

Role of activin A in carbon tetrachloride-induced acute liver injury

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Abstract

AIM: To investigate the expression and role of activin A in a mouse model of acute chemical liver injury.

METHODS: Acute liver injury in C57BL/6 male mice was induced by intraperitoneal injection with carbon tetrachloride (CCl₄) (0.5 mL/kg, body weight) dissolved in olive oil (1:19 v/v). Mice were sacrificed 1, 3, 5 and 7 d after the treatment. The levels of alanine aminotrans-

ferase (ALT) and aspartate aminotransferase (AST) in serum were examined and pathological changes of liver observed by hematoxylin and eosin staining to evaluate the liver injury. Activin A protein levels in serum and hepatic tissue homogenate of mice were detected by enzyme-linked immunosorbent assay, and the expression pattern of activin A protein in livers of mice was examined by immunohistochemistry. Activin type II A receptor (ActR II A) and Smad3 expressions in the liver were analyzed by real-time quantitative reverse transcription-polymerase chain reaction. In order to further investigate the role of activin A, we also utilized activin A blocking experiment by anti-activin A antibody (500 µg/kg, body weight) injection into mouse tail vein.

RESULTS: In CCl₄-treated mice, serum ALT and AST levels were significantly increased, compared with that in control mice ($P < 0.01$). Furthermore, the serious necrosis was observed around hepatic portal areas in CCl₄-treated mice. Simultaneously, activin A levels in serum and hepatic tissue homogenate of mice treated with CCl₄ for 1, 3 and 5 d increased significantly, compared with that in control mice ($P < 0.01$). Activin A protein expression in hepatocytes not within the necrotic area was also upregulated in mice following CCl₄ treatment. Not only activin A, but also ActR II A and activin signaling molecule Smad3 mRNA expressions in injury liver induced by CCl₄ were significantly higher than that in control liver. In addition, levels of serum ALT and AST in CCl₄-treated mice were significantly decreased by injection of anti-activin A antibody to block endogenous activin A action, compared with that in CCl₄-treated mice by injection of immunoglobulin G instead of anti-activin A antibody ($P < 0.01$), and the severity of liver injury was also reduced remarkably.

CONCLUSION: These data show that activin A is involved in CCl₄-induced acute liver injury. Blocking activin A actions may be a therapeutic approach for acute liver injury.

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Key words: Liver injury; Carbon tetrachloride; Activin A; Immunohistochemistry

Core tip: The objective of this study was to investigate the expression and role of activin A in acute liver injury. A carbon tetrachloride (CCl₄)-induced acute liver injury mouse model was used. Liver injury effects were examined by measuring alanine aminotransferase and aspartate aminotransferase levels in serum and liver pathological changes. Activin A protein expression levels were detected by enzyme-linked immunosorbent assay and immunohistochemistry. Activin type II A receptor and Smad3 expressions in liver were analyzed by real-time quantitative reverse transcription-polymerase chain reaction. We found that activin A is involved in CCl₄-induced acute liver injury, suggesting activin A could be a potential therapeutic option for acute liver injury diseases.

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INTRODUCTION

Activin A, a member of the transforming growth factor- β (TGF- β) superfamily, has a wide range of biological roles^[1-3]. It is distributed widely in various tissues and produced by numerous cells including macrophages, T-helper 2 cells and hepatocytes^[4-6]. It plays important roles in regulation of pituitary hormone release, neuron survival, hematopoiesis and the early development of embryos^[7-10]. The expression of activin A is closely related to liver diseases, and abnormal expression of activin A and its signal proteins are found during the development of virus hepatitis, hepatic fibrosis, liver cancer and other diseases^[11-14]. Activin A affects the function of hepatocytes by inhibiting DNA synthesis while stimulating the synthesis of the extracellular matrix of hepatic stellate cells, which can result in the hepatic fibrosis^[2,11,12].

The liver is an important metabolic organ in the body, and can be injured by various factors, which include viral infection, trauma, and chemical reagents. Carbon tetrachloride (CCl₄)-induced liver injury is a classic model of chemical liver injury in mice^[15]. Activin A expression increased significantly in CCl₄-induced chronic liver injury in mice, marking it as an important factor in liver fibrosis development^[16]. However, it is still unclear whether activin A is involved in the process of CCl₄-induced acute chemical liver injury.

In the present study, the expression and role of activin A were investigated in CCl₄-induced acute chemical liver injury in mice. Further, the role of activin A in the process of acute liver injury was confirmed by *in vivo* blockade of activin A action.

