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Evidence for the participation of endogenous activin A/erythroid differentiation factor in the regulation of erythropoiesis

(follicle-stimulating hormone-releasing protein/inhibin/burst-promoting activity/hemopoiesis/follistatin)

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ABSTRACT **Activin A/erythroid differentiation factor (EDF) is a human protein that induces differentiation of a murine erythroleukemia cell (the Friend cell). In this study, we demonstrate that endogenous activin A/EDF activity is present in murine bone marrow and spleen. In addition, this activity is secreted by bone marrow and spleen cells in primary culture. Administration of follistatin (a specific binding protein for activin A/EDF) to mice results in a decrease of erythroid progenitors in the bone marrow and spleen. These findings support the concept that activin A/EDF and follistatin have opposing actions in the regulation of erythropoiesis.**

Erythrocytes are generated from progenitor cells that are found in the bone marrow and, in the case of mice, also in spleen. The process of erythrocyte generation is called erythropoiesis, and it is regulated both positively and negatively by various humoral factors (1–4). The late stage of erythropoiesis is dependent on erythropoietin and is also influenced by several other factors, including testosterone, estrogens, and erythroid-potentiating activity, while the early stage of erythropoiesis is dependent on burst-promoting activity in addition to erythropoietin (2). Hemopoietic factors such as interleukin 3, granulocyte–macrophage colony-stimulating factor, and interleukin 9 are known to have burst-promoting activity (5–7), but it remains unclear whether they have a physiological role in supporting early erythropoiesis. To better understand the mechanisms of erythropoiesis, it is important to identify the physiological regulators of the early and late stages of erythrocyte development.

We recently identified a protein called erythroid differentiation factor (EDF) in the culture supernatant of a human leukemia cell line (THP-1), which acted as a differentiation inducer of murine erythroleukemia (Friend) cells (8). EDF is a member of the transforming growth factor β (TGF-β) peptide family and has been found to be identical to activin A (=FRP, follicle-stimulating hormone-releasing protein), a factor present in gonadal fluid that promotes follicle-stimulating hormone (follitropin) release from primary cultured pituitary cells (9–11). Activin A/EDF causes the *in vitro* differentiation of Friend cells in a dose-dependent manner over a concentration range of 0.5–10 ng/ml.

The effect of activin A/EDF on normal erythropoiesis was initially demonstrated by investigating the *in vitro* erythropoiesis of human bone marrow cells in an erythroid colony assay. In human bone marrow cell cultures containing erythropoietin, activin A/EDF increased the number of hemoglobin-synthesizing colonies derived from erythroid progenitors in both the early stage (erythroid burst-forming units, BFU-E) (12) and the late stage (erythroid colony-forming units, CFU-E) (13) of erythropoiesis. A stimulatory effect of activin A/EDF on erythropoiesis has also been shown *in vivo*, since its administration to mice results in an increase in the number of erythroid progenitors in the bone marrow and spleen (14). These findings, taken together with the detection of mRNA for this factor in several organs and tissues (including bone marrow and spleen) (15), raise the possibility that endogenous activin A/EDF has a role in normal erythropoiesis *in vivo*.

Our previous study showed that follistatin is identical to the specific activin A/EDF-binding protein (16). Follistatin is a 32-kDa protein that was discovered in follicular fluid and is an inhibitor of follicle-stimulating hormone secretion by primary cultured pituitary cells (17). Follistatin binds to activin A/EDF (the dissociation constant is 590 ± 230 pM), but it does not bind to peptides such as inhibin and TGF-β1 even though they are structurally related to activin A/EDF (18). The finding of this specific activin A/EDF-binding protein suggested that some regulatory system for activin A/EDF activity exists. Accordingly, the current study investigated the possible physiological role of endogenous activin A/EDF and also assessed the *in vivo* effect of follistatin.

MATERIALS AND METHODS

Activin A/EDF and Other Peptides. Recombinant human activin A/EDF was purified from the culture supernatant of Chinese hamster ovary cells (CHO cells) bearing an expression vector for the cDNA of this peptide (8, 9, 19). The recombinant peptide was structurally identical to natural activin A/EDF, and its biological activity was confirmed in primary cultures of pituitary cells by the promotion of follicle-stimulating hormone secretion as well as by induction of the differentiation of Friend cells (19, 20). The amino acid sequence of activin A/EDF is completely conserved among humans, cows, pigs, and rats (9, 21–23).

