

• CLINICAL RESEARCH •

Quantitative analysis of transforming growth factor beta 1 mRNA in patients with alcoholic liver disease

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Abstract

AIM: To investigate the expression of the transforming growth factor beta 1 (TGF- β 1) mRNA in different stages of alcoholic liver disease (ALD) and its clinical value.

METHODS: One hundred and seven male alcoholics were grouped by clinical findings into four groups: alcohol abusers without liver impairment ($n=22$), alcoholic steatosis ($n=30$), alcoholic hepatitis ($n=31$), and alcoholic cirrhosis ($n=24$). Using peripheral blood mononuclear cells (PBMC) as samples the gene expression of TGF- β 1 was examined quantitatively by reverse transcription polymerase chain reaction (RT-PCR) and dot blot. There are 34 healthy subjects served as control.

RESULTS: The expression of TGF- β 1 from all ALD patients was significantly greater than that in controls (1.320 ± 1.162 vs 0.808 ± 0.276 , $P<0.001$). The differences of the expressions were significant between the patients from each groups (alcoholic steatosis, alcoholic hepatitis and alcoholic cirrhosis) and the controls (1.168 ± 0.852 , 1.462 ± 1.657 , 1.329 ± 0.610 vs 0.808 ± 0.276 , $P<0.050$). No significant differences of TGF- β 1 mRNA expression were observed between alcohol abusers without liver impairment and controls. The expressions in patients with alcoholic hepatitis and alcoholic cirrhosis were significantly greater than that in alcohol abusers respectively (1.462 ± 1.657 , 1.329 ± 0.610 vs 0.841 ± 0.706 , $P<0.050$). No significant differences of TGF- β 1 mRNA expression were observed between alcoholic fatty liver men and alcohol abusers.

CONCLUSION: TGF- β 1 expression level can be a risk factor for alcoholic liver disease and might be related to the inflammatory activity and fibrosis of the liver in patients.

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INTRODUCTION

Alcoholic liver disease (ALD) is the most common cause of liver disease in late stage in the developed world^[1], and ranked as the second cause of hepatic cirrhosis in China now. The increased deposition of extracellular matrix in the liver is a key factor in the developing of the disease.

In recent years, the role of TGF- β 1 in ALD has been much emphasized^[2,3], but the study of expression of TGF- β 1 in peripheral blood mononuclear cells (PBMC) from ALD patients has

scarcely been performed. This study was aimed at quantitative analysis of the expression of TGF- β 1 mRNA in PBMC of ALD patients through reverse transcription-polymerase chain reaction (RT-PCR) technique and dot blot.

MATERIALS AND METHODS

Subjects

The subjects were from the epidemiologic survey of ALD in Zhejiang province including 107 male alcoholics, aged 25~70 years ($\chi=43.32\pm 10.39$), who had ingested more than 40g of alcohol a day for more than 5 years. According to the diagnostic criteria of Nanjing conference, there were 22 alcohol abusers without hepatic impairment, 30 alcoholic steatosis, 31 alcoholic hepatitis and 31 alcoholic cirrhosis. Percutaneous liver biopsy was performed in all cases, C and B hepatitis were ruled out by appropriate serological tests. 34 healthy subjects served as control, with age range from 26 to 68 years ($\chi=45.60\pm 10.08$). There were no significant differences in their ages among the alcoholics and healthy controls.

Design and synthesis of primers

TGF- β 1 primers and probe were synthesized by Gibco BRL Co. The forward primer, reverse primer and probe were 5'GGA CAC CAA CTA TTG CTT CAG3', 5'TCC AGG CTC CAA ATG TAGG3' and 5'CAG CTG TAC ATT GAC TTC CGC AAG GAC CT3' respectively. β -actin primers were synthesized by the Academy of Microorganism Science of Lubeck University, and kindly provided by Prof. Chen Zhi. The sequence of the primers were 5'TTC CAG CCT TCC TTC CTGG3' (forward primer) and 5'TTG CGC TCA GGA GGA GCA AT3' (reverse primer). The Primers were designed according to the previously reported sequence as shown in Roulot's report^[4].

Samples preparation and RNA extraction

Blood samples 3ml were mixed with Ficoll fluid to isolate the PBMC and then 150 μ l Trizol Reagent (Gibco BRL Co.) was added. Total cellular RNA was extracted by the guanidinium thiocyanate/phenol chloroform single-step method. RNA was washed in ethanol and dissolved in diethyl pyrocarbonate (DEPC)-treated water.

