Comparison of the Function of the β_C and β_E Subunits of Activin in AML12 Hepatocytes

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Abstract. To investigate the function of the β_C and β_E subunits of activin, we overexpressed these subunits in AML12 cells, a normal hepatocyte cell line, using adenovirus vector. Overexpression of the β_C subunit increased [3 H]thymidine incorporation and the cell number. In contrast, both [3 H]thymidine incorporation and the cell number were reduced in the β_E overexpressing cells. When AML cells overexpressing the β_E subunit were cultured in medium containing 1% serum for 48 h, many of the cells died by apoptosis, whereas cells overexpressing the β_C subunit or β -galactosidase survived in the same condition. To examine dimer formation, the β_C and β_E subunits were expressed in AML12 cells. In these cells, the β_C homodimer, the β_E homodimer and the β_C - β_E heterodimer were detected. When the expression level of the β_E subunit was increased, formation of the β_E homodimer was increased, while formation of the β_C - β_E heterodimer was slightly reduced. Overexpression of the β_E subunit did not significantly affect the formation of the β_C homodimer. These results indicate that the β_C and β_E subunits form homo- and heterodimers, and that the functions of the two subunits are quite different.

Key words: Activin, Liver, Growth, Heterodimer

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ACTIVINS are multifunctional cytokines belonging to the transforming growth factor- β (TGF- β) superfamily [1] and regulate growth and differentiation of various types of cells [2–4]. Structurally, activins are dimeric proteins comprising two β subunits of inhibin. There are at least four types of β subunits in mammals [1, 5–7], namely β_A , β_B , β_C and β_E . Consequently, there are many possible combinations of homo- and heterodimers for these subunits. It is, however, not totally clear how many types of activins are actually formed in the body. It has been shown that β_A and β_B subunits form homo- and heterodimers, activin A, activin B and activin AB [1, 8]. It has been also shown that β_A and β_C subunits form heterodimer activin AC [9] but it is still uncertain whether or not other types of heterodimers

are actually formed.

Four of the β subunits are expressed in the liver [7, 10]. Regarding their function, the function of activin A is well known. Activin A is produced dominantly in parenchymal cells and acts as an autocrine inhibitor of hepatocyte growth [11]. The expression of activin A is relatively low in normal liver, but the production of activin A is increased 12 to 24 hrs after partial hepatectomy [11], which tonically inhibits liver regeneration [12]. Activin A also modulates differentiation during liver regeneration. For example, we recently reported that activin A acts coordinately with vascular endothelial growth factor and augments formation of hepatic sinusoid during liver regeneration [13]. Activin A is also produced in non-parenchymal cells such as hepatic stellate cells. These cells are activated by activin A and the production of collagen is markedly augmented [14], which may lead to hepatic fibrosis. In addition to the β_A subunit of activin, the β_C and β_E subunits are abundantly expressed in the liver [7, 10]. Under normal conditions, the expression of the β_C and β_E sub-

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units is high but is reduced after partial hepatectomy [7, 10, 15]. Hence, the expression pattern of the β_C and β_E subunits is reciprocal to that of the β_A subunit. This raises the possibility that the functions of these subunits may be different from that of the β_A subunit. In this regard, we reported recently that the β_C subunit antagonizes the β_A subunit in many respects [16]. Intracellularly, the β_C subunit forms a heterodimer with the β_A subunit and thereby reduces the amount of the β_A - β_A homodimer activin A. The β_C - β_C dimer, activin C, also acts as a ligand and promotes cell growth [16]. It is not yet known whether the β_E subunit has a similar function. In the present study, we investigated the function of the β_E subunit using AML12 cells, a normal hepatocyte cell line [17].

Materials and Methods

Materials

[³H]Thymidine was purchased from NEN Life Science Products, Inc. (Boston, MA, USA). Anti-FLAG M2 monoclonal antibody was purchased from Sigma (St. Louis, MO, USA) and anti-His (C-term) antibody was from Invitrogen (Carlsbad, CA, USA).

