Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 347-352

Specific Erythroid Differentiation of Mouse Erythroleukemia Cells by Activins and Its Enhancement by Retinoic Acids

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Received July 10, 1992

Summary: Activin A has been shown to induce hemoglobin production in various hematopoietic cells. Such activities of three structurally distinct activins (activin A, activin AB, and activin B) were compared using F5-5 mouse erythroleukemia cells. Activin A and AB had similarly potent inducing activities whereas that of activin B was much lower. The erythroid inducing activity of activins was suppressed by follistatin, an activin-binding protein but not by inhibin A and inhibin B. Retinoic acids (both *all-trans* and *13-cis*) had weak erythroid differentiation activity. In addition, clear synergistic erythroid induction occurred when retinoic acid and activin A were mixed together. These results indicate that retinoic acid may modulate activin-induced erythropoiesis *in vivo*.

Erythroleukemia cells differentiate into more mature erythroid cells and accumulate hemoglobin by various low molecular weight compounds including dimethylsulfoxide (1), HMBA (2), butyric acid (3, 4), and hemin (5). Although these compounds induce erythrodifferentiation *in vitro*, the mechanism of erythroid differentiation in the mammalian bone marrow has not fully been elucidated partly because these substances are not of natural origin and do not have defined biological activities. More recently, genistein, an inhibitor of tyrosine protein kinases and phosphatidyl cholines have been shown to induce erythrodifferentiation in Friend cells (6-8).

EDF, a factor which is secreted by several myelomonocytic cells responsible for induction of erythroid differentiation in MEL cells turned out to be activin A, known as a stimulator for the secretion of pituitary FSH (9,10). The effectiveness of activin in the erythrodifferentiaion of K562 erythroleukemia cells and multipotential hematopoietic progenitor cells was also confirmed (11,12). Activin is composed of

Abbreviations used are: EDF, erythroid differentiation factor; MEL, mouse erythroleukemia; PBS, phosphate-buffered saline; RA, retinoic acid; HMBA, hexamethylene bisacetamide; FGF, fibroblast growth factor; TGF, transforming growth factor; EGF, epidermal growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; IL, interleukin; EPO, erythropoietin.

0006-291X/92 $4.00

two inhibin β chains. By combining either two subunits of β_A or β_B chains, three isotypes of activins, activin A, activin AB, and activin B are generated. Recombinant human activin A and activin B have been reported to be equally effective in the differentiation of K562 cells (13). In the present study we compared the erythroid differentiation activities of three isotypes of activins on F5-5 MEL cells, and further describe synergistic erythroid induction by activin and RA.

Materials and Methods

Activins, inhibins, EDF and follistatin: Activins A, AB, B (14) and follistatin (15) purified from porcine ovarian follicular fluid were provided by Dr. Sugino at the Institute of Physical and Chemical Researches. EDF (human recombinant activin A) purified from CHO cells expressing inhibin β_A chain gene (10) was also used. Each of these activins gave a single band in SDS-PAGE in nonreducing conditions, indicating no contamination of inhibins and follistatin (14). Bovine inhibin A and human recombinant inhibin B are gifts from Dr. Hasegawa in the Gumma Univ. The activins, follistatin, and inhibins were determined by A_{280}. Stock solutions of these substances were diluted to the final concentration just before the assay with a medium for Friend F5-5 cells as described below.

Chemicals: Following growth factors were purchased and used according to the manufacturer's instructions. acidic FGF from bovine brain (BT-104, Biomedical Technologies Inc., Stoughton, USA); basic FGF from bovine brain (133-FB, R&D Systems Inc., Minneapolis, USA); human recombinant TGF-β1 (King Brewing Inc., Japan); TGF-β2 from porcine platelets (102-B2, R&D Systems Inc.); EPO from human urine (051-04011, Wako Chemicals, Osaka, Japan); recombinant mouse IL3 (MIL-3, Genzyme Inc., Boston, USA); human recombinant EGF (GE002, Wakunaga Pharmaceuticals, Hiroshima, Japan); NGF from mouse submaxillaries (BT-206, Biomedical Technologies Inc.); PDGF from human platelets (BT-208, Biomedical Technologies Inc.); somatostatin (S9129, Sigma, St. Louis, USA).