RT-PCR

The RT-PCR mixture consisted of RNA of each sample 5 μ l, 2mM dNTP 5 μ l, 5 \times first stand buffer 5 μ l, 100mM TT 2.5 μ l, 100pmol/ μ l oligod(T) 15 μ l, MMLV-RT100 unit, Rnase 1 μ l (Pharmacia Biotech) to a total volume of 20 μ l. The reaction was allowed to proceed at 41 $^{\circ}$ C for 1 hour.

The PCR mixture consisted of RT-PCR products 5 μ l, 10 \times PCR buffer 5 μ l, 20 pmol of each primer, 2mM dNTP 5 μ l and Taq polymerase 1.25 unit (Gibco BRL Co.) to a total volume of 40 μ l. This reaction mixture was overlaid with 1 drop of mineral oil.

PCR was carried out in a DNA thermal cycler (Perkin-Elmer Cetus) for 30 cycles; after initial denaturation by heating to 95 $^{\circ}$ C for 2 minutes, each cycle consisted of denaturation at 94 $^{\circ}$ C for 60 seconds, annealing at 56 $^{\circ}$ C for 60 seconds and extension at 72 $^{\circ}$ C for 45 seconds. An extra extension at 72 $^{\circ}$ C for 10 minutes was performed after the final cycle.

Dot blot

The probe was labelled using a DIG oligonucleotide 3'-end labeling kit (Boehringer Mannheim). The efficiency of the labeling reaction was checked by comparing with the labelled control-oligonucleotide by direct detection. 5µl PCR products was dropped to on a nylon membrane. The membranes were hybridized at 60°C for 6h after being denatured, fixed, prehybridized and then washed and stained.

Quantitative analysis

Quantification of TGF-β1 mRNA was carried out using β-actin mRNA as an internal standard because the transcription levels of β-actin were very stable in all types of tissues. The dots on the membranes were quantitatively analyzed and pictures were taken using IS-1000 multifunction agarose imaging analysis system (Alpha Innotech Co.). The relative index (RI) of mRNA was calculated using a formula as RI = scan value of TGF-β1 mRNA dot / scan value of β-actin mRNA dot^[5]. Here RI might represent the relative levels of TGF-β1 mRNA in PBMC.

Statistics

The data were presented as means ± s, all the statistics were done with the software Statistical Package for the Social Sciences (SPSS) and the P value was considered significant when it was less than 0.05.

RESULTS

The expression of TGF-β1 from all ALD patients was significantly greater than that in the controls (1.320 ± 1.162 vs 0.808 ± 0.276, $t=3.811$, $P=0.0001$, Figure 1).

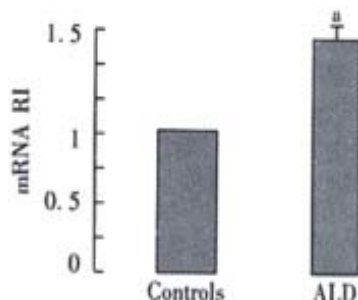


Figure 1 TGF-β1 mRNA RI values in ALD and Controls' * $P < 0.05$ vs controls

The expressions in patients with the alcoholic steatosis, alcoholic hepatitis and alcoholic cirrhosis were significantly greater than the expression in the controls respectively (1.168 ± 0.852, 1.462 ± 1.657, 1.329 ± 0.610 vs 0.808 ± 0.276, $t=2.213$, $P=0.017$, $t=2.171$, $P=0.019$, $t=3.915$, $P=0.0002$). No significant differences of TGF-β1 mRNA expression were observed between alcohol abusers and the controls (Figure 2).

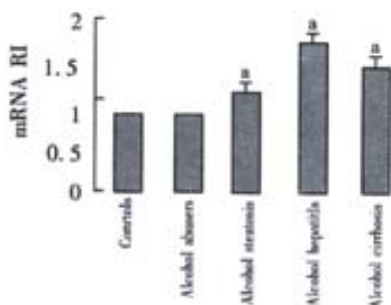


Figure 2 The TGF-β1 mRNA in alcohol abusers, alcohol steatosis, alcohol hepatitis, alcohol cirrhosis and controls. * $P < 0.05$ vs controls

The expression in patients with alcoholic hepatitis and alcoholic hepatic cirrhosis was significantly greater than that in alcohol abusers respectively (1.462 ± 1.657, 1.329 ± 0.610 vs 0.841 ± 0.706, $t=1.859$, $P=0.035$, $t=2.495$, $P=0.008$). No significant differences of TGF-β1 mRNA expression were observed between alcoholic fatty liver males and alcohol abusers (Figure 3).