Cell culture

AML12 cells, a hepatocyte cell line derived from a TGF- α transgenic mouse [17], were provided by Dr. Hitoshi Takagi of Gunma University, who received the cells from Professor Nelson Fausto of Brown University. AML12 cells were cultured in Dulbecco's modified eagle's medium (DMEM)/F-12 (GIBCO-BRL, NY, USA) containing 5 mM NaHCO₃, 100 U/ml penicillin, 0.1 μ M streptomycin, ITSTM premix (5 mg/l insulin, 5 mg/l transferrin and 5 μ g/l selenous acid) (Becton Dickinson, MA, USA) and 10% FBS at 37°C under a humidified condition of 95% air and 5% CO₂ [17]. They were routinely subcultured using trypsin-EDTA every 5 or 7 days.

Preparation of adenovirus vectors

To introduce the His tag into the carboxyl terminus of β_E , we amplified a fragment of β_E by PCR using the following primers for mouse inhibin β_E : a 5' end-specific forward primer (5'-CACCATGAAGCTTCCA

AAAGCCCAG-3') and a 3' end-specific reverse primer (5'-CTAGTGATGGTGATGGTGATGGCTGCAGCCACAGGCCTCTACTAC-3'). Reactions contained 5 μ l of 10 $\times$ PCR buffer, 1 μ l of MgCl₂ (50 mM), 1.5 μ l of dNTP mixture, 1 μ l of 5'-primer, 1 μ l of 3'-primer, 0.5 μ l of PLATINUM[®] Pfx DNA polymerase (Invitrogen), and 1 μ l of cDNA. Samples were incubated at 94°C for 5 min, followed by 5 cycles at 94°C for 1 min, at 70°C for 1 min and 72°C for 1 min and followed by 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min with final extension at 72°C for 10 min in a Perkin-Elmer DNA thermal cycler. The inhibin β_E cDNA clone was subcloned into the GatewayTM entry-Vector (Invitrogen). The PCR-generated constructs were verified by sequencing the amplified region. The β_E entry vectors were purified using ConcertTM High Purify Plasmid Purification System (Marligen Bioscience, Ijamsville, MD, USA) according to the manufacturer's instructions.

The cDNA clones for the mouse β_C [16] and β_E subunits were subcloned into the ViraPowerTM adenoviral vector (Invitrogen) according to the manufacturer's instructions. Briefly, 300 ng of the entry clone containing inhibin β_C or β_E cDNA was mixed with 150 ng of the ViraPowerTM Adenoviral vector, 4 μ l of 5X LR ClonaseTM reaction buffer (Invitrogen) and TE buffer (pH 8.0). The solutions were mixed with 4 μ l of LR ClonaseTM enzyme (Invitrogen) and incubated at 25°C for 60 min. Two μ l of the proteinase K solution was added to each sample to terminate the reaction and incubated at 37°C for 10 min. For transformation, 1 μ l of each sample was mixed with 50 μ l of competent cells, incubated on ice for 30 min and incubated at 42°C for 30 sec for heat-shock. The transformed cells were plated onto an LB plate containing 100 μ g/ml ampicillin. The adenovirus containing the β_C or β_E subunit was purified using the ConcertTM High Purity Plasmid Purification System (Marligen Bioscience). To expose the viral inverted terminal repeats, the adenovirus was digested with Pac I restriction enzyme, and the adenovirus was transfected into 293 cells with LipofectamineTM 2000 (Invitrogen). The adenovirus vectors containing the β_C (Adex- β_C) and β_E (Adex- β_E) were purified using CsCl gradient centrifugation and the titer was measured in 293 cells. To determine the optimal multiplicity of infection (M.O.I.) the adenovirus expressing β -galactosidase (Adex-LacZ) [16] was transfected.

