RAPID COMMUNICATION

Reciprocal Expression of mRNA for Inhibin β_C and β_A Subunits in Hepatocytes

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Abstract. Messenger RNA expression of activin β_C subunit in the liver was compared with that for β_A subunit before and after 70% hepatectomy. mRNA for β_C was abundantly expressed in the liver but decreased at 12 h and later after 70% hepatectomy, whereas that for β_A was increased 12 h after the hepatectomy. We also compared the expression of mRNA for β_A and β_C in cultured rat hepatocytes. mRNA for β_C subunit was abundantly expressed in the beginning of the culture but was reduced gradually after stimulation with epidermal growth factor. In contrast, mRNA for β_A subunit was undetectable before stimulation and was increased 24 to 48 h after stimulation with the mitogen. These results indicate that expression of mRNA for β_C and β_A is regulated differently. The role of activin C may be different from that of activin A in the liver.

Key words: Activin, Liver regeneration, Hepatocyte

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ACTIVINS belong to the transforming growth factor- β (TGF- β) supergene family and elicit diverse effects in various cell systems [1, 2]. In particular, activins modify growth, differentiation and survival of epithelial and mesenchymal cells. In the liver, activin A is expressed predominantly in parenchymal cells and acts as an autocrine inhibitor of cell growth [3]. Activin A also induces apoptosis in parenchymal liver cells [4, 5]. In cultured rat hepatocytes, the expression of activin β_A subunit is low at the beginning of the culture, but is increased 24 h after the stimulation by epidermal growth factor (EGF). The peak of the expression is observed in the middle to late G₁ phase [3]. Activin A released from hepatocytes exerts tonic inhibition of DNA synthesis [3, 6] so that activin A acts as a brake system for the G₁ progression of hepatocytes.

Expression of activin A is relatively low in intact liver, reduced immediately after 70% hepatectomy, and markedly increased 24 h after the hepatectomy [3]. When the effect of endogenous activin was blocked by an administration of follistatin, liver regeneration was accelerated [7]. Therefore, activin A also acts as a brake on liver regeneration *in vivo*.

Other subunits of activin have recently been identified [8–10]. Hötten *et al.* isolated a cDNA for the activin β_C subunit [9]. The amino acid sequence of activin β_C deduced from cDNA has 51% homology with that of activin β_A . Of particular interest is the fact that β_C mRNA is expressed predominantly in the liver [11]. This raises the possibility that activin β_C plays a significant role in the regulation of liver cell function including growth and differentiation. As an initial step in elucidating the role of activin β_C subunit in the liver, we studied the expression of mRNA for β_C subunit in the liver of the rat and mouse before and after hepatectomy. The results indicate that the pattern of expression mRNA for β_C is a mirror image of that for β_A .

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Materials and Methods

Materials

Recombinant human activin A was provided by Dr. Y. Eto of the Central Research Laboratory, Ajinomoto Inc. (Kawasaki, Japan). EGF was purchased from Collaborative Research (Lexington, MA). cDNA for the human β_C subunit was provided by Dr. G. Hötten of Biopharm (Heidelberg, Germany). Phenylephrine, glucagon, dexamethasone and insulin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical reagent grade.

Culture of parenchymal liver cells

Parenchymal liver cells were prepared from male Wistar rats (170–200g) by the methods described by Berry and Friend [12]. Cells were plated in 100-mm collagen-coated dishes at a density of 5×10^6 cells/well in William's E medium containing 10% fetal bovine serum, 10 nM insulin, 1 nM dexamethasone, streptomycin and penicillin. The cells were allowed to attach for 3–4 h, after which the medium was changed to serum free William's E medium containing 0.1 nM insulin, 1 nM dexamethasone, 0.1% bovine serum albumin and test agents. At the time points indicated, total RNA was extracted as described below. Cells were incubated at 37 °C under a humidified atmosphere of 95% air and 5% CO₂. Non-parenchymal cells were prepared as described by Shimaoka *et al.* [13]. Contamination of parenchymal cells in the preparation of non-parenchymal cells was less than 5%.

