Effect of Activins AB and B on DNA Synthesis Stimulated by Epidermal Growth Factor in Primary Cultured Rat Hepatocytes

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The effect of activins AB and B on DNA synthesis stimulated by epidermal growth factor (EGF) was studied in primary cultured rat hepatocytes and compared with the effect of activin A, a suppressor of DNA synthesis. Activin AB inhibited DNA synthesis as assessed by [³H]thymidine incorporation. The inhibition by activin AB was detected at 6 ng/ml, and the 12.5 ng/ml concentration produced almost maximal inhibition, approximately 40%, almost the same as that produced by activin A. Inhibition by activin A was detected at 3 ng/ml, and the 6 ng/ml concentration produced almost maximal inhibition. Activin B, on the other hand, had no effect on DNA synthesis up to 50 ng/ml. The increase in labeling index by EGF was also reduced to about 20% by 25 ng/ml activin A and activin AB, but not by activin B. Activin B, however, inhibited the binding of [¹²⁵I]activin A to hepatocytes, but had no effect on the inhibition of DNA synthesis by activin A, even at 3-fold excess concentrations. These findings suggest that activin AB may act in the same manner as activin A does in terms of EGF's inhibitory effect on DNA synthesis, although the effective concentration is higher than that of activin A. The findings also suggest that activin B receptors are present in hepatocytes but that they do not mediate signal transduction leading to the inhibition of DNA synthesis.

Key words activin; cultured rat hepatocyte; DNA synthesis; epidermal growth factor (EGF)

Activins are members of the transforming growth factor- β superfamily and are homodimeric proteins composed of two inhibin/activin β -subunits linked by a disulfide bridge.^{1–5)} Four β -subunits have been identified in mammals thus far: β_A , β_B , β_C , and β_E . Of all of the dimer that are theoretically possible, only activin A (β_A , β_A), activin AB (β_A , β_B), activin B (β_B , β_B) and activin C (β_C , β_C) have actually been isolated.^{6–11)}

Activin was originally identified as a stimulator of FSH (follicle-stimulating hormone) secretion.^{6–8)} Later, activins were shown to be expressed in various organs during development and in acquired conditions, and to have a wide variety of biological functions, including regulation of cell proliferation and differentiation, and induction of mesodermal tissues during amphibian development.^{12,13)}

Recent studies have shown that activin A plays several roles in rat liver *in vivo* and *in vitro*.^{13–17)} It inhibits mitogen-induced DNA synthesis *in vitro*,¹⁴⁾ induces hepatocellular apoptosis *in vivo* and *in vitro*,^{15–17)} and stimulates glycogenolysis *in vitro*.¹⁸⁾ Activin C has been reported to have no effect on DNA synthesis by liver tumor cell line HepG2.¹¹⁾ However, there have been no reports on the actions of activin AB and B in rat liver either *in vivo* or *in vitro*. It, therefore, seemed meaningful to examine the effect of activins AB and B on the liver functions that have been shown to be regulated by activin A. In this regard, we were especially interested in mitogen-stimulated DNA synthesis as a model system for its biological significance, because expression of β_A and β_B mRNA in rat liver has been found to change markedly in rat models of liver regeneration.^{14,19)} In this paper, we examined the effect of activins AB and B on DNA synthesis stimulated by epidermal growth factor (EGF) in primary cultured rat hepatocytes and compared their effect with that of activin A.

MATERIALS AND METHODS

Materials The materials used for cell isolation and cell culture were the same as reported by Tanaka *et al.*²⁰⁾ Recombinant human (rh) activin A was kindly provided by Dr. Y. Eto (Ajinomoto Co., Inc., Japan). Porcine activin A, activin AB, and activin B were purchased from Wako (Osaka, Japan). EGF was obtained from Toyobo (Osaka). [³H]thymidine (89.9 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, U.S.A.).

