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THE EXISTENCE OF ACTIVIN A / ERYTHROID DIFFERENTIATION FACTOR AND ITS INHIBITOR IN HUMAN SERUM : COMPARISON OF NORMAL AND CHRONIC RENAL FAILURE SERA

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SUMMARY: Activin A / EDF, initially found as a differentiation inducer of murine Friend erythroleukemia, also has a stimulatory effect on erythropoiesis in vitro and in vivo. Here we proved activin A / EDF activity in human serum. The activin A / EDF level in 18 normal human serum samples was measured by a specific bioassay and was found to be 8.3 ± 4.6 ng/ml, indicating that there exists sufficient activity to affect erythropoiesis in normal serum. In contrast, activin A / EDF activity was reduced in the chronic renal failure patients and 23 / 26 serum samples examined showed levels below 1.2 ng/ml. Further analysis using HPLC revealed that chronic renal failure serum actually contained as much activin A / EDF as normal serum, and that the difference between normal and patient serum existed in the content of a specific inhibitor of activin A / EDF. This observation suggests the possibility that the inhibitor is participating in the regulation of activin A / EDF activity in vivo in chronic renal failure patients and also the possibility of activin A / EDF could be utilized in the therapy of the anemia of such patients.

Erythroid differentiation factor (EDF) is a protein which we found in the culture medium of a phorbol ester-stimulated human monocytic cell line (THP-1) as a differentiation inducer of murine Friend erythroleukemia (1,2) and is a member of the TGF-β family. Activin A, a protein which was independently found in gonadal fluid as a promoter of follicle-stimulating hormone release in primary pituitary cell cultures, is identical to EDF (3,4).

ABBREVIATIONS: EDF, erythroid differentiation factor; CRF, chronic renal failure; CFU-E, erythroid colony-forming units; BFU-E, erythroid burst-forming units.

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A recent study revealed that activin A / EDF not only promotes the differentiation of Friend erythroleukemia but also acts on normal erythropoiesis. Namely, this protein stimulates in vitro colony formation of erythroid progenitors (CFU-E and BFU-E) in response to erythropoietin (5,6). Activin A / EDF has also been shown to independently stimulate in vivo erythropoiesis, since treatment of mice with this protein resulted in an increase of the number of erythroid progenitors (CFU-E and BFU-E) in bone marrow (7). These data indicate that activin A / EDF acts as a regulator of erythropoiesis.

In this study, we investigated the activin A / EDF activity in the serum of healthy volunteers and patients with chronic renal failure (CRF). We compared normal and CRF serum to study the relationship between endogenous activin A / EDF activity and the occurrence of anemia, as CRF produces a type of anemia which is called renal anemia (8,9).

MATERIALS AND METHODS

Serum samples: Serum was obtained from 18 healthy volunteers among the medical staff of the University of Tokushima Hospital. Their ages ranged from 20 to 39 years (8 females and 10 males) and all of them had a normal hematocrit, hemoglobin concentration and normal renal function as determined by blood tests and urinalysis. Serum was also obtained before hemodialysis from 26 CRF patients who were on chronic dialysis treatment at the University of Tokushima Hospital. Their ages ranged from 22 to 79 (10 females and 16 males). Mean levels of their hemoglobin, blood urea nitrogen, and creatinine were 8.5 g/dl, 76.5 mg/dl, and 11.3 mg/dl respectively. Informed consent was obtained from both the CRF patients and the normal volunteers. Each serum sample was treated at 56℃ for 30 min and then filtered through a membrane filter with 0.22 μm pores (Myrex GT filter, Millipore, Japan) before assay.

Bioassay of activin A / EDF: Activin A / EDF activity was measured by a bioassay using the induction of differentiation in Friend cells (1,10). Serially diluted samples were added to cultures of Friend F5-5 cells ($1x10^4$/ml) in Ham's F12 medium, with 10% fetal bovine serum. After 6 days of culture at 37℃ in a humidified incubator with 5% CO_2, hemoglobin staining was performed using dianisidine solution. Then the hemoglobin-positive cells (differentiated cells) were counted under a microscope and the percentage of these cells was determined. Further identification of the differentiation-inducing activity as activin A / EDF was performed using follistatin, a protein that binds specifically to activin A / EDF and neutralizes it (11,12). The differentiation-inducing activity detected in the bioassay was confirmed to be as activin A / EDF activity if it was neutralized by the addition of 100 ng/ml of follistatin at the start of the assay. A follistatin sample was prepared from porcine ovary by a method described previously (11).

Purification of activin A / EDF from serum: One milliliter of each serum sample was precipitated by the addition of 3.35 volumes of 0.7% trifluoroacetic acid/ 99.3% acetonitrile followed by hard mixing. After removing the precipitate by centrifugation, the supernatant was chilled to -20℃ for one hour. The lower of the separated layers was collected, diluted with two volumes of distilled water containing 0.1% trifluoroacetic acid, and loaded into a

COSMOSIL 5TMS-300 HPLC column (4.6 X 250 mm). The column was eluted with a linear gradient of 0 - 80% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The single active peak of activin A / EDF was collected and then lyophilized. After resolving in 200 μl of distilled water, samples were filtered and subjected to bioassay to determine the activin A / EDF concentration.