RNA extraction and Northern blot analyses

Total RNA was extracted from hepatocytes cultured with EGF, activin A or other agents for the time indicated with TRIzol Reagent (GIBCO BRL, Grand Island, NY, USA). Poly(A)⁺RNA was then isolated by using Oligotex-dT30 (Japan Synthetic Rubber, Tokyo, Japan). Total poly(A)⁺RNA (2 μ g) was denatured on a 1.2% agarose gel containing 2.2 mol/L formaldehyde and blotted onto a nylon membrane (Hybond-N+, Amersham Japan, Tokyo, Japan) by a capillary

blotting technique with 10 $\times$ sodium citrate buffer (SSC). The filter was prehybridized for 2 h at 42 °C in 50% (vol/vol) formamide, 5 $\times$ standard saline phosphate-EDTA (SSPE), 5 $\times$ Denhardt's solution, 100 μ g/ml denatured salmon sperm DNA, and 0.5% sodium dodecyl sulfate, and then hybridized for 16 to 24 h at 42 °C in the same buffer with a 0.37 kb EcoRI-XhoI fragment of cDNA for β_C that had been labeled with ³²P by random priming with a DNA labeling kit (-dCTP) (Pharmacia, Biotech Inc., USA). The same membrane was then washed and hybridized with a 1.9-kb XhoI fragment of cDNA of human β_A provided by Dr. Y. Eto. The membranes were then washed to a stringency of 0.1 $\times$ SSC and 0.1% SDS at room temperature for 5–10 min then at 55 °C for 30 min and subjected to autoradiography, analyzed with a FUJIX BAS2000 and photographed with a FUJIX Pictography 3000 (Fuji Photo Film, Japan).

Partial hepatectomy

Partial hepatectomy (70%) was performed in the rat and mouse according to Higgins and Anderson [14]. Under diethylether anesthesia, the peritoneal cavity was entered through a median incision. After hepatectomy, blood coagula was wiped thoroughly with gauze, and the incision was closed with sutures. At various times indicated after the operation, the remaining liver tissue was removed and total RNA was extracted, and then total poly(A)⁺ RNA (2 μ g) was isolated by the methods described above.

Results

mRNA expression of β_C subunit in remnant liver after hepatectomy

We studied the steady state expression of mRNA for β_C and β_A subunits in the liver of the rat before and after 70% hepatectomy. As shown in Fig. 1, the expression of mRNA for β_C was high in the liver before hepatectomy. Expression of β_C was decreased by 12 h after the hepatectomy, became minimal by 48 h, and recovered slightly by 72 h. In contrast, mRNA for β_A was undetectable before hepatectomy, and became detectable by 12 h after and later. The pattern of expression of β_C subunit

was nearly identical in the mouse liver before and after the hepatectomy (data not shown).

Expression of mRNA for β_C and β_A in cultured rat hepatocytes

We then studied the expression of mRNA for β_C and β_A subunits in cultured rat hepatocytes. As shown in Fig. 2, the expression of mRNA for β_C is

high at the beginning of the culture. When hepatocytes were incubated with 1 nM EGF, mRNA for β_C expression was gradually reduced. Reduction of β_C expression was evident at 12 h and became undetectable at 72 h. In contrast, the expression of mRNA for β_A is quite low at the beginning of the culture. Consistent with our previous report [3], mRNA for β_A subunit was increased when cells were incubated with 1 nM

Fig. 1. Time course of changes in expression of mRNA for β_C and β_A subunits in the liver before and after 70% hepatectomy. Seventy percent hepatectomy was performed in the rat. mRNA was extracted from the liver before and after the hepatectomy. The expression of mRNA for β_C and β_A subunits was then measured.

Fig. 2. Time course of changes in the expression of mRNA for β_C and β_A subunits in cultured hepatocytes stimulated by EGF. Rat hepatocytes were incubated with 1 nM EGF for the indicated. mRNA was then extracted and the expression of mRNA for β_C and β_A subunits was determined by Northern blotting.