Cell Isolation and Monolayer Culture Parenchymal hepatocytes were isolated from adult male Wistar strain rats weighing 180–200 g by *in situ* perfusion of the liver with collagenase,²⁰⁾ and they were suspended, 2×10^5 cells/ml, in Williams' E medium containing 5% fetal bovine serum, 10 nM insulin, and 1 nM dexamethasone, and cultured at 0.2×10^5 cells/cm² in a 48-well and 6-well microplate (IWAKI) precoated with collagen type-1 AC in a humidified chamber at 37 °C under 5% CO₂ and 30% O₂ in air. Cells plated in the 48-well and 6-well plate were used to measure DNA synthesis and [¹²⁵I]rh activin A binding, respectively. After 2.5 h, the medium was replaced with serum- and hormone-free medium containing aprotinin (1 μ g/ml).

Measurement of [³H]thymidine Incorporation After 15 h, the medium was replaced with hormone-free medium containing aprotinin (1 μ g/ml) and 0.1% bovine serum albumin (BSA), and various hormones were added. After another 24 h, [³H]thymidine (0.626 μ Ci) and thymidine (67.6 ng) were added, and 10 μ g/ml aphidicolin was added at the same time. [³H]thymidine incorporation was measured by a modification of the method of Takai *et al.*²¹⁾ After 24 h, the cells were washed twice with cold phosphate-buffered saline and fixed with 0.25 ml of 10% trichloroacetic acid (TCA) overnight. The hepatocytes were solubilized in 0.25 ml of 1 N NaOH at 37 °C for 1 h, and 0.1 ml of the solution was re-

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moved for protein assay. The rest of the solution was mixed with 0.65 ml of 1 N NaOH, 12.8 μ l of bovine serum albumin (10 mg/ml), and then 0.2 ml of 100% TCA. The precipitate was washed with 10% TCA, and the DNA was hydrolyzed by heating the precipitate at 100 °C for 15 min in 1.0 ml of 10% TCA. The difference between the radioactivity in the hot-TCA soluble fraction with and without aphidicolin was calculated as dpm/mg protein, and the values were subtracted from the control value, and divided by the value from cells treated with EGF, and they are shown as percentages of [3 H]thymidine incorporation. Cell protein was measured by the method of Bradford.²²⁾

Measurement of Labeling Index After 15 h, the medium was replaced with hormone-free medium containing aprotinin (1 μ g/ml), and various hormones were added. Bromodeoxyuridine (Amersham Corp., Arlington Heights, IL, U.S.A.) was included in the medium from 24 to 48 h, and labeled nuclei were stained with anti-bromodeoxyuridine antibody according to the manufacturer's instructions.

Iodination of rh Activin A and Determination of Binding rh activin A (5 μ g/ml) was iodinated to a specific activity of approximately 10000 cpm/ng by the chloramine T method, as described previously.^{23,24)} Binding of [125 I]rh activin A to hepatocytes was measured by the modification of the method of Yasuda *et al.*¹⁴⁾ After 15 h of culture, hepatocytes were washed with 1 ml of binding buffer containing 50 mM Hepes (pH 7.4), 128 mM NaCl, 5 mM KCl, 1.2 mM CaCl_2 , 5 mM MgSO_4 , and 0.5% BSA, incubated with 0.6 ml of the binding buffer for 30 min at 37 °C, and then cooled down to 4 °C. [125 I]rh activin A (25 ng/ml) and various amounts of unlabeled activins were added to the hepatocytes, and after incubating them for 3.5 h at 4 °C, and they were washed four times with 1 ml of the ice-cold binding buffer and solubilized with 1 ml of 1 N NaOH for 1 h at 37 °C. Non-specific binding determined in the presence of a 10-fold excess of unlabeled rh activin A was measured. Specific binding was defined as the difference between the total binding observed and the nonspecific binding. The differences between the total binding observed and the binding determined in the presence of various concentrations of activins were divided by the specific binding. Values are shown as percentages of [125 I]rh activin A bound.