EDF test: Mouse F5-5 erythroleukemia cells (16) were maintained in Ham's F12 nutrient mixture (GIBCO 430-1700) supplemented with 10% fetal calf serum under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. One thousand cells in logarithmic growth phase were suspended in 50 μl of the medium and added to a well of 96-well multititer plate (Falcon 3072) containing 50 μl of the same medium in which various concentrations of test substances were included. For each test substance, serial 12-step twofold dilution series was prepared. The plate was kept still under the above culture conditions. Six days later, the medium was replaced with PBS to reduce the serum concentration to 1/125 of original. Cells in each well were suspended in 100 μl PBS and immediately 10 μl saturated solution of o-dianisidine (D9123, Sigma) in 3% acetic acid and 3% hydrogen peroxide was added. 30 min later, peroxidase-positive cells were scored using a hemacytometer or an ocular micrometer. More than 500 cells were counted for each well. In the coincubation experiment of activin and follistatin, 50 ng/ml follistatin was mixed with various concentrations of activins and reacted at room temperature for 60 min prior to the addition of F5-5 cells.

Results

F5-5 Friend erythroleukemia cells were cultured with serial twofold dilution series from 250 ng/ml to 0.122 ng/ml of activins A, B, and AB, and were stained with dianisidine to demonstrate hemoglobin production. All activins had EDF activities (Fig. 1A). The ratio of dianisidine-positive cells increased steadily with increasing amount of activins. The curve reached a plateau at about 10 ng/ml for activin A and

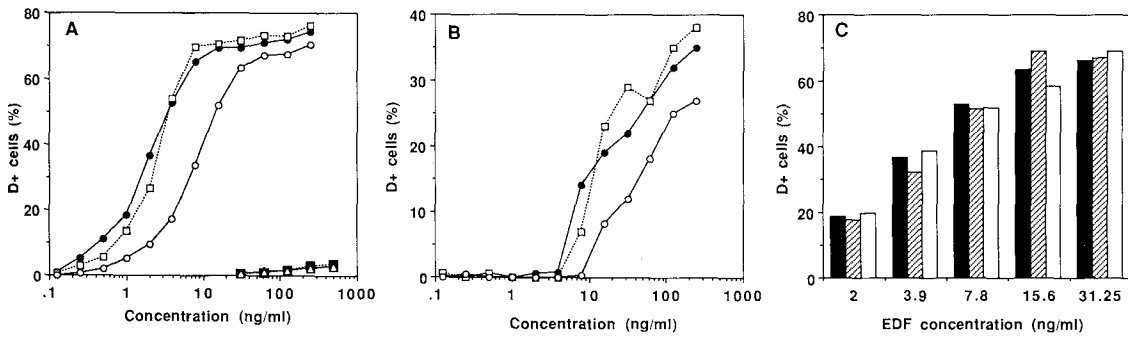

Fig. 1. (A) EDF activity of activins and inhibins. The percentage of dianisidine-positive cells was plotted. (●) activin A; (□) activin AB; (○) activin B; (■) inhibin A; (△) inhibin B. (B) Preincubation of activins with 50 ng/ml of follistatin prior to the inoculation of F5-5 cells resulted in the suppression of EDF activities of activins. (●) activin A; (□) activin AB; (○) activin B. (C) Ineffectiveness of inhibins for the suppression of EDF-induced erythroid differentiation. Black bar: EDF alone; hatched bar: EDF + 20 ng/ml inhibin A; white bar: EDF + 20 ng/ml inhibin B.

AB and at about 50 ng/ml for activin B. The maximal differentiation ratio was about 70%. The ED_{50} (concentration needed for 35% of cells to become dianisidine-positive) of activins A, B, and AB was 1.8 ng/ml, 2.4 ng/ml, and 8.3 ng/ml, respectively. Thus, EDF activities of activin A and AB were very similar in contrast to the low activity of activin B.