Follistatin was prepared from porcine ovary by the method described previously (18). In brief, the supernatant of an ovarian homogenate was subjected to activin A/EDF affinity chromatography and successive HPLC purification. Recombinant human erythropoietin was purchased from the Kirin Brewery (Tokyo).

Bioassay of Activin A/EDF Activity (Friend Cell Assay). Activin A/EDF activity was measured by a bioassay based on the induction of Friend cell differentiation. Closely related peptides such as TGF-β1 and inhibin do not have any effect on this assay system (8). Unrelated peptides, such as tumor

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Abbreviations: EDF, erythroid differentiation factor; BFU-E, erythroid burst-forming unit(s); CFU-GM, granulocyte–macrophage colony-forming unit(s); CFU-E, erythroid colony-forming unit(s); TGF-β, transforming growth factor β.

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necrosis factor α, epidermal growth factor, and interleukin 3, also do not have any effect on this assay system (unpublished data).

Serially diluted samples were added to cultures of Friend F5-5 cells (1×10^4 cells per ml) in Ham's F-12 medium with 10% fetal bovine serum. After culture for 6 days at 37°C in a humidified incubator containing 5% CO_2 and hemoglobin staining with dianisidine solution, the hemoglobin-positive cells (differentiated cells) were counted under a microscope and the percentage of such cells was calculated (24). The activin A/EDF activity of each sample was then determined from a standard curve giving the percentage of hemoglobin-positive cells induced by serial dilutions of recombinant activin A/EDF. Results were expressed as activin A/EDF units, with 1 unit corresponding to 0.5 ng of recombinant activin A/EDF (8).

Measurement of Activin A/EDF Activity in Murine Hemopoietic Organs. To measure activin A/EDF activity in the femoral bone marrow, six femurs were obtained from three male 10-week-old C3H/HeJ mice. Both ends of the bones were cut and the marrow was immediately flushed out with 1 ml of chilled saline. The marrow was then dispersed into a single-cell suspension by repeated pipetting. This suspension was centrifuged and the activin A/EDF activity of the supernatant was measured by a bioassay (Friend cell assay) after membrane concentration (molecular weight cutoff = 5000). Spleen supernatant was prepared in the same way after dispersion.

For the measurement of activin A/EDF activity in cultures, bone marrow or spleen cells were cultured at a density of 5×10^6 nucleated cells per ml in RPMI 1640 medium containing 15% fetal bovine serum. On day 4, the cells were collected and centrifuged, and then the supernatant was assayed. Data are expressed as the mean value of three independent cultures.

Methylcellulose Culture of Hemopoietic Progenitor Cells (Colony-Forming Assay). The *in vitro* culture of murine hemopoietic progenitor cells (colony-forming assay) was performed by a modification of the methylcellulose method (25). The culture medium for the assay consisted of α medium with 1% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol, and erythropoietin at 2 units/ml. Nucleated bone marrow cells were added at 2×10^5 per ml for the CFU-E colony assay and 2×10^6 per ml for the BFU-E colony assay. The same medium without bovine serum albumin and 2-mercaptoethanol was used for the granulocyte–macrophage colony-forming unit (CFU-GM) assay, with the cell density altered to 5×10^4 per ml and 2.5% medium conditioned by pokeweed mitogen-stimulated mouse spleen cells [PWM-SCM (26)] being added instead of erythropoietin. Culture dishes were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2 and 7% O_2. CFU-E colonies were counted by microscopy on day 2, CFU-GM colonies on day 7, and BFU-E colonies on day 9.

***In Vivo* Treatment Schedule.** Ten-week-old male C3H/HeJ mice (Clea Japan, Tokyo) were used for *in vivo* follistatin treatment. Follistatin was dissolved in 10 mM sodium acetate buffer, pH 5.5, containing 0.2% syngenic mouse serum and was given for 13 days by continuous infusion at 120 ng/hr. Osmotic minipumps (Alza, Palo Alto, CA; model 2002) that had been implanted intraperitoneally into animals under anesthesia with diethyl ether were used to deliver the follistatin. Control mice were administered buffer solution without follistatin in the same manner. On day 13, the femurs and spleens were removed, and the hemopoietic progenitor levels in the spleen and marrow were determined by the colony-forming assay.