Immunohistochemistry

Cells transfected with Adex- β_E and Adex-LacZ were cultured on coverglasses, fixed for 30 min in 3% paraformaldehyde/PBS, washed twice with 50 mM glycine/PBS, blocked for 60 min with Blocking Ace (Snow Brand, Tokyo, Japan), and incubated with Anti-His (C-term) antibody (Invitrogen) at room temperature for 1 h. After washing twice with PBS and once with 0.1% Tween 20/PBS, the coverglasses were incubated with secondary antibody conjugated with Alexa Fluor 488 obtained from Molecular Probes, Inc. [18] (Eugene, OR, USA).

Measurement of DNA synthesis and apoptosis

DNA synthesis was assessed by measuring [3 H]thymidine incorporation as described previously [16]. Changes in the viable cell number were determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [19]. To measure cell growth, cells were plated at a density of 10^5 cells/well in a 24-well plate in DMEM/F12 medium containing 10% FBS. At 6 h after seeding, the cells were incubated for 2 h with serum-free DMEM containing Adex- β_C , Adex- β_E or Adex-LacZ. Infected cells were then washed and cultured for 48 h in medium containing 1% FBS by changing the medium every 12 h. [3 H]Thymidine was added to the last 6 h and [3 H]thymidine incorporation was measured as described previously [16]. Apoptosis was assessed by TUNEL staining using an apoptosis *in situ* detection kit (Wako, Osaka Japan). At 6 h after seeding on coverglass, cells were infected with Adex- β_E , Adex- β_C or Adex-LacZ as described above and cultured for 72 h in medium containing 1% FBS by changing the medium every 12 h.

Immunoblotting

Cells were washed twice with ice-cold PBS, suspended in $2\times$ sample buffer (0.125 M Tris-HCl (pH 6.8), 4% sodium dodecylsulfate (SDS), 20% glycerol) and heated to 100°C for 3 min. After centrifugation at room temperature, the supernatant was collected, and the protein concentration was determined by using BCATM Protein Assay Reagent (Pierce, Rockford, IL, USA). Seventy micrograms of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10–20% gradi-

ent gel and transferred to a polyvinylidene difluoride membrane (Nihon Millipore, Yamagata, Japan) by electroblotting. To reduce nonspecific antibody binding, the membrane was blocked with 3% BSA at room temperature for 1 h. The membrane was then washed with TBST buffer and incubated with Anti-His antibody at room temperature for 1 h. After washing with TBST, the membrane was incubated with peroxidase-linked secondary antibody (Amersham Biosciences, Bucks, UK) and imaged by LAS 3000 (Fuji Film, Kanagawa, Japan) using ECL Western blotting detection reagent (Amersham).

Results

Transfection of the β_C or β_E subunit

Transfection of adenovirus encoding β -galactosidase (Adex-LacZ) did not affect DNA synthesis (Fig. 1A). Note that at 30 M.O.I., more than 95% of the cells expressed β -galactosidase (data not shown). We then infected the adenovirus vector encoding the β_E subunit (Adex- β_E) to AML12 cells. As shown in Fig. 1A, transfection of the β_E subunit inhibited DNA synthesis as assessed by measuring [3 H]thymidine incorporation at a virus titer of 30 M.O.I. At 50 M.O.I., DNA synthesis was approximately 50% of that of the control. In contrast, infection of the adenovirus encoding the β_C subunit (Adex- β_C) increased the DNA synthesis (Fig. 1A). At 50 M.O.I., DNA synthesis was approximately 150% of that of the control. Transfection of Adex- β_E also reduced the cell number, whereas transfection of the β_C subunit significantly increased the cell number (Fig. 1B).

To examine whether transfection of the β_E subunit induced apoptosis, infected cells were incubated for 48 h in medium containing 1% serum. Cells infected with Adex-LacZ or Adex- β_C survived for 48 h whereas some of the cells infected with Adex- β_E died. Nuclear fragmentation was observed in Adex- β_E -transfected cells (Fig. 2A). Fig. 2B shows the number of TUNEL-positive cells in β_E -transfected cells. At 50 M.O.I., more than 50% of the cells became TUNEL-positive. In sharp contrast, TUNEL-positive cells were not detected in β_C -transfected cells (Fig. 2B).