RESULTS

Effect of Activins A, AB, and B on DNA Synthesis Stimulated by EGF Figure 1 shows the dose-dependence of the effect of activin A, AB, and B on DNA synthesis stimulated by EGF and assessed by [3 H]thymidine incorporation. Activin AB at 6 ng/ml inhibited DNA synthesis, and almost maximal inhibition, approximately 40% inhibition was observed at 12.5 ng/ml. Activin A also inhibited DNA synthesis, consistent with another report.¹⁴⁾ The inhibition by activin A was detected at 3 ng/ml, and the inhibitory effect at 6 ng/ml was comparable to that of activin AB at 12.5 ng/ml, although the extent of the maximal inhibition by activin A and activin AB was almost same. Activin B, on the other hand, did not inhibit DNA synthesis up to 50 ng/ml. The effect of these activins on DNA synthesis was also examined by nuclear labeling. As shown in Table 1, activin A and AB (25 ng/ml) inhibited the increase in nuclear labeling induced

Fig. 1. Effect of Activins A, AB, and B on DNA Synthesis Stimulated by EGF

After 15 h culture, insulin (0.1 nM), EGF (20 ng/ml), and various concentrations of activins A (○), AB (△), and B (□) were added to the culture medium. Hepatocyte culture and the assay of DNA synthesis were performed as described in Materials and Methods. Values are means $\pm$ S.D. of the percentage of [3 H]thymidine incorporation in duplicate dishes in three experiments. [3 H]thymidine incorporation in the presence and absence of insulin and EGF was approximately 700000–850000 and 80000–120000 dpm/mg protein, respectively.

Table 1. Effect of Activins A, AB and B on the Increase in Nuclear Labeling in Response to EGF

Substance added	Nuclear labeling (%)
None	2.48 $\pm$ 0.346
EGF	18.8 $\pm$ 0.759
EGF+activin A	6.16 $\pm$ 1.44
EGF+activin AB	4.96 $\pm$ 0.881
EGF+activin B	18.1 $\pm$ 1.37

After 15 h culture, insulin (0.1 nM), EGF (20 ng/ml), and activins A, AB, and B (25 ng/ml) were added to the culture medium. Hepatocyte culture and measurements of nuclear labeling were performed as described in Materials and Methods. Values are means $\pm$ S.D. for duplicate dishes in three experiments.

Fig. 2. Effect of Activins A, AB, and B on [125 I]rh Activin A Binding to Hepatocytes

After 15 h culture, hepatocytes were incubated with [125 I]rh activin A in the presence of various concentrations of unlabeled activins A (○), AB (△) and B (□). Determination of binding was performed as described in Materials and Methods. Values are means $\pm$ S.D. for duplicate dishes in three experiments. Specific binding of [125 I]rh activin A was approximately 2000 cpm/plate.

by EGF. The extent of the inhibition by activin A and AB was almost same as shown by [3 H]thymidine incorporation. Activin B, on the other hand, had no effect on the increase in nuclear labeling. These findings are consistent with the results obtained by [3 H]thymidine incorporation.

Effect of Activins A, AB, and B on [125 I]rh Activin A Binding to Hepatocytes Figure 2 shows the effect of activins A, AB, and B on the binding of [125 I]rh activin A to hepatocytes. [125 I]rh activin A binding was not only inhibited

Fig. 3. Effect of Activin B on Inhibition of DNA Synthesis by Activin A

After 15 h culture, insulin (0.1 nM), EGF (20 ng/ml), activin A (12.5 ng/ml), and various concentrations of activin B were added to the culture medium. Hepatocyte culture and assay of DNA synthesis were performed as described in Materials and Methods. Values are means $\pm$ S.D. and shown as percentages of [³H]thymidine incorporation in duplicate dishes in three experiments.

by activin A and AB in a dose-dependent manner, but by activin B as well. At concentrations below 25 ng/ml the extent of the inhibition by activin B was even greater than by activin A and AB.

Effect of Activin B on the Inhibition of DNA Synthesis by Activin A As shown in Fig. 3, activin B had no effect on the inhibition of DNA synthesis by activin A up to 3-fold excess concentrations.

DISCUSSION

The results of this study show that stimulation of DNA synthesis by EGF was inhibited by activin AB (β_A, β_B) as well as activin A (β_A, β_B), but not by activin B (β_A, β_B) (Fig. 1, Table 1). These results indicate that the β_A subunit is essential for inhibition of DNA synthesis by activins. Furthermore, the detailed dose-dependency study showed that the increase in β_A subunit in the composition of activin results in reduction of the effective concentration for inhibition, because activin A inhibited DNA synthesis at lower concentrations than activin AB. Since β_A mRNA was increased at 24 h in rat liver after partial hepatectomy and remained high for at least 72 h,¹⁴ the β_A subunit seems to play an important role as a component of activin A and AB in the cessation of rat liver regeneration *in vivo* in the later phase.