RESULTS

Activin A / EDF bioassay of normal and CRF serum: The serum activin A / EDF activity in 18 healthy volunteers was determined by a bioassay using Friend erythroleukemia cells without any pretreatment to purify the serum samples. All 18 serum samples induced the differentiation of Friend cells and this differentiation-inducing activity was blocked in the presence of follistatin, a specific binding protein that neutralizes activin A / EDF. Thus, the serum differentiation-inducing activity was identified as activin A / EDF. Serum activin A / EDF activity in the volunteers ranged from 1.7 to 19.6 ng/ml, with the average being 8.3 ± 4.6 ng/ml. No obvious sex difference was noted (Fig.1). The 26 CRF patients showed lower serum levels of differentiation-induction activity for Friend cells; in all cases the activity was lower than 4.0 ng/ml (Fig.1). No detectable activity was seen in all of 13 patients with their hemoglobin level being lower than 8.5 mg/dl.

Purification of activin A / EDF from serum: The low activin A / EDF activity shown in CRF patients by bioassay raised two possibilities. One was that CRF serum did not contain as much activin A / EDF as normal serum and the other was that CRF sera contained some unknown soluble factor which interferes with the bioassay. To investigate these possibilities, further analysis of normal and CRF serum was done after purification of activin A / EDF. After HPLC fractionation following trifluoroacetic acid / acetonitrile precipitation, activin A / EDF activity was recovered from serum as a single peak. Bioassay of this purified fraction revealed that the same level of activin A / EDF as in healthy volunteers could be recovered from CRF serum, even though activin A / EDF activity was undetectable without pretreatment. As seen in Table 1, the activin A / EDF activity in two normal serum samples was 4.5 and 2.1 ng/ml respectively, while that recovered from CRF serum ranged from 1.7 to 2.9 ng/ml. Considering the yield of the purification procedure, the original activin A / EDF concentration in CRF serum was comparable with that in normal serum. Thus, the results obtained here suggested that CRF serum contained some soluble factor interfering with the activin A / EDF bioassay.

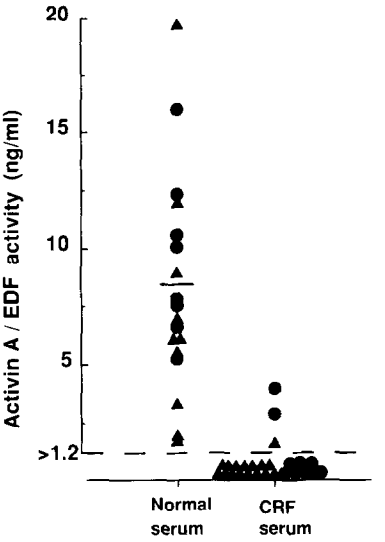

Fig 1. Activin A / EDF bioassay of normal and CRF serum samples without pretreatment.
Each serum sample was serially diluted without pretreatment and was assayed for activin A / EDF activity. The activity of each sample was calculated using a standard curve for authentic recombinant activin A / EDF and was expressed in ng/ml. ▲: male, ●: female. Bar: average activin A / EDF activity in normal serum.

Demonstration of an activin A / EDF inhibitor in CRF serum: The interfering factor was shown out to be an inhibitor of activin A / EDF by the following experiment using Friend erythroleukemia cells. As seen in Fig.2a, CRF serum inhibited the differentiation-inducing activity of exogenously added activin A

Table 1. Recovery of activin A / EDF activity from normal and CRF sera using HPLC fractionation

	Activin A / EDF activity (ng/ml)	
	Serum without pretreatment	HPLC-fractionated serum *
Healthy volunteers		
No. 1	10.6	4.5
No. 2	4.8	2.1
CRF patients		
No. 1	<1.2	1.8
No. 2	<1.2	2.3
No. 3	<1.2	2.9
No. 4	<1.2	1.7

* Activin A / EDF activity recovered by HPLC fraction converted to the activin A / EDF concentration in the original serum volume.