Fig. 1. Comparison of the effects of transfection of the β_C and the β_E gene on cell growth. A: AML12 cells were infected with different dose of Adex-LacZ (Δ), Adex- β_C ($\circ$) and Adex- β_E ($\bullet$). The cells were incubated in 1% serum containing medium and [3 H]thymidine incorporation was measured 48 h later. Values are the mean $\pm$ S.E. for three experiments each done in triplicate. *: $p < 0.05$ vs Adex-LacZ. B: AML12 cells were infected with 50 M.O.I. Adex-LacZ, Adex- β_C and Adex- β_E , and the number of viable cells was assessed by MTT assay. Values are the mean $\pm$ S.E. for four experiments. *: $p < 0.05$ vs Adex-LacZ.

Fig. 2. Apoptosis induced by transfection of the β_E gene. A: AML12 cells were infected with 10 M.O.I. of Adex- β_E and the cells were incubated for 24 h in medium containing 1% serum. The cells were then stained with anti-His antibody (red). Nuclei were stained with DAPI. Arrows show condensation of chromatin and nuclear fragmentation. A cell not expressing the β_E subunit (*) has a normal nucleus. B: AML12 cells infected with different doses of Adex- β_C ($\circ$) or Adex- β_E ($\bullet$) were incubated in serum-free medium for 48 h and the number of TUNEL-positive cells was counted. Values are the mean $\pm$ S.E. for four experiments. *: $p < 0.01$ vs Adex- β_C .

Transfection of the β_C and β_E subunit

We then transfected the β_C and β_E subunits simultaneously to examine interaction of the two subunits. Fig. 3 depicts the changes in the DNA synthesis in cells transfected with 30 M.O.I. Adex- β_C and different doses of Adex- β_E . In cells transfected with 30 M.O.I. Adex- β_C , Adex- β_E reduced DNA synthesis in a dose-dependent manner. When cells were transfected with 30 M.O.I. Adex- β_E and different doses of Adex- β_C , DNA synthesis was only slightly affected by the Adex- β_C (data not shown).

Dimer formation

We next assessed the dimer formation of the β_E subunit. To determine whether or not the β_C and β_E subunits form a heterodimer, we infected the cells with 30 M.O.I. Adex- β_C and 10 M.O.I. Adex- β_E , and immunoblotting of the cell lysate was performed using anti-His antibody. As shown in Fig. 4A, a significant amount of β_C - β_E heterodimer was observed. Also, the β_E - β_E homodimer as well as the β_E monomer and pro- β_E were observed. When the titer of Adex- β_E was increased to 50 M.O.I., the amount of β_E - β_E dimer, the β_E

monomer and pro- β_E increased significantly while the amount of β_C - β_E heterodimer was slightly reduced. Formation of the β_C homodimer measured using anti-FLAG antibody was not affected significantly by the overexpression of the β_E subunit (Fig. 4B).

Discussion

In the present study, we compared the function of the β_C and β_E subunits of activin. To this end, we overexpressed the β_C and β_E genes in AML12 hepatocytes by using adenovirus vector. The results clearly show that the effects of overexpression of the two subunits are quite different. First, overexpression of the β_C subunit promoted DNA synthesis and increased the cell number, whereas overexpression of the β_E subunit inhibited DNA synthesis and reduced the cell number. These effects were dependent on the titer of the adenovirus

Fig. 3. Effect of transfection of β_C and β_E subunits on DNA synthesis

AML12 cells were infected with 30 M.O.I. Adex- β_C and various doses of Adex- β_E . The cells were incubated for 48 h in medium containing 1% serum and [³H]thymidine incorporation was measured 48 h later. Values are the mean $\pm$ S.E. for four experiments. *: $p < 0.05$ Adex- β_C alone.