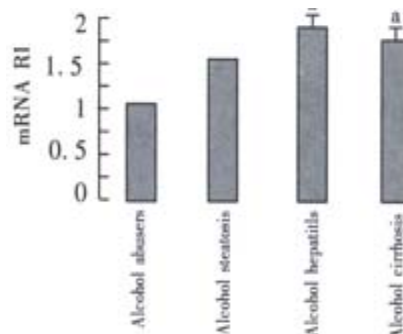


Figure 3 The TGF-β1 mRNA in alcohol abusers, alcohol steatosis, alcohol hepatitis, alcohol cirrhosis and alcohol abusers. * $P < 0.05$ vs alcohol abusers

DISCUSSION

In Western societies roughly 50% of all cases of liver cirrhosis are related with alcohol abuse. The increased deposition of extracellular matrix in the liver is a key factor in the morbidity and mortality of alcoholic liver disease^[6]. This increased fibrosis may be due to a superabundance of profibrogenic factors such as transforming growth factor-beta 1 and/or relative decrease of the factors that inhibit fibrogenesis such as collagenase or interferon^[7-10]. Transforming growth factor beta 1 (TGF-β1) is believed to play a key role in enhancing fibrogenesis and inhibiting extracellular matrix degradation^[11]. Acetaldehyde, the oxidative metabolite of ethanol damages cell membranes, initiates lipid peroxidation and forms noxious protein adducts, which result in the activation of Kupffer cells and perisinusoidal lipocytes/portal fibroblasts^[12-18]. The activation of lipocytes and fibroblasts to a proliferative and collagen-producing myofibroblast-like phenotype is triggered by the release of TGF-β1 from the activated Kupffer cells. In the liver, TGF-β1 is primarily responsible for activation of lipocytes, which are the main source of extracellular matrix proteins. Their deposition play a key role in the development of alcoholic liver cirrhosis^[19-21].

A number of investigators have shown increase of TGF-β1 mRNA and protein levels in animal models of hepatic fibrosis^[22-27]. A few studies also demonstrated increased levels of TGF-β1 mRNA and protein in animal models of ALD. Matsuoka *et al*^[28] examined Kupffer cells and hepatic lipocytes isolated from a rat model of alcoholic liver fibrosis. The collagen formation was increased significantly in alcohol-fed group more than the control, which was completely inhibited by anti-transforming growth factor beta IgG. The major peak of the molecular weight was about 25000 which was revealed by high-performance liquid chromatography and demonstrated with Northern blotting and hybridization. Kamimura *et al*^[29] examined Kupffer cell gene expression of TGF beta 1 in the rat model of ALD. Kupffer cells were isolated from the model after 10 and 17 weeks of intragastric ethanol infusion, the protein and mRNA levels of TGF beta 1 were significantly increased by 143% and 204% at 10 weeks and 238% and 295% at 17 weeks respectively in the ethanol-fed rats.

A number of studies have suggested a role for TGF-β1 in human liver disease^[30]. In our study, the expression of TGF-β1 was significantly greater in all the ALD patients than in the controls and the expression in the patients with the alcoholic steatosis, alcoholic hepatitis and alcoholic cirrhosis was significantly greater than

their expressions in the controls respectively. No significant differences of TGF- β 1 mRNA expression were observed between alcohol abusers and the controls. The results demonstrated that the expression of TGF- β 1 was related with ALD. Our study also showed that the expression of patients with alcoholic hepatitis and alcoholic cirrhosis was significantly greater than their expression in alcohol abusers respectively, and no significant differences of TGF- β 1 mRNA expression were observed between alcoholic steatosis men and alcohol abusers. These results indicated that the expression of TGF- β 1 was higher in more active and advanced ALD patients. Santos *et al.*^[31] also demonstrated similar results in liver specimens. The presence of TGF- β 1 expression could be recognized as a risk factor for active and advanced alcoholic liver disease.

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