MATERIALS AND METHODS

Reagents

CCl₄ was purchased from Beijing Chemical Factory (batch number 20050106). Olive oil was obtained from Beijing KeLipei Tsui olive oil Development Centers. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kit was provided by NJBI (Nanjing, China). Anti-activin A antibody was obtained from Sigma Company. Trizol reagent was provided by Invitrogen Corporation. SYBR fluorescence quantitative reverse transcription-polymerase chain reaction (PCR) kit was purchased from Takara Company.

Animals

C57BL/6 male mice were provided by Animal Center of Jilin University (Changchun, China). All animal experiments were performed following an institutionally approved protocol in accordance with the Jilin University Guide for the care and use of laboratory animals.

Preparation of CCl₄-induced acute liver injury mouse model

C57BL/6 mice were randomly divided into the olive oil control group and the CCl₄ group ($n = 24$). In the control group, mice were treated with olive oil (10 mL/kg) by intraperitoneal injection; in the CCl₄ group, mice were injected intraperitoneally with CCl₄ (0.5 mL/kg) + olive oil (9.5 mL/kg) (1:19 v/v). Mice were executed 1, 3, 5 and 7 d after the treatment, serum was collected for the determination of transaminases and activin A levels, and hepatic tissues were obtained for pathological examination and immunohistochemical staining.

Determination of serum transaminases ALT and AST

The serum transaminases ALT and AST levels were detected by assay kit according to the manufacturer's protocol (NJBI, Nanjing, China). Briefly, 25 μ L AST or ALT substrates and 5 μ L serum were added into one well of polystyrene microtiter plates at 37 °C for 30 min. And then 25 μ L of 2,4-dinitrophenylhydrazine was added in to all wells at 37 °C for 30 min. Finally, 250 μ L of 0.4 mol/L sodium hydroxide was added to stop the reactions at room temperature for 15 min, and the absorbance at 510 nm in each well was measured with an enzyme-linked immunosorbent assay (ELISA) reader (BioRad Laboratories, Hercules, CA, United States). The levels of AST or ALT are expressed in U/L.

Pathological examination

The right lobe of each mouse liver was collected, fixed with 40 g/L paraformaldehyde for 24 h, embedded in paraffin, and sliced into a thickness of 3-4 μ m. The sections were deparaffinized and pathological liver change were examined by hematoxylin and eosin (HE) staining.

Detection of activin A levels

To prepare the mouse hepatic tissue homogenate, 50 mg mouse liver was added to 1 mL lysis buffer (1% Triton

Table 1 Primer sequences for polymerase chain reaction

Genes	Primers	Sequences	Product size (bp)
GAPDH	Sense	5'-GATTGTTGCCATCAACGACC-3'	371
	Antisense	5'-GTGCAGGATGCATTGCTGAC-3'	
Activin A	Sense	5'-GAGAGGAGTGAACGTGTGCT-3'	514
	Antisense	5'-ATGACTGTGAGTGGAAAGG-3'	
ActR II A	Sense	5'-ATTGCCAGCATCCATCTCTTG-3'	296
	Antisense	5'-TGCCACCATCATAGACTAGATTC-3'	
Smad3	Sense	5'-CGGTCAAGAGCTGGTCAAGA-3'	241
	Antisense	5'-TTGAAGGCGAACTCACACAGC-3'	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ActR II A: Activin type II A receptor.

X-100, pH 7.5, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium fluoride, 4 µg/mL leupeptin, 1 µg/mL aprotinin). The lysate was centrifuged for 30 min at 12000 rpm at 4 °C and the supernatant was harvested. The levels of activin A in serum and hepatic tissue homogenates of mice were determined by ELISA according to the manufacturer's protocol (R and D).

Real-time quantitative reverse transcription-PCR

Total hepatic tissue RNA was extracted using the Trizol reagent according to the manufacturer's protocol (Invitrogen), and then activin βA, Activin type II A receptor (ActR II A) and Smad3 mRNA expressions were examined by SYBR real-time-PCR kit using a ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems) in a two-stage, single-tube reaction. The following reaction conditions were used: stage one, 95 °C, 10 s for 1 cycle; stage two, 95 °C, 5 s and 60 °C, 31 s for 40 cycles, collecting fluorescence in this phase; stage three, 95 °C, 15 s, 60 °C 1 min, 95 °C 15 s for 1 cycle. Primers were synthesized by BECL (Shanghai, China), and the sequences are shown in Table 1. The reverse transcription (RT)-PCR products were quantitatively analyzed according to a standard cDNA calibration curve^[17].

