Acetaldehyde stimulates the activation of latent transforming growth factor-β1 and induces expression of the type II receptor of the cytokine in rat cultured hepatic stellate cells

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Acetaldehyde, the major active metabolite of alcohol, induces the activation of hepatic stellate cells (HSC), leading to overproduction of α1(I) collagen and ultimately causing hepatic fibrosis. The underlying mechanisms of this process remain largely unknown. Transforming growth factor-β1 (TGF-β1) is a potent inducer of α1(I) collagen production. Accumulating evidence has shown a potential role for TGF-β1 in alcohol-induced hepatic fibrogenesis. The aims of this study were to determine the effect of acetaldehyde on TGF-β signalling, to elucidate the underlying mechanisms as well as to evaluate its role in expression of α1(I) collagen gene in cultured HSC. It was hypothesized that acetaldehyde activated TGF-β signalling by inducing the expression of elements in the TGF-β signal transduction pathway, which might contribute to α1(I) collagen gene expression in cultured HSC. Initial results revealed that acetaldehyde activated TGF-β signalling in cultured HSC. Additional studies demonstrated that acetaldehyde stimulated the secretion and activation of latent TGF-β1, and induced the expression of the type II TGF-β receptor (Tβ-RII). Further experiments found *cis*- and *trans*-activating elements responsible for Tβ-RII gene expression induced by acetaldehyde. Activation of TGF-β signalling by acetaldehyde contributed to α1(I) collagen gene expression in cultured HSC. In summary, this report demonstrated that acetaldehyde stimulated TGF-β signalling by increasing the secretion and activation of latent TGF-β1 as well as by inducing the expression of Tβ-RII in cultured HSC. Results from this report provided a novel insight into mechanisms by which acetaldehyde stimulated the expression of α1(I) collagen in HSC and a better understanding of effects of alcohol (or acetaldehyde) on hepatic fibrogenesis.

Key words: alcohol, gene expression, hepatic fibrogenesis, myofibroblast-like cell.

INTRODUCTION

Cirrhosis is one of the leading causes of death. It is estimated that approx. 50 % of all deaths due to cirrhosis are caused by alcohol abuse [1]. Without an effective treatment at an early stage, reversible hepatic fibrosis progresses to irreversible cirrhosis [2]. Hepatic fibrogenesis is a process where production of extracellular matrix (ECM) surpasses degradation. Hepatic stellate cells (HSC) are the primary source of excessive production of ECM during liver fibrogenesis [2]. During liver injury, HSC are activated and undergo profound phenotypic changes (transdifferentiation) from vitamin A-storing cells to myofibroblast-like cells. These changes include increases in cell proliferation, morphological alterations, *de novo* expression of α-smooth muscle actin and excessive production of ECM, including α1(I) collagen. Activation of HSC is associated with the sequential expression of several key cytokines and their surface receptors, including transforming growth factor-β (TGF-β)1 and its receptors [3,4]. The underlying mechanisms that drive the progression of HSC activation remain incompletely understood.

Cytokines of the TGF-β family influence a wide spectrum of cellular processes, including differentiation, proliferation, apoptosis and migration [5]. Of the known TGF-β isoforms, TGF-β1, which has been most extensively studied, is the most abundant isoform in both normal and fibrotic liver [6]. TGF-β1 is synthesized and secreted in a latent, biologically inactive form, which must be activated before binding to TGF-β receptors. TGF-β signalling is initiated by binding of this active cytokine to the type II TGF-β receptor (Tβ-RII), which phosphorylates and activates the type I TGF-β receptor [5,7]. The latter, in turn, phosphorylates Smad 2/3 proteins, which subsequently form a complex with Smad 4 and migrate to the nucleus to regulate the expression of target genes, including α1(I) collagen [8,9]. In normal liver, TGF-β1 is strongly expressed by Kupffer cells. In contrast, in fibrotic liver, the level of TGF-β1 expression increases selectively in HSC [6], suggesting an autocrine/paracrine action for TGF-β1 and an important role of this cytokine in activation of HSC. Results from over-expression of TGF-β1 in transgenic mice demonstrated the profibrogenic role of TGF-β1 in hepatic fibrogenesis [10]. In addition, introduction of dominant negative Tβ-RII, or soluble TGF-β receptors, resulted in a marked reduction in the development of hepatic fibrosis and an enhancement of hepatocyte regeneration in rats [11–13]. All these observations have, therefore, led to a widely accepted model in which persistent autocrine/paracrine stimulation of HSC by TGF-β1 plays a key role in liver fibrogenesis [14].