Follistatin suppressed EDF activities of every activin (Fig. 1B). Follistatin (50 ng/ml) suppressed EDF activities of activin A, B, or AB almost completely below 3.9 ng/ml of activins. EDF activities of activins appeared abruptly over 7.8 ng/ml. The differentiation ratio of each activin increased steadily thereafter.

Both bovine inhibin A and human recombinant inhibin B, which are structurally related natural antagonists of activin on FSH secretion, had extremely low EDF activities (Fig. 1A). The concentration needed for 1% of cells to become dianisidine-positive was 62.5 ng/ml for inhibin A, which was about a 500-fold concentration compared to activin A. EDF activity of inhibin B was similar to that of inhibin A. When applied together with EDF, 20 ng/ml of inhibin A and B did not antagonize EDF activities of activin from 1.9 ng/ml to 31 ng/ml (Fig. 1C).

TGF-β1 and -β2 (0.5-1250 ng/ml) had no EDF activities. Other substances that gave negative results in the EDF test were EGF (1-2000 ng/ml), NGF (1-2000 ng/ml), acidic FGF (0.5-1000 ng/ml), basic FGF (2.5-5000 ng/ml), EPO (0.025-50 u/ml), IL3 (0.5-1000 u/ml), PDGF (0.125-250 u/ml), and somatostatin (1-2000 ng/ml). IL3 and EPO had no synergistic effect when these two chemicals were mixed together with EDF (data not shown).

Both *all-trans* and *13-cis* RAs had weak EDF activities (Fig. 2). From nM to μM concentrations, both RAs induced erythrodifferentiation in steadily increasing proportions of cells. However, even at 10 μM, the ratio of induced cells was only about 30%. When EDF and

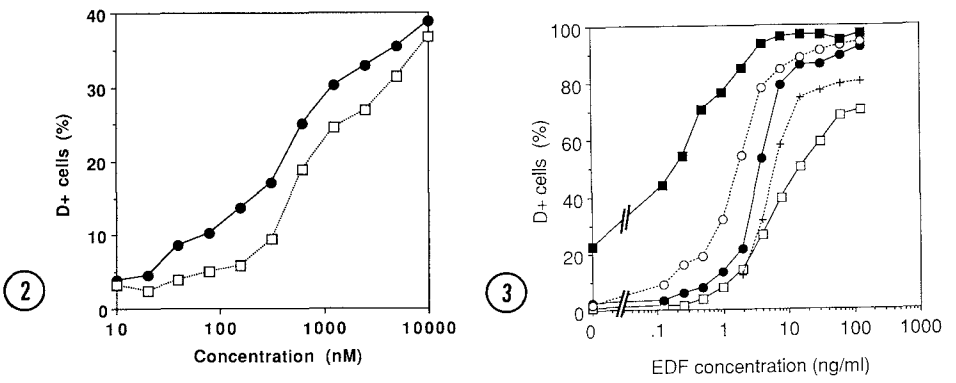

Fig. 2. EDF activity of RAs. The percentage of dianisidine-positive cells was shown. (●) *all-trans* RA; (□) *13-cis* RA.

Fig. 3. Synergistic erythroid induction by EDF and *all-trans* RA. The percentage of dianisidine-positive cells was plotted. (□) RA = 0 M; (+) RA = 10^{-12}M; (●) RA = 10^{-10}M; (○) RA = 10^{-8}M; (■) RA = 10^{-6}M.

all-trans RA were mixed together, a clear synergistic effect was observed (Fig. 3). The maximum erythroid differentiation ratio increased to nearly or over 90% of cells in contrast to 70% in case of EDF alone. The increase in differentiation ratio was marked when the activin A concentration was 1.9–15.6 ng/ml. When RA concentration was low, the maximum synergism was observed at a relatively high concentration of activin A. In contrast, when RA concentration was high, maximal synergism was obtained at a low concentration of activin A.

