RAPID COMMUNICATION

Expression of Activin A/Erythroid Differentiation Factor in Murine Bone Marrow Stromal Cells

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Accumulating evidence suggests that activin A/erythroid differentiation factor (EDF), a homodimer of βA chain, is a physiologic hematopoietic factor, particularly of erythroid lineage. Media conditioned by phorbol myristate acetate (PMA)-stimulated murine bone marrow stromal cell lines, MC3T3-G2/PA6 and ST2, contained activin A/EDF, assayed as the activity inducing erythroid differentiation of an activin A/EDF-responsive murine erythroleukemia cell clone F5. Follistatin, a protein specifically binding to activin A/EDF, abolished this erythroid differentiation. Northern blot analysis showed that PMA rapidly increased βA chain messenger RNA levels in MC3T3-G2/PA6 and ST2 cells. In a search for natural stimulators, we found that tumor necrosis factor-α, in itself and synergistically with interleukin-1β, induced activin A/EDF production in both cell lines. These results indicate that stromal cells produce activin A/EDF in bone marrow.

From THE CULTURE supernatant of the phorbol ester-treated human monocyctic leukemia cell line THP-1, Eto et al purified a protein that induces erythroid differentiation in a murine erythroleukemia (MEL) cell line F5. They designated this protein as erythroid differentiation factor (EDF). Subsequent study showed that EDF is identical to activin A, which was originally purified from porcine ovarian fluid as a protein that enhances the secretion of follicle-stimulating hormone from cultured pituitary cells. This protein is a homodimer of the βA chain with about 40% homology to a subunit of transforming growth factor β-1. Several lines of evidence indicate that activin A/EDF is an important hematopoietic factor; it induces hemoglobin (Hb) synthesis in the human erythroleukemic cell line K562 as well as in MEL cells and enhances the growth of normal erythroid precursor cells not only in culture systems, but also when administered to rodents in vivo. These observations strongly suggest an erythropoietic action for activin A/EDF. Recent reports from our laboratory and that of others showed that activin A/EDF also induces megakaryocytic differentiation in murine leukemic cells. In addition, Brommeyer et al reported a stimulating action on multipotential hematopoietic precursors. Although βA chain messenger RNA (mRNA) was shown to be present in bone marrow, little is known of the production of activin A/EDF in hematopoietic tissue except that several myeloid cell lines were found to produce this factor.

Increasing attention has focused on the role of bone marrow stromal cells in the regulation of hematopoiesis. A number of hematopoietic colony-stimulating factors produced by stromal cells include macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), interleukin-6 (IL-6), and IL-7. IL-11 and stem cell factor/c-kit ligand were identified as novel stromal cell factors. These factors presumably play an important role in stromal cell-mediated regulation of hematopoiesis. We now report that murine bone marrow stromal cell lines, ST2 and MC3T3-G2/PA6 (PA6), can produce activin A/EDF. These findings provide new insight into the physiologic actions of activin A/EDF and the function of bone marrow stromal cells.

MATERIALS AND METHODS

Materials. Phorbol myristate acetate (PMA) was obtained from Sigma (St Louis, MO). Follistatin, purified from porcine ovary, was a kind gift from Dr Y. Eto (Ajinomoto Co, Kawasaki, Japan) with the permission of Dr H. Sugino (Institute of Physical and Chemical Research, Saitama, Japan). Recombinant human (rh) activin A/EDF, IL-1β, and tumor necrosis factor-α (TNF-α) were kindly provided by Ajinomoto Co, Otsuka Pharmaceutical Co (Tokushima, Japan), and Santoco Co (Tokyo, Japan), respectively.

Cell culture. ST2 and PA6 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and Dulbecco’s modified Eagle’s medium with FCS, respectively, in a humidified atmosphere of 5% CO₂. Both cell lines were passaged every 3 to 4 days at a split ratio of 1:4, using 0.02% trypsin containing 0.25% EDTA. MEL cell clone F5 was originally established from a DDS mouse by Ikawa et al and subcloned by Eto et al as a clone highly sensitive to activin A/EDF in erythroid differentiation. These cells were cultured in Ham’s F-12 supplemented with 10% FCS and passed every 3 to 4 days at a cell density of about 5 × 10⁵ cells/mL.

Preparation of conditioned medium (CM). ST2 or PA6 cells at 4-day culture were seeded at 1 × 10⁶ cells per well in 1 mL of medium in 24-well plates. The cells were cultured overnight at 37°C and then media were replaced by 0.5 mL of the fresh media containing various agents. CM was collected at indicated times and used for the determination of activin A/EDF activity.

Assay of activin A/EDF activity. This activity was assayed as described elsewhere. In brief, activin A/EDF-responsive MEL cell clone F5 was cultured with various concentrations of samples at 1 × 10⁴ cells per well in 100 μL of medium in 96-well plates and the percentages of Hb-positive cells were scored after a 5-day culture.

Northern blot analysis. Northern blot analysis of βA, βB, and α chain mRNA was performed as described previously. Human βA chain mRNA was performed as described previously.
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Figure 1. Production of activin A/EDF in stromal cells. (A) EDF activity in CM taken from the PMA-treated stromal cells. F5 cells were cultured in the presence of various concentrations of CM taken from (●) ST2 and (▲) PA6 cells treated with 100 nmol/L PMA for 96 hours. (B) Effects of follistatin on F5 cell erythroid differentiation. F5 cells were treated with 5 mol/L HMBA, 1 nmol/L rh activin A, or 25% CM of PMA-treated ST2 and PA6 cells in the presence (+) or absence (−) of 100 ng/mL follistatin. Values are the percentages of Hb-positive (Hb+) cells determined as described in Materials and Methods. Data are means ± SEM of triplicate experiments.