Immunohistochemical staining

The right lobe of the liver was fixed by 40 g/L paraformaldehyde for 24 h, embedded in paraffin, and then sliced into a thickness of 3-4 µm. The sections were deparaffinized, and 3% hydrogen peroxide (H₂O₂)-methanol was used to block endogenous peroxidase at room temperature for 30 min. Two percent of bovine serum albumin in 0.01 mol/L phosphate-buffered saline (PBS) was used to block nonspecific reactivity by a preincubation of section for 30 min. The sections were then incubated in anti-activin A antibody (1:400) at 4 °C overnight. The sections were washed with PBS, bound antibodies were detected with SP1 kit (ZSJQ, Beijing, China) and immunoreactive products were visualized in 0.05% diaminobenzidine and 0.03% H₂O₂. The sections were counterstained with hematoxylin, dehydrated, cleared, counted

and observed under an Olympus microscope (BX51). In control staining, the sections were incubated with normal mouse immunoglobulin G (IgG) instead of anti-activin A antibody^[18].

Blocking experiment

C57BL/6 mice adaptive feeding for 7 d were randomly divided into four groups ($n = 12$). The IgG control group (IgG Cont), in which each mouse was injected intraperitoneally with olive oil (10 mL/kg), 2 h later, injected by tail vein with normal IgG (500 µg/kg); the antibody control group (Anti-A Cont), in which each mouse was injected intraperitoneally with olive oil (10 mL/kg), 2 h later, injected by tail vein with anti-activin A antibody (500 µg/kg); the CCl₄ group (IgG + CCl₄), in which each mouse was injected intraperitoneally with CCl₄ (0.5 mL/kg) + olive oil (9.5 mL/kg), 2 h later, injected by tail vein with IgG (500 µg/kg); the antibody plus CCl₄ group (Anti-A + CCl₄), in which each mouse was injected intraperitoneally with CCl₄ (0.5 mL/kg) + olive oil (9.5 mL/kg), 2 h later, injected by tail vein with anti-activin A antibody (500 µg/kg). Sera and livers of these mice were collected 3 d after CCl₄ injection for determination of serum ALT and AST levels and pathological examination of livers.

Statistical analysis

The statistical software SPSS 10.0 was used to analyze all data. $P < 0.05$ was considered statistically significant.

RESULTS

The establishment of the acute liver injury mouse model

Change of serum transaminases ALT and AST levels is an important indicator of liver injury. Our results revealed that serum ALT and AST levels were significantly elevated on days 1, 3, 5 and 7 after the administration of CCl₄, compared with that in control group, $P < 0.01$ (Figure 1A). Furthermore, lobular architecture in the control mouse liver was clear and the hepatic cells arranged in neat rows by HE staining. In mouse liver 1 d following CCl₄ treatment, there were round necrotic lesions around the hepatic lobule portal area, and 3 d after CCl₄ treatment, there was inflammatory cell infiltration in the hepatic lobule portal area (Figure 1B).

Increase of activin A levels in mice treated with CCl₄

Activin A levels in serum and hepatic homogenates of experimental mice were determined by ELISA. The results showed that activin A levels increased significantly in serum and hepatic homogenate from CCl₄-induced acute liver injury mice for 1, 3 and 5 d, compared with that in control group, $P < 0.01$ (Figure 2). These levels peaked on days 1 and 3, and returned to normal levels by day 7.

Elevation of activin A protein expression in liver of mice treated with CCl₄

To explore the reason for increased activin A levels in the serum of CCl₄-treated experimental mice and the