There have been a few reports comparing the biological potency of activin A with that of activins AB and activin B. The ED₅₀ values of activin A have been found to be almost same as those of activin AB in several assays, including stimulation of FSH secretion, FSH receptor expression and luteinizing hormone receptor expression.¹⁰ The ED₅₀ value of activin A was also shown to be the same or about 25% lower than that of activin AB with regard to the induction of mouse erythroleukemia cell F5-5 differentiation.^{10,25} Compared with these findings, the biological potency of activin A in our study was greater than that of activin AB. The ED₅₀ values of activin B, on the other hand, have been found to be about 5-fold higher than those of activin A and AB in these assays.^{10,25} There is also a report that rh activin B (40 ng/ml) inhibits DNA synthesis by approximately 80 and 30%, by human prostate tumor cell lines LNCaP and DU145 respectively.²⁶ The magnitude of the inhibitions was almost the same or the same as that of rh activin A, depending on the cell type. These findings conflict with the results of our study

because activin B did not inhibit DNA synthesis even at 15-fold the ED₅₀ value of activin A (about 3 ng/ml). The source of the activins used in this study was the same as in several other studies.^{10,25} In the study using human prostate tumor cell lines, the effect of activin B was compared with that of activin A using the same recombinant human derived activins.²⁶ Therefore, these differences are probably attributable to differences in the cell models used in the studies.

To elucidate the mechanism of the unresponsiveness to activin B, we examined the effect of activin B on the binding of [¹²⁵I]rh activin A to hepatocytes. Our results showed that activin B is more effective at inhibiting the binding of [¹²⁵I]rh activin A than activins A or AB (Fig. 2), indicating the presence of common receptors with a higher affinity for activin B than for activins A or AB. However, activin B did not have any effect on DNA synthesis or the inhibitory action of activin A (Figs. 1, 3, Table 1), suggesting that the common receptors do not mediate the signal transduction leading to the inhibition of DNA synthesis. Therefore, additional receptors that are specific to activins A and AB may be present and may be responsible for mediating the signal transduction. Zhang *et al.* reported the existence of two classes of binding sites having apparent K_d values of 3×10^{-10} and 3.5×10^{-9} M in cultured rat hepatocytes.¹⁷ They suggested that the high-affinity binding sites may represent an oligomeric complex of the type I and type II receptor and that at least part of the low affinity binding site may represent cell-bound follistatin.¹⁷ A preliminary dose-response study showed that [¹²⁵I]rh activin A binding was almost saturated at 25 ng/ml, which is approximately equal to 1×10^{-9} M (data not shown). The ED₅₀ value of rh activin A for inhibition of DNA synthesis was approximately 25 ng/ml (data not shown), and higher than that of porcine activin A. Based on this evidence, the common receptors seem to represent part of the oligomeric complex rather than cell-bound follistatin. Interestingly, multiple subtypes in both type I and type II receptor have been identified,^{27–30} but the significance of this remains unclear. The specificity of receptor binding and the receptor-mediated signal transduction may depend on the subtype expressed in hepatocytes. Further study is required to clarify the mechanism of the inability of activin B to inhibit DNA synthesis, including characterization of common and specific receptors.

We recently found that the β_B mRNA level had increased at 1 h in the rat carbon tetrachloride model of liver regeneration, and that it peaked at 3 h and remained constant up to 24 h.¹⁹ This finding suggests that apart from the effects of activin B on DNA synthesis, activin B may play some other role in rat liver regeneration *in vivo*. Other possible regulatory activities of activin B include the modulation of hepatocyte mitogen levels, such as that of hepatocyte growth factor (HGF), which is synthesized in mesenchymal cells.³¹ In fact, we found that activin B decreased the elevation of HGF protein levels produced by phorbol ester in MRC-5 human lung fibroblasts (Niimi, S., unpublished results). Therefore, activin B may suppress rat liver regeneration *in vivo* by decreasing the level of HGF.

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