Statistical Evaluation. The results were evaluated by the Student *t* test. Differences between the groups were considered significant if the *P* value of comparison was lower than 0.05.

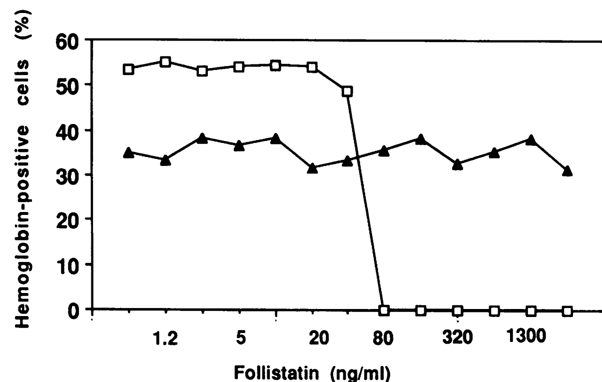

FIG. 1. Neutralizing effects of follistatin on activin A/EDF activity in the Friend cell assay. Friend F5-5 cells were cultured in the presence of recombinant activin A/EDF at 25 ng/ml (□) or 1% dimethyl sulfoxide (▲), together with serial dilutions of follistatin. After 6 days of culture, the percentage of hemoglobin-positive cells (differentiated cells) was plotted as a function of follistatin concentration.

RESULTS

Neutralizing Effect of Follistatin on Activin A/EDF Activity. A neutralizing effect of follistatin on recombinant activin A/EDF activity was demonstrated in the Friend cell bioassay (Fig. 1). The differentiation of Friend cells induced by 1% dimethyl sulfoxide (a known differentiation inducer) was not affected by follistatin, but the differentiation induced by activin A/EDF at 25 ng/ml was completely suppressed by follistatin at 80 ng/ml. It has been shown that follistatin does not bind to the surface of Friend cells (unpublished data), so this assay suggested that follistatin had a specific neutralizing effect on activin A/EDF by some direct molecular interaction mechanism.

Existence of Activin A/EDF Activity in Hemopoietic Organs. The existence of endogenous activin A/EDF activity in mouse bone marrow was demonstrated by using the Friend cell bioassay. The freshly prepared femoral bone marrow extract had a differentiation-inducing effect on Friend cells that was completely neutralized by follistatin at 100 ng/ml, suggesting that it was identical to activin A/EDF activity. This endogenous activity was determined to be 1.4 activin A/EDF units per femur, which corresponds to 0.7 ng of recombinant activin A/EDF (Table 1). The same experiment showed that the spleen also contained activin A/EDF activity

Table 1. Existence and production of endogenous activin A/EDF activity in murine hemopoietic organs

	Activin A/EDF activity			
	In organ, units/organ		In 4-day cultures, units/5×10^6 cells	
Organ	Without follistatin	With follistatin	Without follistatin	With follistatin
Bone marrow (femur)	1.4	<0.4	19.0	<1.2
Spleen	7.6	<2.6	8.0	<1.2

Data represent the activin A/EDF activity in units per single femur or spleen. One activin A/EDF unit corresponds to 0.5 ng of recombinant activin A/EDF. Activin A/EDF activity in the 4-day cultures of bone marrow cells and spleen cells was also measured. Samples were also assayed in the presence of follistatin at 100 ng/ml. The detectable level of activin A/EDF activity varied according to the extent of membrane concentration in each sample performed prior to assay.

Table 2. *In vitro* effects of activin A/EDF and follistatin on colony formation by erythroid and nonerythroid hemopoietic progenitors

Additions	Experiment 1			Experiment 2	
	CFU-E, colonies/ 2×10^5 cells	BFU-E, colonies/ 2×10^6 cells	CFU-GM, colonies/ 5×10^4 cells	CFU-E, colonies/ 2×10^5 cells	BFU-E, colonies/ 2×10^6 cells
Control medium	538 ± 38 (100%)	77 ± 8 (100%)	162 ± 12 (100%)	297 ± 10 (100%)	54 ± 4 (100%)
Follistatin (100 ng/ml)	300 ± 24* (56%)	39 ± 4* (51%)	180 ± 2 (111%)	227 ± 27‡ (76%)	37 ± 10‖ (69%)
Activin A/EDF (25 ng/ml)	874 ± 23* (163%)	113 ± 15‡ (147%)	166 ± 17 (102%)	NT	NT
Activin A/EDF (25 ng/ml) + follistatin (100 ng/ml)	355 ± 72† (66%)	50 ± 5§ (65%)	NT	NT	NT
Activin A/EDF (400 ng/ml)	NT	NT	NT	439 ± 12* (148%)	80 ± 5** (150%)
Activin A/EDF (400 ng/ml) + follistatin (100 ng/ml)	NT	NT	NT	451 ± 23¶ (152%)	75 ± 9†† (140%)