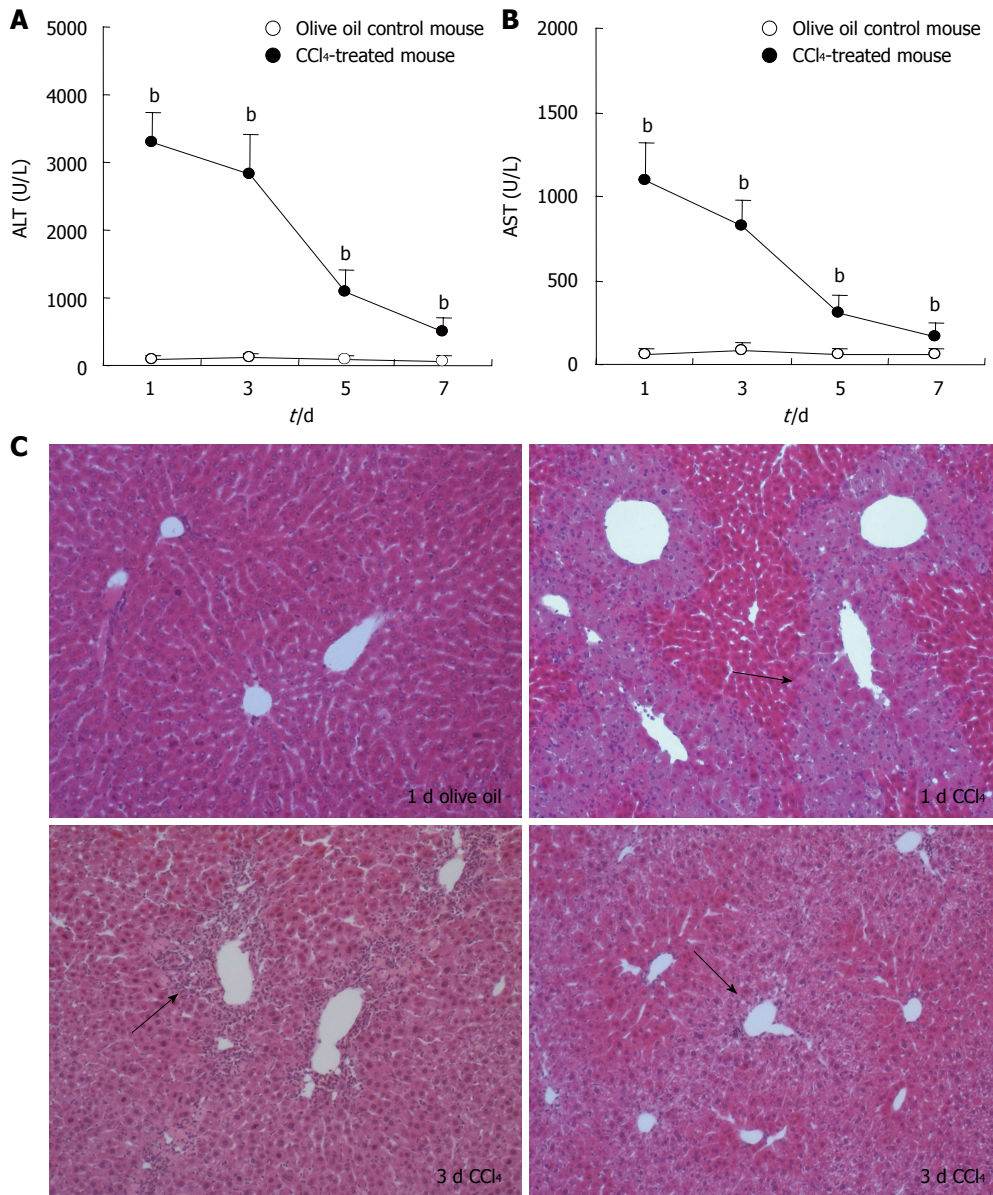


Figure 1 Examination of serum alanine aminotransferase and aspartate aminotransferase levels and pathological changes of liver in carbon tetrachloride-treated mice. **A:** Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were detected by enzyme-linked immunosorbent assay in olive oil control mouse and carbon tetrachloride (CCl₄)-treated mouse. ^b*P* < 0.01 vs control; **B:** Pathological change of liver was analyzed by hematoxylin and eosin staining. Arrows represent lesion area (magnification, × 100).

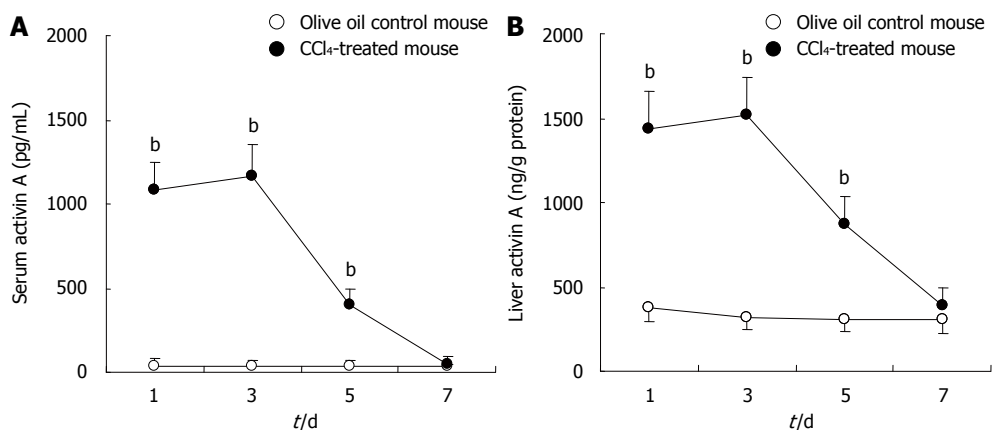


Figure 2 Detection of levels of activin A in serum and hepatic homogenates of mouse treated with carbon tetrachloride by enzyme-linked immunosorbent assay. CCl₄: Carbon tetrachloride. ^b*P* < 0.01 vs control.