Rh activin A/EDF and CM taken from the PMA-treated stromal cells. F5 cells were cultured in the presence of various concentrations of CM taken from (●) ST2 and (▲) PA6 cells treated with 100 nmol/L PMA for 96 hours. (B) Effects of follistatin on F5 cell erythroid differentiation. F5 cells were treated with 5 mol/L HMBA, 1 nmol/L rh activin A, or 25% CM of PMA-treated ST2 and PA6 cells in the presence (+) or absence (−) of 100 ng/mL follistatin. Values are the percentages of Hb-positive (Hb+) cells determined as described in Materials and Methods. Data are means ± SEM of triplicate experiments.

RESULTS AND DISCUSSION

Rh activin A/EDF and CM taken from the PMA-treated PA6 and ST2 cells induced erythroid differentiation of F5 cells in a dose-dependent manner (Fig 1A). In both lines, activin A/EDF activity was constitutively expressed at very low levels, and PMA markedly augmented this expression. In media conditioned by PMA-treated ST2 and PA6 cells, the concentrations of activin A/EDF were estimated to be about 1 to 1.3 nmol/L and 0.5 to 0.8 nmol/L, respectively. To confirm that the erythroid differentiation of F5 cells induced by CM of the stromal cells depends on activin A/EDF, we examined the effects of follistatin, a gonadal protein with a molecular weight of 32 to 35 kd; this protein binds to activin A/EDF with a high affinity (kd = 590 pmol/L) and neutralizes its activity. As shown in Fig 1B, follistatin completely inhibited the erythroid differentiation induced by CM of the stromal cells, whereas it had little effect on that induced by a chemical differentiation inducer, hexamethylenebisacetamide (HMBA).

Figure 2 shows Northern blot analysis of mRNA of bone marrow stromal cells with βA chain cDNA. In both ST2 and PA6 cells, PMA markedly increased the levels of βA chain mRNA of 6.0 kb; a maximum was reached at 4 hours, followed by a decrease. We and other groups reported multiple species of βA chain mRNA. While species of 3 kb and 4.5 kb are the main forms in THP-1 monocytic cells, those of 6.0 kb and 4.5 kb are the main forms in HL-60 cells. On the other hand, 7.2-kb and 4.5-kb species are major forms in gonadal tissue. The molecular basis for this multiplicity of βA chain mRNA and its heterogeneity depending on cell types remains unknown. Differential splicing event or choice of a different polyadenylation signal might explain the existence of multiple mRNA species. The βA chain can form dimer proteins other than activin A/EDF, ie, activin AB (βA βB) and inhibin A (αβA). In no samples, was βB or α chain mRNA detected (data not shown), thereby indicating that neither activin AB nor inhibin A was expressed.

To search for natural inducers for activin A/EDF expression in stromal cells, we examined the effects of TNF-α and IL-1β, because these cytokines are known to stimulate bone marrow stromal cells. TNF-α alone induced a moderate expression of activin A/EDF activity in ST2 cells (Fig 3A). IL-1β in itself was deficient in this capacity, but it did enhance, synergistically, the effects of TNF-α. The responses to TNF-α or TNF-α plus IL-1β were evident after a lag time of 24 to 48 hours, whereas those to PMA were seen within 12 to 24 hours. Similar observations were made for PA6 cells (Fig 3B). The erythroid differentiation induced by these media were also inhibited by follistatin (data not shown).

Our results indicate that stromal cells produce activin A/EDF in bone marrow. The ST2 cell line was derived from the Whitlock-Witte-type long-term culture of murine bone marrow, while the PA6 cell line was developed from newborn mouse calvaria. Although both lines carry the potential to support long-term hematopoiesis, there are functional differences between these cells; ST2 cells but not PA6 cells can support lymphopoiesis by producing IL-7. The finding that such independent cell clones can produce activin A/EDF in a similar manner suggests that this factor plays a fundamental role in the function of stromal cells.

We show that PMA induces activin A/EDF production through an increase of its mRNA. This effect of PMA is probably mediated by the direct activation of protein kinase C. It was reported that IL-1 and TNF, inflammatory mediators secreted from monocytes/macrophages, stimulate CSF and IL-6 production in stromal cells. Our
Fig 2. Expression of βA chain mRNA in stromal cells. Confluent cultures of ST2 and PA6 cells were stimulated by 100 nmol/L PMA for the indicated time. Four micrograms of poly(A) RNA was isolated, electrophoresed, and blotted onto a nylon membrane filter, then the filter was hybridized with 32P-labeled cDNA probes for βA chain and β-actin. Other details are described in Materials and Methods.

Fig 3. Kinetics of activin A/EDF expression induced by PMA, TNF-α, and IL-1β. ST2 and PA6 cells were cultured with (C) 100 nmol/L PMA, (■) 10 ng/mL TNF-α, (▲) 1,000 U/mL IL-1β, (●) TNF-α + IL-1β, or (▲) control medium. CM was harvested at the indicated time. Values are the percentages of Hb-positive (Hb+) cells of F5 cells after culture with 25% CM, as described in Materials and Methods. Data are means ± SEM of triplicate experiments. Significance of the differences from control values was estimated by Student's t-test. *, P < .05. The results were reproducible in three separate experiments.
results suggest that these cytokines upregulate activin A/EDF production in stromal cells. Although the physiologic role of activin A/EDF in inflammatory states is unknown, it may compensate for the suppressive effects of inflammatory cytokines on erythropoiesis or may stimulate multipotential or megakaryocytic precursors. Alternatively, it is possible that this cytokine network is involved in steady state hematopoiesis. The kinetics of the IL-1/TNF-α/FN-mediated and cytokine network on erythropoiesis, thereby suggesting that these agents exert their actions via separate pathways.

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**REFERENCES**


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