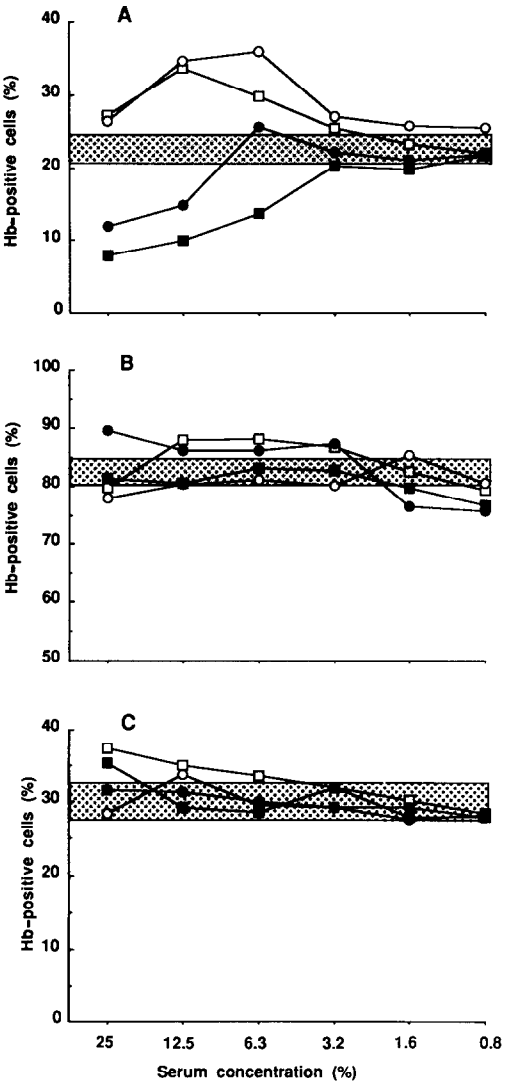

Fig 2. Effects of normal and CRF sera on the differentiation of Friend erythroleukemia cells induced by activin A / EDF or dimethylsulfoxide.
Friend cells were cultured with serially diluted normal or CRF sera in the presence of 2 ng/ml of activin A / EDF (A), 100 ng/ml of activin A / EDF (B), or 0.5% dimethylsulfoxide (C). After six days of culture, cells were stained with dianisidine and the percentage of hemoglobin-positive cells was counted. ○, normal serum (No.2); □, normal serum (No.1); ●, CRF serum (No.7); ■, CRF serum (No.9). Shaded region is the mean ± S.D. of the percentage of hemoglobin-positive cells in the absence of serum.

/ EDF (2 ng/ml), while normal sera did not do so. This inhibition of differentiation by CRF serum was overcame by adding an excess of activin A / EDF (100 ng/ml) to the culture, as seen in Fig.2b. Furthermore, the differentiation of Friend cells induced by dimethylsulfoxide, a known chemical

inducer of Friend erythroleukemia differentiation, was not inhibited by CRF serum (Fig.2c). The neutralization of inhibition by excess activin A / EDF and the selective mode of inhibition suggested that a specific inhibitor of activin A / EDF existed in CRF serum.

DISCUSSION

In this study, we demonstrated the existence of activin A / EDF in human serum. The concentrations are thought to be sufficient to affect in vivo erythropoiesis, since comparable serum levels achieved by activin A / EDF administration to mice caused an increase in the number of bone marrow erythroid progenitors (CFU-E and BFU-E) (Shiozaki, unpublished data). Together with our observation that neutralization of endogenous activin A / EDF in mice by treatment with follistatin resulted in a decrease in the number of erythroid lineage progenitors in both the spleen and the bone marrow (13), endogenously produced activin A / EDF appears to support erythropoiesis in vivo. As previous study revealed that endogenous activin A / EDF also exists in several organs including the spleen and the bone marrow (13,14), there should exist independent physiological role in both of locally existing and systemically circulating activin A / EDF.

We also found that an inhibitor of activin A / EDF exists in the serum, and we suggest the possibility of its participation in the development of renal anemia. Further study is needed to clarify whether or not the inhibitor is actually concerning in renal anemia. It is reported that CRF serum contains uremic toxins which inhibits erythropoiesis, such as parathyroid hormone and spermine (15-17). We confirmed that sufficient dose of parathyroid hormone (3 μg/ml) and spermine (100 nM) to inhibit in vitro erythropoiesis does not affect the differentiation of Friend cells induced by 2 ng/ml of activin A / EDF (data not shown). We previously reported that CRF serum also contains an activity to suppress the colony formation of human erythroid cells (CFU-E and BFU-E), which is counteracted by activin A / EDF (18). The relationship between the activity and the inhibitor found in this study remains to be elucidated.

It is of interest whether the inhibitor is a toxic product of abnormal metabolism in CRF patients or is a specific physiological regulator of activin A / EDF activity which is acting also in normal erythropoiesis. Two protein factors (follistatin and inhibin) have been reported so far to counteract the

action of activin A / EDF. Both of them were initially found in follicular fluid (19,20) and suppress the effect of activin A / EDF on primary cultures of pituitary cells, i.e., the promotion of follicle stimulating hormone secretion. We confirmed that follistatin neutralizes the differentiation of Friend cells induced by activin A / EDF, but inhibin does not (unpublished data). Therefore, inhibin could not be a candidate for the inhibitor in CRF serum. Identification of the inhibitor of activin A / EDF in CRF serum, including assessment of whether follistatin is the agent in question, and measurement of its serum level in normal and pathological states remains to be performed.

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