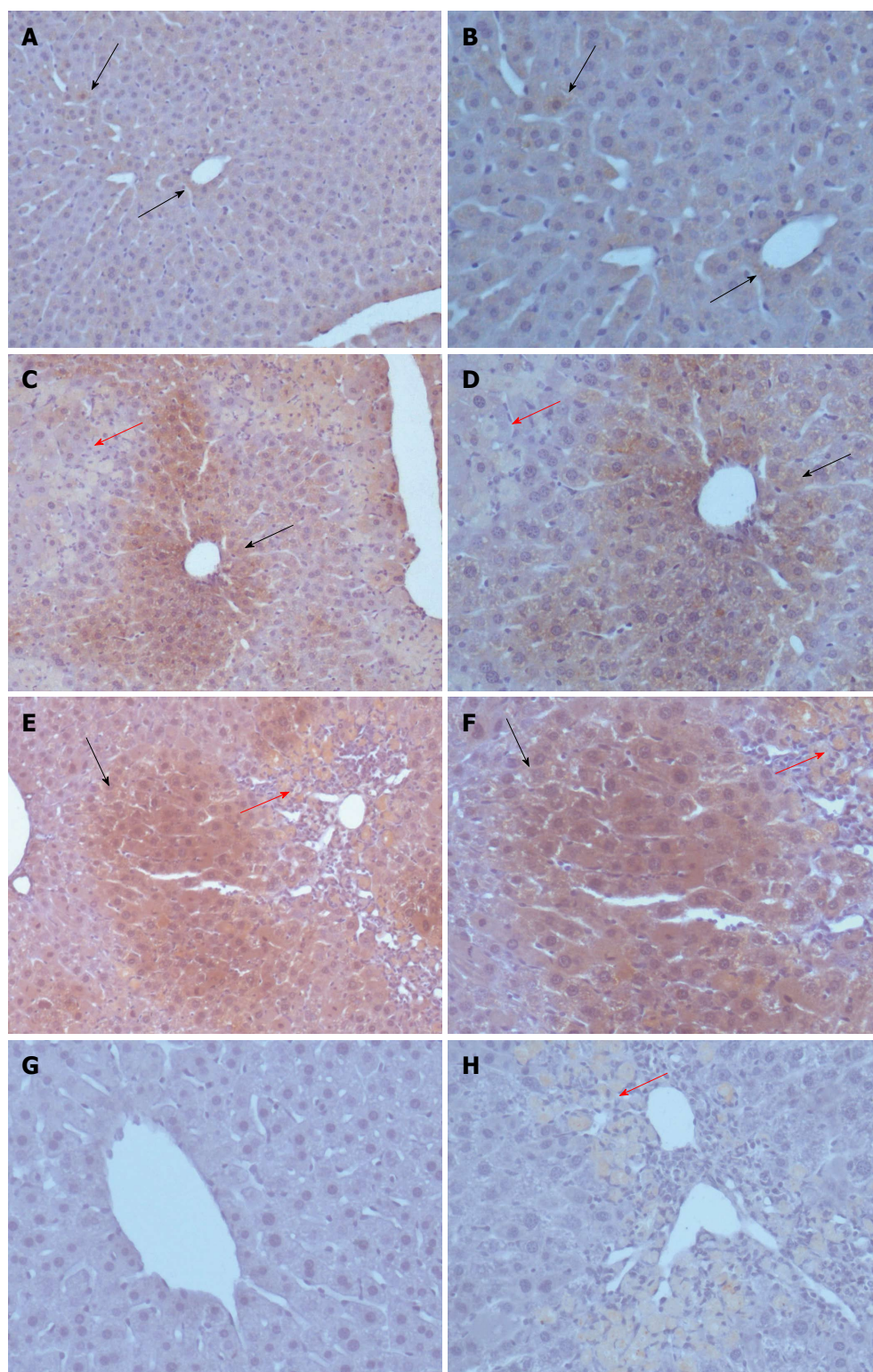


Figure 3 Expression of activin A protein in liver of mouse assessed by immunohistochemical staining. A, B: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 1 after olive oil treatment; C, D: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 1 after carbon tetrachloride (CCl₄) treatment; E, F: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 3 after CCl₄ treatment; G, H: The procedural background control staining was represented by using normal mouse immunoglobulin G instead of anti-activin A antibody in livers of olive oil-treated and CCl₄-treated mice. Red arrows represent lesion area and black arrows represent positive staining for activin A. A, C, E: Magnification × 100; B, D, F, G, H: Magnification × 200.