Bone marrow cells used in the colony assay were obtained from femurs of 9-week-old male C3H/HeJ mice. Each additive was included at the beginning of the culture together with erythropoietin at 2 units/ml (for CFU-E and BFU-E colony assay) or PWM-SCM at 2.5% (for the CFU-GM assay). Each value represents the mean ± SD of the number of colonies in three dishes. Values are also expressed as a percentage of the respective control (control = 100%). NT, not tested. Data were statistically evaluated by the Student *t* test: *, $P < 0.001$ vs. control; †, $P < 0.025$ vs. control and $P < 0.001$ vs. with activin A/EDF; ‡, $P < 0.025$ vs. control; §, $P < 0.001$ vs. control and $P < 0.005$ vs. with activin A/EDF; ¶, $P < 0.01$ vs. control and vs. with follistatin; ‖, $P < 0.05$ vs. control; **, $P < 0.005$ vs. control; ††, $P < 0.025$ vs. control and $P < 0.01$ vs. with follistatin.

(7.6 units), which could be neutralized by follistatin. The level of activity in the spleen was lower than that in the bone marrow on an equivalent volume basis.

The ability of bone marrow cells to produce the same activity was demonstrated by assay of the conditioned medium obtained from primary bone marrow cultures (Table 1). Bone marrow cells (5×10^6) secreted 19.0 units of activin A/EDF activity after 4 days of culture, and spleen cells secreted 8.0 units of activity under the same conditions. In both types of culture, the activity was neutralized by follistatin at 100 ng/ml. These results suggested that the hemopoietic organs contained endogenous activin A/EDF activity and that at least part of this activity was produced locally.

Suppression of Erythropoiesis *in Vitro* by Follistatin. The effect of follistatin on erythropoiesis *in vitro* was investigated by using the erythroid colony assay to determine how its neutralization of endogenous activin A/EDF activity affected erythropoiesis. The addition of follistatin to the colony assay medium at 100 ng/ml resulted in a decrease in the number of CFU-E and BFU-E colonies that formed in response to erythropoietin (Table 2, experiment 1). Exogenous activin A/EDF at 25 ng/ml stimulated CFU-E and BFU-E colony formation; its effect was abolished by follistatin at 100 ng/ml in the same experiment. A suppressive effect of follistatin at 100 ng/ml on CFU-E and BFU-E colony formation observed in the absence of activin A/EDF was overcome by adding excess activin A/EDF (400 ng/ml) (Table 2, experiment 2). These data indicate that erythropoietin-dependent colony formation by erythroid lineage cells was partly supported by activin A/EDF secreted by bone marrow cells, since the culture medium used in this assay did not initially contain activin A/EDF activity.

Neutralization of endogenous activin A/EDF activity appeared to affect only cells of the erythroid lineage, since colony formation by cells derived from a granulocyte-macrophage progenitor (CFU-GM) was not affected by the addition of follistatin (Table 2, experiment 1). This result was in good agreement with the observation that the stimulatory effect of exogenous activin A/EDF was restricted to cells of the erythroid lineage.

Suppression of *in Vivo* Erythropoiesis by Follistatin. The effect of follistatin was further investigated *in vivo*. Continuous intraperitoneal administration of follistatin to normal mice resulted in a decrease of erythroid progenitors in the hemopoietic organs. The CFU-E levels in the bone marrow and spleen of follistatin-treated mice were respectively 72% and 28% of those in control mice (Table 3). BFU-E did not change significantly in the bone marrow, but they fell to 27% of the control level in the spleen. CFU-GM were not changed in either the bone marrow or the spleen (Table 3). The number of nucleated cells in the bone marrow and spleen and the erythrocyte, leukocyte, and platelet counts in the peripheral blood were also not affected by follistatin (data not shown).