EGF. mRNA for β_A was increased at 24 h and peaked at 48 h after the stimulation with EGF. Expression of mRNA for β_A and β_C is reciprocal. It should be noted that mRNA for β_C was detected mainly in parenchymal cells and the expression of β_C in non-parenchymal liver cells was much lower (Fig. 3). Under the same conditions, mRNA for β_A was not detected by Northern blotting either in parenchymal or non-parenchymal cells.

To examine the possibility that β_C mRNA was decreased by activin A produced by hepatocytes, we studied the effect of activin A on the expression of mRNA for β_C . Activin A did not affect the expression of β_C (data not shown). In the next set of experiments, we studied the effect of α -adrenergic stimulant. Norepinephrine acts as a co-mitogen in hepatocytes and plays a significant role in liver regeneration after hepatectomy [14, 15]. As shown in Fig. 4, the expression of mRNA for β_C was reduced after stimulation with phenylephrine. The reduction was significant at 2 h and later. Note that the mRNA level did not change significantly in the absence of phenylephrine. mRNA for β_A remained undetectable before and after the stimulation with phenylephrine.

Similarly, the expression of mRNA for β_C was reduced by the addition of glucagon, a co-mitogen known to act via the cyclic AMP messenger system (data not shown). Again, mRNA for β_A remained undetectable under the same conditions.

Discussion

The present results show that activin β_C subunit is expressed predominantly in parenchymal liver cells and the expression levels are changed by various agents that modify hepatocyte growth. Most importantly, the pattern of expression of activin β_C subunit is quite different from that of activin β_A subunit. In the normal liver, β_C subunit is expressed abundantly whereas mRNA expression of the β_A subunit is quite low. Twelve hours after partial hepatectomy, mRNA for β_A subunit is increased but mRNA for β_C subunit is down-regulated. Similar patterns of expression of β_C and β_A subunits are observed in cultured hepatocytes stimulated by EGF. mRNA for β_A and β_C subunits is therefore regulated differently during the cell-cycle of hepatocytes *in vivo* and *in vitro*. Previous studies in our laboratory provided

Fig. 3. Expression of mRNA for β_C and β_A subunit in liver cells. Parenchymal (PC) and non-parenchymal cells (NPC) were prepared from the rat as described in Methods, and the expression of mRNA for β_C and β_A measured by Northern blotting.

Fig. 4. Time course of changes in the expression of mRNA for β_C and β_A subunits in cultured hepatocytes stimulated by phenylephrine. Rat hepatocytes were incubated for indicated time with 10 μ M phenylephrine for the time indicated. The expression of mRNA for β_C and β_A subunits was determined by Northern blotting.

evidence that activin A acts as an autocrine growth inhibitor of hepatocytes and attenuate G_1 progression both *in vivo* and *in vitro* [3, 6, 7, 17]. Indeed, activin A is released in the middle to late G_1 phase and inhibits the entry of hepatocytes into the S phase [3].

It is possible that β_C subunits form dimers, that is, activin C, but it is not yet known whether β_C subunits actually form dimers *in vivo* or not. Moreover, the function of β_C dimer, activin C, is not known. Nevertheless, the present results suggest that the function of activin C, if any, would be different from that of activin A. It has been proposed that the mass of the liver is regulated by a soluble inhibitory factor released from the liver. This putative factor, called 'liver chalone' [18], inhibits growth of hepatocytes. According to the chalone hypothesis, the liver chalone is expressed predominantly in the liver and tonically blocks cell growth of hepatocytes under normal circumstances [18]. Upon reduction of the liver mass, either the production or action of the chalone is attenuated and thereby growth of hepatocytes is initiated.

Several observations indicate that activin C may be a good candidate for the liver chalone. For example, expression of β_C subunit is predominant in the liver [11]. mRNA for β_C is abundant in intact liver but is markedly reduced shortly after partial hepatectomy. Furthermore, mRNA for β_C is reduced *in vitro* by liver cell mitogens. Given that members of the TGF- β superfamily regulate growth and differentiation in various cell systems, it is tempting to speculate that activin C acts as the putative liver chalone. Clearly further studies are necessary to elucidate the role of activin C in the liver.