expression pattern of activin A in acute liver injury, the expression of activin A protein was examined by immunohistochemical staining. The results revealed that activin A protein expression was detectable in the livers of olive

oil-treated mice and in injured livers from CCl₄-treated mice. In addition, activin A protein expression in hepatocytes with non-necrotic area was up-regulated on days 1 and 3 after CCl₄ treatment (Figure 3).

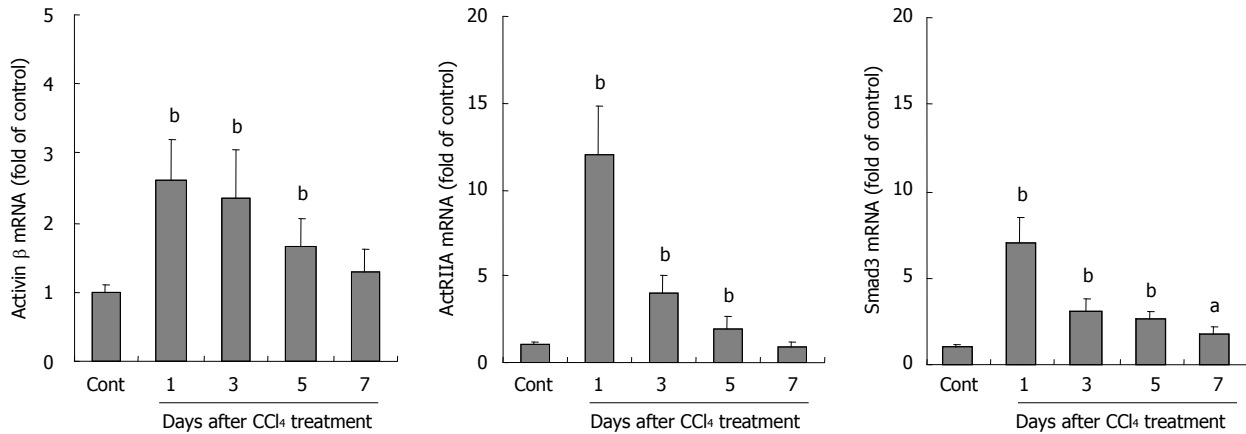


Figure 4 Assay of mRNA expressions of activin β A and activin signal molecules in liver of mouse by real-time quantitative reverse transcription-polymerase chain reaction. The mRNA levels in olive oil control group (Cont) were adjusted to 100%. All values (mean \pm SD) were expressed as % of that in control. ^a $P < 0.05$, ^b $P < 0.01$ vs control.

Up-regulation of activin β A, ActRIIA and Smad3 mRNA expressions in liver of mice treated with CCl₄

Activin A is a dimeric glycoprotein formed by two β A subunits. To further investigate activin and its signal molecule expression in liver injury, activin β A, ActRIIA and Smad3 mRNA expressions were examined by real-time quantitative RT-PCR. The results showed that not only activin β A mRNA, but also ActRIIA and intracellular signal protein Smad3 mRNA expressions were significantly increased in livers from CCl₄-treated mice when compared with that in control group, $P < 0.01$ (Figure 4).

Protective effect of anti-activin A antibody on CCl₄-treated mouse liver

In order to further confirm that activin A was involved in acute liver injury, pathological changes of livers in CCl₄-treated mice were examined after injection of an anti-activin A antibody to block endogenous activin action. The results revealed that serum ALT and AST levels in mice treated with anti-activin A + CCl₄ were significantly lower than that in IgG + CCl₄ control mice (Figure 5A, $P < 0.01$). Furthermore, the results showed that the necrotic severity around the hepatic lobule portal area in mice treated with anti-activin A + CCl₄ was remarkably reduced compared with that in IgG + CCl₄ control mice (Figure 5B).