DISCUSSION

Our present study confirmed the participation of endogenous activin A/EDF in murine erythropoiesis. Neutralization of endogenous activin A/EDF activity in mice by follistatin administration resulted in a significant decrease of splenic BFU-E. Taken together with a previous study that demonstrated an increase in the number of BFU-E in activin A/EDF-treated mice (14), this result indicates that activin A/EDF has burst-promoting activity *in vivo*. Follistatin treatment also decreased the number of CFU-E in both the spleen and the bone marrow. This may not have resulted from the decrease in BFU-E, since our previous study showed that an increase of CFU-E in mice was promoted by activin A/EDF independent of any effect on BFU-E (14). As follistatin treatment decreased both the splenic CFU-E and BFU-E, it

Table 3. Changes in the number of hemopoietic progenitors in follistatin-treated mice

Addition	Bone marrow			Spleen		
	CFU-E, colonies/ 2×10^5 cells	BFU-E, colonies/ 2×10^6 cells	CFU-GM, colonies/ 5×10^4 cells	CFU-E, colonies/ 2×10^5 cells	BFU-E, colonies/ 2×10^6 cells	CFU-GM, colonies/ 5×10^4 cells
Control	276 ± 13 (100%)	111 ± 21 (100%)	133 ± 32 (100%)	205 ± 44 (100%)	27 ± 7 (100%)	15 ± 7 (100%)
Follistatin	198 ± 59* (72%)	98 ± 18 (88%)	142 ± 34 (107%)	58 ± 13‡ (28%)	10 ± 4† (37%)	15 ± 1 (100%)

After follistatin was administered to mice continuously for 13 days at 120 ng/hr, femurs and spleens were removed and the levels of hemopoietic progenitors were estimated. Control mice received buffer solution without follistatin in the same manner. Each value represents the mean ± SD of the data from four mice. Results are also expressed as a percentage of the respective control (control = 100%). Data were statistically analyzed by the Student *t* test: *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ vs. control.

seems possible that endogenous activin A/EDF supports the maturation from BFU-E to CFU-E as well as erythropoiesis preceding the BFU-E stage. Thus, activin A/EDF appears to have burst-promoting activity that also stimulates the later stage of erythropoiesis.

This study also showed that activin A/EDF activity is produced in the bone marrow and spleen in mice. It remains uncertain whether endogenous activin A/EDF circulates in the peripheral blood as a hormonal factor, but it is supplied to the hemopoietic tissues at least partly by cells within the bone marrow and spleen. The results of the *in vitro* study (Table 2) suggested the importance of activin A/EDF produced in the bone marrow for erythropoiesis, since neutralization of the activin A/EDF activity secreted by bone marrow cells resulted in a decrease in the number of erythroid colonies. Activin A/EDF therefore seems to be an autocrine or paracrine factor that acts physiologically to support the growth and maturation of erythroid lineage progenitors in the hemopoietic microenvironment. Granulocyte-macrophage colony-stimulating factor is known to have burst-promoting activity and is reported to be produced constitutively in the bone marrow (27). Whether the production of activin A/EDF in the hemopoietic organs is constitutive or is induced by certain physiological circumstances remains to be elucidated, and the identification and characterization of the cells producing this activity will be necessary for such studies.

In vivo follistatin administration had less effect on CFU-E and BFU-E numbers in the bone marrow than on those in the spleen (Table 3), but the reason for this differential response is currently unknown. We suggest that there may be a difference in the concentration of endogenous activin A/EDF in bone marrow and spleen or in the follistatin levels achieved in each tissue by intraperitoneal administration. The amount of activin A/EDF activity produced by bone marrow cells was twice that produced by the equivalent number of spleen cells (Table 1), a finding that supports the first suggestion.

The decrease in the number of erythroid progenitors in follistatin-treated mice also suggests the possibility that follistatin is a negative regulator of erythropoiesis. An interesting feature of the negative effect of this factor is its ability to act on both the early and late stages of erythropoiesis. Follistatin was discovered in the ovary, and its mRNA has been found in both the kidney and the ovary (28). Follistatin has not been found in hemopoietic tissues, and it is not known whether it acts only locally at the site of production or whether it circulates and acts systemically. The physiological role of follistatin remains to be elucidated.

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