta$  subunits ( $\beta_A$  or  $\beta_B$ ) and activins are homo- or heterodimers of the  $\beta$  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  $\beta_A$  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<sub>3</sub> (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  $\mu$ g of RNAs was run in 1.2% formaldehyde-agarose gels, and transferred on nylon membranes according to standard methods (9). In vitro transcribed [<sup>32</sup>P]UTP-labeled antisense cRNA probes from full-length human inhibin  $\alpha$ , and  $\beta_A$  and  $\beta_B$  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  $\beta$ -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  $2 \times 10^7$  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  $\mu$ g of each plasmid were hybridized to  $2.0 \times 10^6$  cpm/ml of <sup>32</sup>P-labeled nuclear RNAs for 48 h, washed, and RNase A treated as previously described (12). Reverse-transcription (RT)-

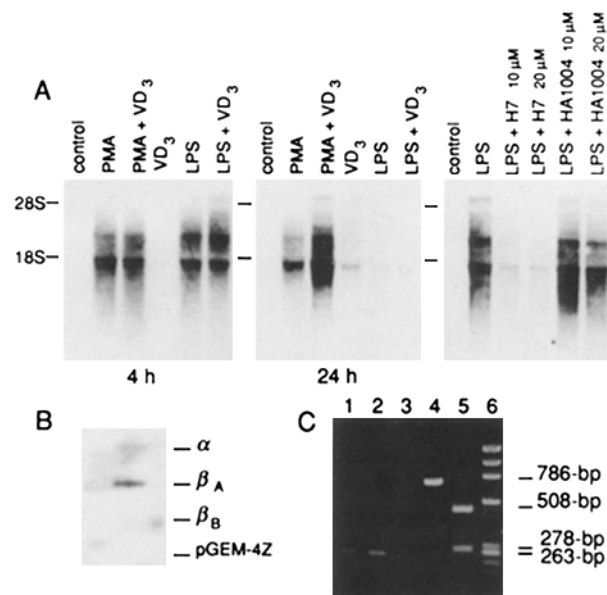
PCR analyses from 1  $\mu\text{g}$  of monocyte total RNAs were performed essentially as described by Rappolee et al. (13). 40 95°C (30 s)–54°C (30 s)–72°C (90 s) PCR cycles were performed in a 50- $\mu\text{l}$  vol with primers 5'-GTTTGGCCGAGTCACGGAACAG-3' and 5'-GAGGTTGGCAAAGGGGCTATGGCCCCGCAT-3' for human  $\beta_A$  subunit (10), and 5'-CCCAGGCACCAGGGCGTGAT-3' and 5'-TCAAA-CATGATCTGGGTCAT-3' for human  $\beta$ -actin (14). The monocyte  $\beta_A$  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- $\beta_A$  Abs, the synthetic peptide Cys-Gly-Gly-( $\beta_A$  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  $\mu\text{g}$  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 unstimulated or LPS-stimulated cells (cells cultured at  $10^6/\text{ml}$ ) were concentrated with the use of Centricon 10 (Amicon, Beverly, MA). Duplicate 30- $\mu\text{l}$  aliquots equivalent to 500  $\mu\text{l}$  of medium ( $\sim 180$   $\mu\text{g}$  of total proteins) were run on reducing SDS-PAGE and transferred to nitrocellulose. Blots were incubated overnight at 4°C with a 1:2,000 dilution of the rabbit no. 560 anti- $\beta_A$ (93–105) antipeptide antiserum. The immunoreactive bands were visualized with  $^{125}\text{I}$ -protein A.

**Activin A/EDF Bioassay.** 1-ml test samples of monocyte-conditioned media were concentrated to 60  $\mu\text{l}$  as above and divided into 30- $\mu\text{l}$  heat-treated (5 min at 95°C) and nontreated aliquots. 10  $\mu\text{l}$  of test samples and their threefold serial dilutions was added in duplicate to  $10^4$  target K562 cells in 90  $\mu\text{l}$  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