Fig. 4. Dimer formation of the β_C and β_E subunits

AML12 cells were infected with various doses of Adex- β_C and Adex- β_E . Cells were incubated for 24 h and cell lysate was separated by SDS-PAGE. Immunoblotting was performed using anti-His antibody (A) and anti-flag antibody (B).

vectors. Secondly, overexpression of the β_E but not the β_C subunit induced apoptosis in AML12 cells cultured in medium containing low concentration of serum.

In our experimental condition, the expression level of the endogenous β_A subunit was rather low and the expression of β_B was not detected [16]. Therefore, interaction of overexpressed β_C and β_E subunits with endogenous β_A or β_B subunit may have been minimal. Given that the β subunit monomer has little affinity with activin receptors [20], it can be concluded that the dimers containing the β_C and/or β_E subunits altered cell growth and survival of AML12 cells. As shown in Fig. 4, the β_C and β_E subunits form a heterodimer, activin CE, in hepatocytes. This is the first demonstration showing that the β_C and β_E subunits form a heterodimer. Consequently, three types of dimers, β_C - β_C , β_C - β_E and β_E - β_E were actually formed in AML12 cells. Because the β_C and β_E subunits form a heterodimer, the composition of dimers produced in hepatocytes is influenced by the relative amount of the subunits expressed. We have reported that activin C, the β_C - β_C dimer, increases DNA synthesis in AML12 cells [16]. The present observation that overexpression of the β_C subunit increases DNA synthesis is in agreement with our previous report [16]. When the β_E subunit is overexpressed, it did not significantly affect the amount of the β_C - β_C dimer formed. In this regard, the inhibition of DNA synthesis by overexpression of the β_E subunit cannot be explained by reduction of the β_C - β_C homodimer. It rather suggests that the β_E - β_E homodimer activin E has an inhibitory effect on DNA synthesis. In addition, the apoptosis-inducing activity of the β_E subunit overexpression cannot be explained by the reduction of the β_C - β_C homodimer. It is therefore likely that the β_E - β_E homodimer activin E has its own function. Presumably, activin E may act to inhibit DNA synthesis in serum-containing medium and induce apoptosis in medium containing low concentrations of serum. Hence, the action of activin E resembles that of activin A. At present, we are not certain whether or not activin E acts on the activin receptor. Further study is neces-

sary to address this issue.

As shown in Fig. 3, overexpression of the β_E gene reduced DNA synthesis in β_C -expressing cells while overexpression of the β_C gene in β_E -expressing cells did not affect DNA synthesis. The difference may result from the difference in the potency of activin C and activin E. Results of our previous study indicate that activin C has a stimulatory effect on DNA synthesis but the effect is rather small [16].

The present results provide new insights into the role of the β_C and β_E subunits during liver regeneration. The β_C and β_E subunits of activin are abundantly expressed in normal liver [7, 10], but after partial hepatectomy, the expression of the β_C and β_E subunits is reduced gradually [7, 10, 15]. If the two subunits are expressed to the same extent in normal liver and are reduced proportionally after partial hepatectomy, it is reasonable to assume that the dominant form of activin dimer is the β_C - β_E heterodimer. On the other hand, if the expression levels of the β_C and β_E differ considerably, either the β_C or β_E homodimer is formed, which would promote or inhibit proliferation of hepatocytes depending upon the type of dimer synthesized. Accordingly, the relative amount of the β_C and β_E subunits is critical. Changes in the expression of the β_C and β_E subunits need to be elucidated more precisely to better understand the significance of these subunits during liver regeneration. Matzuk *et al.* generated mice lacking the β_C and/or β_E gene and found no abnormality in the liver [21]. Given that multiple subunits of activin and related ligands are expressed in the liver, it is possible that the defect in the β_C and/or β_E subunit gene may have been compensated for in their knock out mice. We thus need to assess the functions of these subunits by different approaches.

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