Discussion

In this study, we screened several growth factors for their erythroid differentiating potency on F5-5 MEL cells. We focused on FGFs, TGF-βs and activins because these growth factors are all mesoderm-inducing factors on presumptive ectodermal cells of *Xenopus* embryos, and induce blood-like cells (17). Other factors related to erythroid differentiation were also tested: namely, IL3 and EPO (18). Among these and other growth factors, only activins had potent EDF activities. Therefore, F5-5 MEL cells offer a good assay system for quantitating activin.

It turned out that among three isotypes of activins, activin B had several-fold lower activity than activin A and activin AB. This does not agree with the previous reports on the erythroid differentiation test using K562 cells and recombinant human activin A and activin B (13). The mesoderm-inducing activity of activin B used in the present study was quite similar to activin A and activin AB (15). Therefore, it is unlikely that activin B used in this study had lost much of its activity. Inhibin A has been shown to antagonize activin A on the erythroid differentiation of K562 cells (11,12). However, both bovine inhibin A and human recombinant inhibin B had no effect on F5-5 cells when cocultured with EDF.

Additionally, EPO was effective in the differentiation of K562 cells (10), whereas it was ineffective for F5-5 cells. Therefore, F5-5 Friend cells are quite different from K562 cells. There are at least two genes for mouse activin receptors (actR-II and actR-IIB), and actR-IIB gene undergoes alternative splicing to generate four distinct types of activin receptors with different affinity to activin A (19,20). It has been reported that there are 3200 activin binding sites of single class on F5-5 cells and the binding constant is 310 pM (21). Although it is not known which activin receptor gene is expressed in F5-5 cells, the difference of biological activity of activin isotypes as observed here could be attributed to the difference of affinity between the activin receptor and activin isotypes.

The EDF activities of *all-trans* RA and *13-cis* RA were extremely low compared to activins. For 35% of cells to become dianisidine-positive, μM order of RA was necessary, which was about 5000-fold higher concentration as activin A. However, pM to nM concentration of RAs was sufficient for the synergistic erythroid induction when mixed with activin A. It has been shown that HMBA acts synergistically with activin on the erythroid differentiation of F5-5 cells (22). In this case, mM concentration of HMBA was necessary for the synergism. Apart from the concentration, the synergism of HMBA and activin is overall similar to that of RAs and EDF, suggesting the possible similarity of the target molecule(s) for HMBA and RAs.

Marked inhibition of proliferation was observed at more than 1 μM of RA (data not shown). Activin is also inhibitory on F5-5 cell proliferation (9). However, from pM to nM order of RAs where maximum synergism with EDF was observed, suppression of proliferation by RA alone or in combination with activin was not observed. To analyse proliferation further, more sensitive assay such as colony forming assay would be adequate. The maximum differentiation ratio of activin-induced F5-5 cells dramatically rised by the addition of 1-100 pM RA which is in itself not very effective inducer, suggesting that RA may elevate responsiveness of F5-5 cells to activin. Recently, *Xlim-1* which is an activin-responsive homeobox gene in *Xenopus laevis* turned out to be activated synergistically by activin and RA (23). Conversely, RA inhibits mesoderm differentiation in *Xenopus* early embryos (24) and neural differentiation in EC cells (25) which are both induced by activin A. There are several types of RA receptors in the mouse (RAR-α, RAR-β, RAR-γ, and RXRs). By the preliminary results of the immunostaining with anti-RAR-α antibodies, F5-5 cells were negative in contrast to the intensely stained *Xenopus* early embryos. However, we are expecting the presence of RARs in F5-5 cells, considering the wide distribution of RARs in hematopoietic cells including K562 cells (26).

Activin A is participating in the erythropoiesis in vivo (27). The marked enhancement of activin-induced erythroid differentiation by RAs suggests the possible role of RA in the erythropoiesis.

Acknowledgments

We wish to thank Dr. Yuzuru Eto for the kind instruction of the EDF assay, for providing EDF and F5-5 cells, and for encouragement during the course of this study. The authors are also indebted to Dr. Hiromu Sugino and Dr. Kazunori Nakamura for the kind gift of porcine activin A, activin AB, activin B, and follistatin. The authors are grateful to Dr. Yoshihisa Hasegawa for bovine inhibin A and human recombinant inhibin B. This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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