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 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)

Materials and Methods

Materials The materials used for cell isolation and cell culture were the same as reported by Tanaka et al. 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, and they were suspended, 2 × 10⁶ cells/ml, in Williams’ E medium containing 5% fetal bovine serum, 10 nm insulin, and 1 nm dexamethasone, and cultured at 37 °C in a humidified atmosphere containing 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 μg/ml).

Measurement of [3H]thymidine Incorporation After 15 h, the medium was replaced with hormone-free medium containing aprotinin (1 μg/ml) and 0.1% bovine serum albumin (BSA), and various hormones were added. After another 24 h, [3H]thymidine (0.626 μCi) and thymidine (67.6 ng) were added, and 10 μg/ml aphiidicolin was added at the same time. [3H]thymidine incorporation was measured by a modification of the method of Takai et al. After 24 h, the cells were washed twice with cold phosphate-buffered saline and fixed with 0.25 ml of 1 N NaOH at 37 °C for 1 h. 0.1 ml of the solution was re-
moved for protein assay. The rest of the solution was mixed with 0.65 ml of 1 N NaOH, 12.8 μl 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 [3H]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 μg/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 μg/ml) was iodinated to a specific activity of approximately 10000 cpm/ng by the chloramine T method, as described previously.23,24) Binding of [125I]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 CaCl2, 5 mM MgSO4, 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. [125I]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 [125I]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 [3H]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 by EGF. The extent of the inhibition by activin A and AB was almost same as shown by [3H]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 [3H]thymidine incorporation.

Effect of Activins A, AB, and B on [125I]rh Activin A Binding to Hepatocytes  Figure 2 shows the effect of activins A, AB, and B on the binding of [125I]rh activin A to hepatocytes. [125I]rh activin A binding was not only inhibited
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 (βα, ββ) as well as activin A (βα, βα), but not by activin B (βα, ββ) (Fig. 1, Table 1). These results indicate that the βα subunit is essential for inhibition of DNA synthesis by activins. Furthermore, the detailed dose-dependency study showed that the increase in βα 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 βα mRNA was increased at 24 h in rat liver after partial hepatectomy and remained high for at least 72 h,15 the βα 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 Kd 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^-8 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 ββ 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.39 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.

REFERENCES