DISCUSSION

Biologic activity of activin

Activin A as a multifunctional factor plays an important role in acute phase immune response, and its expression levels are increased in several inflammatory diseases such as septicemia, inflammatory bowel disease, and rheumatoid arthritis^[19-22]. Recent studies have reported that activin A is closely related to the liver diseases hepatitis and hepatic fibrosis^[3,14,16]. Activin receptors belong to serine/threonine kinase receptors of the TGF- β superfamily, are divided into ActRI and ActRII. Activin binds directly to the type II receptor on the cell membrane, and then recruits the type I receptor^[23,24]. Further, the activated

type I receptor propagates the signal through a cascade reaction elicited by downstream Smad proteins into the nucleus to activate target genes^[25,26].

Damage effects of CCl₄ on the liver

CCl₄ is a chemical reagent that induces mouse liver injury, a classic chemical model for studying liver injury. The principle of injury is that in the function of cytochrome P450, CCl₄ generates chloromethyl free radicals (-CCl₃), which enable the peroxidation of the membrane lipids on the liver cell membrane or subcellular structure causing the rise of membrane permeability that results in the release of large number of ALT in the cytoplasm into blood. Another possibility is covalent binding of -CCl₃ and hepatic microsomal lipids and proteins that damage the integrity of the structure and function of hepatic cell membranes^[27-29]. Additionally, various factors can further aggravate CCl₄-induced liver injury in mice, such as the increase of activin A expression in chronic liver injury^[16].

Role of activin in liver injury

Abnormal expression of activin A is related to a variety of liver diseases, and activin A can directly induce hepatocyte apoptosis and inhibit hepatocyte proliferation and DNA synthesis^[2,12,30]. To investigate whether activin A is involved in acute chemical liver injury, C57BL/6 mice were injected with CCl₄ through peritoneal cavity to establish acute liver injury model. The results showed that serum ALT and AST increased, and serious necrosis of hepatic tissue was observed by HE staining in CCl₄-treated mice. Simultaneously, activin A levels increased significantly in serum and hepatic tissue homogenate of mice treated with CCl₄. By immunohistochemical staining, the elevated expression of activin A protein was also observed in the injured livers of CCl₄-treated mice. Since CCl₄ induced necrotic death of hepatocytes in large area, which necrotic hepatocytes could not release cytokines, but the decomposition product of necrotic tissues as inflammatory stimulator could induce hepatocytes activation to secrete activin A. Thus, activin A protein was expressed in hepatocytes around the necrotic area and the