Acetaldehyde, the major active metabolite of alcohol, activates intracellular signal transduction pathways, including protein kinase C, Jun N-terminal kinase and extracellular signal-regulated kinase, and induces the expression of collagen genes in

Abbreviations used: AP-1, activator protein-1; BTEB, basic transcription element-binding protein; CAT, chloramphenicol acetyltransferase; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; HSC, hepatic stellate cells; PHMB, *p*-hydroxymercuribenzoate; RPA, RNase protection assay; Tβ-RII, type II TGF-β receptor; TAE, TGF-β-activating element; TGF-β, transforming growth factor-β.

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HSC [15–21]. Accumulating evidence has indicated a close link between alcohol and TGF-β in the process of hepatic fibrogenesis during hepatic injury [22,23]. A significant increase was observed in the expression of all TGF-β isoforms in patients with alcohol-induced cirrhosis [22]. Acetaldehyde, as well as TGF-β1, stimulated the expression of both α1(I) and α2(I) collagen genes [2,16,17,19,24]. Acetaldehyde and TGF-β, however, had no synergistic effects on α2(I) collagen gene promoter activation in HSC [21,24]. The precise intracellular signal pathways involved in inducing the expression of collagen genes by acetaldehyde, however, remain largely unknown. A better understanding of the involved signal pathways may be conducive to discovering potential targets for novel therapeutic treatment and prevention of alcohol-induced cirrhosis.

The focus of the present communication was to determine the effect of acetaldehyde on TGF-β signalling, to elucidate the underlying molecular mechanisms, as well as to explore its role in α1(I) collagen expression in cultured HSC.

MATERIALS AND METHODS

Stellate cell isolation and culture

HSC were isolated from male Sprague–Dawley rats as described previously [25,26]. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum. Cells were serum-starved for 48 h in Dulbecco's modified Eagle's medium with 0.4 % fetal bovine serum to render them quiescent [18,27] prior to the addition of acetaldehyde (200 μM) for the indicated times. A low percentage of serum (0.4 %) in media was used to minimize the formation of adducts between acetaldehyde and serum proteins. The media with acetaldehyde was replaced every 12 h. In some experiments, anti-TGF-β1 antibodies (30 μg/ml; Promega), or *p*-hydroxymercuribenzoate (PHMB; 4 μM; Sigma), an acetaldehyde–protein adduct-formation inhibitor [18], were added to the 0.4 % fetal bovine serum media 30 min prior to the addition of acetaldehyde.

^{125}I-TGF-β1-binding assays

^{125}I-labelled TGF-β1 binding assays were performed as described previously [28]. Briefly, pre-confluent HSC were serum-starved for 48 h prior to the treatment with or without acetaldehyde (200 μM) for an additional 24 h. Cells were washed once with a binding buffer (25 mM Hepes, pH 7.4, 125 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$ and 1 mM $CaCl_2$). The same number of HSC (10^5) in different treatments were subsequently incubated with ^{125}I-TGF-β1 (0.1–3.5 ng/ml) in binding buffer containing 0.2 % BSA at 4 °C for 4 h in the absence or presence of a 50-fold excess of unlabelled TGF-β1 to measure total, or non-specific, binding, respectively. Cells were then washed at least three times with the binding buffer and lysed with 1 % Triton X-100, 0.1 % BSA and 0.1 M NaOH. Radioactivities of the aliquots of soluble fractions were measured using a gamma counter. The difference between radioactivities in cells incubated with (i.e. non-specific binding) and without (i.e. total binding) excessive unlabelled TGF-β1 was considered to be specific binding. Scatchard analysis of ^{125}I-TGF-β1 specific binding data was used to evaluate the effect of acetaldehyde on the affinity of receptors to TGF-β1 [29].