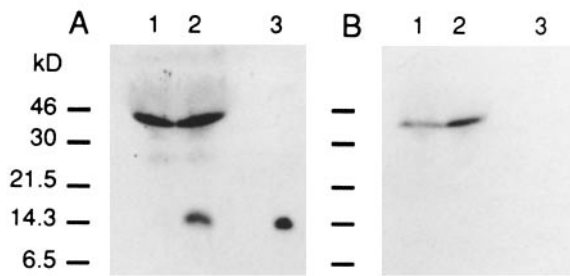
Human peripheral blood monocytes did not spontaneously express any of the inhibin subunit mRNAs in our culture conditions, which are strictly free of LPS. By contrast, in RNA from monocytes activated 4 h with LPS, our  $\beta_A$  subunit cRNA probe detected transcripts of molecular size similar to those previously described in other human tissues (8, 16). PMA, a regulator of protein kinase C (17), mimicked the effect of LPS on  $\beta_A$  mRNA expression (Fig. 1 A). VD<sub>3</sub>, which induces macrophage maturation of monocytes (18), also induced  $\beta_A$  subunit mRNA expression at 24 h after stimulation but not yet at 4 h (Fig. 1 A). The induction of  $\beta_A$  mRNA with LPS was rapid and transient, as the expression level declined in 24-h samples as compared with those at 4 h (Fig. 1 A). At 24 h, PMA-induced  $\beta_A$  mRNA expression was about the same as at 4 h (Fig. 1 A). The effect of LPS on  $\beta_A$  mRNAs was inhibited by H7, a potent inhibitor 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  $\beta_A$  mRNA expression through the protein kinase C-dependent signaling pathway. Nuclear run-off assay with human monocytes showed that PMA activates directly  $\beta_A$  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  $\beta_A$  subunit mRNA expression in human monocytes. (A) *Left*, Northern analysis of  $\beta_A$  subunit mRNA in cultured monocytes stimulated 4 h with PMA (10 ng/ml), LPS (100 ng/ml), VD<sub>3</sub> ( $10^{-8}$  M), and their different combinations: *middle*, same stimulations as in the *left*, but for a 24-h incubation period; *right*, Northern analysis of  $\beta_A$  subunit mRNA in cultured monocytes stimulated 4 h with LPS alone or in combination with H7 or HA1004 (at 10 and 20  $\mu\text{M}$ ). Migration of the 28S and 18S rRNAs is indicated. (B) Autoradiogram of  $^{32}\text{P}$ -labeled nuclear run-off transcripts from nuclei of monocytes stimulated for 4 h with PMA (10 ng/ml) hybridized to  $\alpha$ ,  $\beta_A$ , and  $\beta_B$  subunit cDNAs in pGEM-4Z plasmid or plasmid alone bound to nitrocellulose filter. (C) RT-PCR analyses of human  $\beta$ -actin and  $\beta_A$  subunit mRNAs in monocytes. The figure shows the 263-bp  $\beta$ -actin PCR product used as positive control 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  $\beta_A$  PCR product in unstimulated and PMA-stimulated monocytes, respectively; lane 5, PstI digest of DNA in lane 4 yielding the expected 508- and 278-bp fragments; lane 6, HaeIII digest of  $\phi\text{X174RF}$  DNA used as molecular weight marker.

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

By Western analysis we found that LPS-activated monocytes cultured for 48 h secrete a protein with a molecular mass  $\sim 14$  kD that upon reduction reacts specifically with our rabbit no. 560 anti- $\beta_A$  (93–105) antiserum (Fig. 2). Its comigration with a rh-activin A/EDF standard further indicates that it represents monocyte-secreted activin A/EDF protein (Fig. 2). From these Western analyses, we estimate that LPS-stimulated monocytes secrete  $\sim 20$ –40 ng/ml/ $10^6$  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 hemoglobin synthesis in K562 cells (Table 1). Heat treatment



**Figure 2.** Western analysis of activin A/EDF  $\beta_A$  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 anti $\beta_A$ (93–105) antipeptide antiserum and  $^{125}\text{I}$ -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 anti $\beta_A$ (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.

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  $\sim 60$  and  $\sim 180$  EDF U/ml/ $10^6$  cells, respectively, corresponding to  $\sim 20$  and  $\sim 60$  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  $\beta_A$  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-

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

Sample	EDF titer	
	No treatment	Heat treatment
rh-activin A/EDF (0.3 ng/ml)	1	ND
LPS (0.001–10 $\mu\text{g}/\text{ml}$ )	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  $95^\circ\text{C}$ ). 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  $\sim 40$ – $45\%$  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.

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.

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