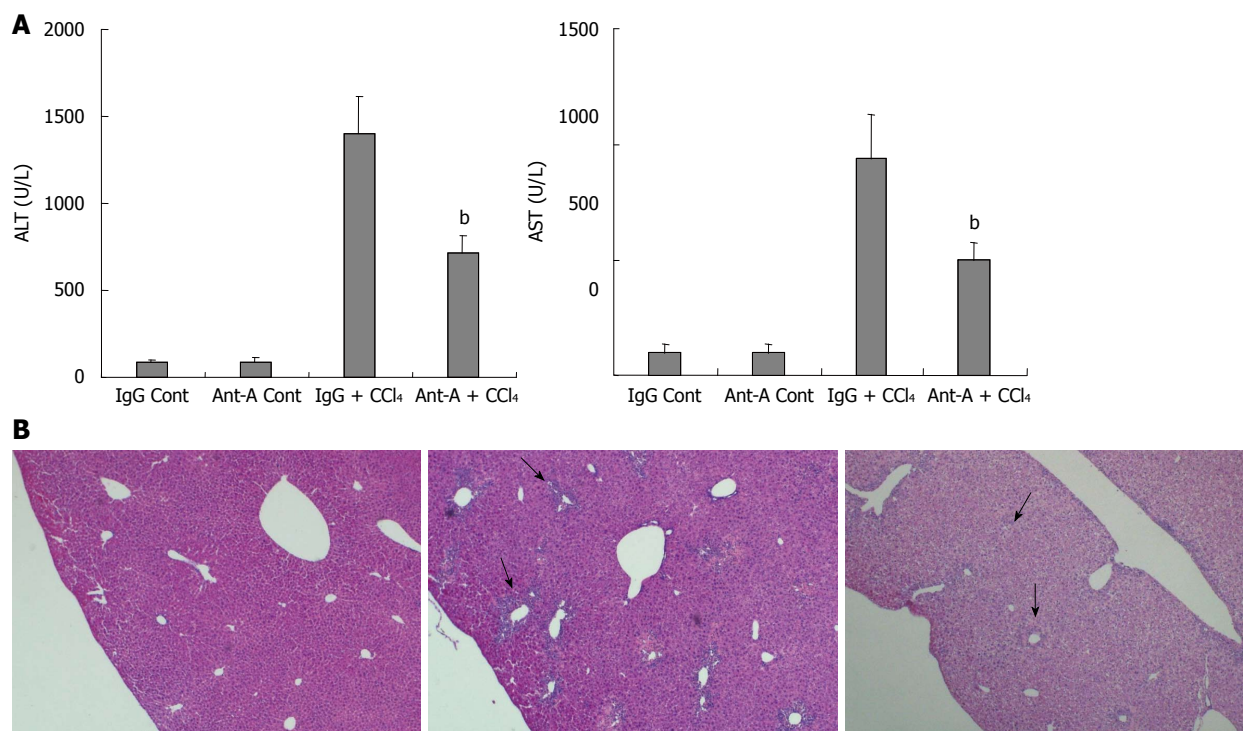


Figure 5 Effects of anti-activin A antibody *in vivo* on serum alanine aminotransferase and aspartate aminotransferase levels and pathological change of liver in carbon tetrachloride-treated mouse. **A:** Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were detected in mouse 3 d after carbon tetrachloride (CCl₄) treatment. Immunoglobulin G (IgG) + Cont, IgG control group; Anti-A + Cont, anti-activin A control group; IgG + CCl₄, IgG plus CCl₄ group; Anti-A + CCl₄, anti-activin A plus CCl₄ group. ^b*P* < 0.01 vs IgG + CCl₄ group; **B:** Pathological change of liver in mouse 3 d after CCl₄ treatment was analyzed by hematoxylin and eosin staining. Arrows represent lesion area (magnification × 40).

activated hepatocytes have larger clear nuclei. In addition, the real-time quantitative RT-PCR results revealed that in CCl₄-treated mice, activin A, ActR II A and intracellular activin signal protein Smad3 mRNA expressions significantly increased. In order to further confirm the role of activin A in CCl₄-induced liver injury, pathological changes of livers in CCl₄-treated mice were examined after injection with anti-activin A antibody to block endogenous activin action. These results revealed that activin A might participate in the process of CCl₄-induced liver injury *via* inhibiting proliferation and DNA synthesis of hepatocytes to aggravate the CCl₄-induced liver injury, and blockade of activin A actions by anti-activin A antibody could significantly reduce the levels of serum transaminases and the necrosis severity of hepatic tissue in CCl₄-treated mice.

In summary, these data suggested that the activin A-ActR II A-Smad signal transduction cascade was induced in CCl₄-treated mice. Further, activin A was involved in the process of CCl₄-induced acute chemical liver injury of mice in an autocrine/paracrine manner. Activin A may be a potential therapeutic target for acute liver injury disease.

COMMENTS

Background

Activin A is an important mediator of liver cells, not only in proliferation of hepatocytes, but also in activation of hepatic stellate cells, secretion of liver extracellular matrix, as well as the formation and development of liver injury.

Carbon tetrachloride (CCl₄)-induced liver injury is a classical model of chemical liver injury in mice. Previous studies reported that the expression of activin A increased significantly in CCl₄-induced chronic liver injury in mice, and it was an important factor leading to liver fibrosis. The effect of activin A on the process of CCl₄-induced acute chemical liver injury has not been reported.

Research frontiers

Previous studies have demonstrated the effect of activin A on CCl₄-induced chronic liver injury in mice, however, the effect and mechanism of activin A on CCl₄-induced acute liver injury remains undefined.

Innovations and breakthroughs

New therapies that aim to block biological role of activin A in liver injury diseases are being actively explored and evaluated. Authors believe this study to be the first to explore the effect of activin A on acute liver injury.

Applications

The findings that block the biological action of activin A may be clinically relevant to potential liver injury therapeutics.

Terminology

Activin is a multifunctional factor of transforming growth factor- β superfamily. There are three types of activins formed by homo- or hetero-dimerization of two inhibin subunits (β A and β B), activin A (β A β A), activin B (β B β B) and activin AB (β A β B). Activin A is an important mediator in CCl₄-induced acute liver injury in mice, and blockade of biological action of activin A may be a potential therapeutics for acute liver injury diseases.

Peer review

This is a good descriptive study in which authors analyze the role of activin A in carbon tetrachloride-induced acute liver injury in mouse. The results are interesting and suggest that activin A is involved in the process of CCl₄-induced acute liver injury in an autocrine/paracrine manner. Further, the authors show that an antibody-mediated blockade of activin A biological action may provide insights into potential therapeutics.

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