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References

1. Ying SY (1988) Inhibins, activins, and follistatins: Gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrine Rev* 9: 267–293.
2. De Paolo LV, Bicsak TA, Erickson GF, Shimasaki S, Ling N (1991) Follistatin and activin: A potential intrinsic regulatory system within diverse tissues. *Proc Soc Exp Biol Med* 198: 500–512.
3. Yasuda H, Mine T, Shibata H, Eto Y, Hasegawa Y, Takeuchi T, Asano S, Kojima I (1993) Activin A; an autocrine inhibitor of initiation of DNA synthesis in rat hepatocytes. *J Clin Invest* 92: 1491–1496.
4. Schwall RH, Robbins K, Jardieu P, Chang L, Lai C, Terrell TG (1993) Activin induces cell death in hepatocytes *in vivo* and *in vitro*. *Hepatology* 18: 347–356.
5. Zhang YQ, Kanzaki M, Mashima H, Mine T, Kojima I (1995) Norepinephrine reverses the effect of activin A on DNA synthesis and apoptosis in rat hepatocytes. *Hepatology* 23: 288–293.
6. Zhang YQ, Mashima H, Kanzaki M, Shibata H, Kojima I (1997) Assessment of the role of activin A and TGF- β in the regulation of AML12 cell growth. *Hepatology* 25: 1370–1375.
7. Kogure K, Omata W, Kanzaki M, Zhang YQ, Yasuda H, Mine T, Kojima I (1995) A single intraportal administration of follistatin accelerates liver regeneration in partially hepatectomized rats. *Gastroenterology* 108: 1136–1142.
8. Oda S, Nishimatsu S, Murakami K, Ueno N (1995) Molecular cloning and functional analysis of a new activin β subunit. *Biochem Biophys Res Commun* 210: 581–588.
9. Hötten G, Neidhardt H, Schneider C, Pohl J (1995) Cloning of a new member of the TGF- β family: A putative activin β_C chain. *Biochem Biophys Res Commun* 206: 608–613.
10. Fang J, Yin W, Smiley E, Wang SQ, Bonadio J (1996) Molecular cloning of the mouse activin β_E subunit gene. *Biochem Biophys Res Commun* 228: 669–674.
11. Schmitt J, Hotten G, Jenkins NA, Gilbert DJ, Copeland NG, Pohl J, Schrewe H (1996) Structure, chromosomal localization, and expression analysis of the mouse inhibin/activin beta C gene. *Genomics* 32: 358–366.
12. Berry MN, Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells. *J Cell Biol* 43: 506–520.
13. Shimaoka S, Nakamura T, Ichihara A (1987) Stimulation of growth of primary cultured adult rat hepatocytes without growth factor by cocultivation with non-parenchymal cells. *Exp Cell Res* 172: 228–242.
14. Higgins GM, Anderson RM (1931) Experimental

- pathology of the liver. *Arch Pathol* 12: 186–202.
15. Cruise JL, Houck KA, Michalopoulos GK (1985) Induction of DNA synthesis in cultured rat hepatocytes through stimulation of α_1 -adreno-receptor by norepinephrine. *Science* 227: 749–751.
 16. Cruise JL, Michalopoulos GK (1985) Norepinephrine and epidermal growth factor: Dynamics of their interaction in stimulation of hepatocytes DNA synthesis. *J Cell Physiol* 125: 45–50.
 17. Kogure K, Zhang YQ, Kanzaki M, Omata W, Mine T, Kojima I (1996) Intravenous administration of follistatin: Delivery to the liver and effect on liver regeneration. *Hepatology* 24: 361–366.
 18. Bullough WS (1962) Control of mitotic activity in adult mammalian tissues. *Biol Res Camb Philos Soc* 37: 307–342.