TGF-β1 immunoassay (ELISA)

Passaged HSC were serum-starved for 48 h prior to the addition of acetaldehyde (200 μM) for an additional 24 h. Conditioned media were collected and centrifuged at 5600 ***g*** for 10 min at 4 °C. The supernatants were analysed for the active form of TGF-β1 by a TGF-β1 E_{max} ImmunoAssay System (ELISA; Promega) following the protocol provided by the manufacturer. This immunoassay system was designed for the sensitive and specific detection of biologically active TGF-β1. The antibodies in the system did not recognize the TGF-β1 precursor, as indicated by the manufacturer. To determine the amount of total TGF-β1 in the conditioned media, samples were pretreated with 1 M HCl for 15 min at room temperature prior to neutralization with 1 M NaOH, as suggested by the manufacturer. This procedure converted all latent TGF-β1 to the active form.

Transient transfection

Semi-confluent HSC in 6-well plastic plates were transiently transfected using the LipofectAMINE reagent (Life Technologies, Grand Island, NY, U.S.A.). Luciferase assays and chloramphenicol acetyltransferase (CAT) assays were performed as described previously [17,25]. Transfection efficiency was determined by co-transfection of a β-galactosidase reporter, pSV-β gal (Promega). β-Galactosidase activities were measured using a chemiluminescence assay kit (Tropix, Bedford, MA, U.S.A.) according to the manufacturer's instructions.

Plasmid constructs

Tβ-RII reporter plasmids, named pTβ-RII-N, contained various portions of the promoter region of the Tβ-RII gene, where N was the distance in nucleotides from the transcription initiation site. They were generously provided by Dr Seong-Jin Kim (Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.) [30]. The plasmd pTβ-RII ($-$219 AP-1 mut.) was derived from pTβ-RII ($-$219/+35) with site-directed mutations (underlined) from TTAGTCA to TTAGTTG ($-$196 to $-$190). The plasmid pTβ-RII ($-$219 GC mut.) was generated by site-directed mutagenesis (CGGGCGGA to CGTTATTA at $-$25 to $-$18) from pTβ-RII ($-$219/+35). The plasmid p3TP-Lux was a TGF-β-inducible luciferase reporter, containing the plasminogen activator inhibitor-1 (PAI-1) gene promoter, kindly provided by Dr Joan Massague (Memorial Sloan-Kettering Cancer Center, NY, U.S.A.). The plasmid pcolCAT 1.7 contained 1.7 kb of the rat α1(I) collagen gene promoter linked to a CAT reporter vector [26]. The plasmid pcolCAT 1.7 (TAE mut) was derived from pcolCAT 1.7 with site-directed mutations in the TGF-β-activating element (TAE) ($-$1629 to $-$1615) from 5′-TGCCCA-CGGCCAAGA-3′ to 5′-TGTGCGCGGCCGCAA-3′ [31]. The expression plasmid of dominant negative c-Fos was originally from Dr R. de Groot. The dominant negative c-Jun was described previously [17].

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously [26]. The integrity of nuclear extracts was tested by EMSA with a ^{32}P-labelled SP-1 consensus probe, resulting in distinct SP-1 shifts from all extracts (results not shown). Epitope-specific polyclonal anti-basic transcription element-binding protein (BTEB) antibodies were commercially generated by Quality Bioresources (Austin, TX, U.S.A.), according to the deduced amino acid sequence of rat BTEB [32]. The following oligonucleotides, synthesized by Gibco-BRL (Grand Island, NY, U.S.A.), were used as probes in EMSA: (i) a DNA fragment containing the activator protein-1 (AP-1) site ($-$219 to $-$172) in the Tβ-RII promoter, 5′-GTT ACT TTG TGA ACT GTG TGC ACT TAG TCA TTC TTG AGT AAA TAC TT-3′; (ii) the GC box located within $-$33 to

-14 in the Tβ-RII promoter, 5′-AGG CTC TCG GGC GGA GAG AG-3′; (iii) a consensus SP-1 probe, 5′-ATT CGA TCG GGG CGG GGC GAG C-3′.

RNA isolation and RNase protection assays (RPA)

Total RNA was isolated using TRI Reagent (Sigma). To prepare a Tβ-RII RNA probe, plasmid pT7-TβRII was linearized with *Eco*RI [33]. A 28 S rRNA probe (115 bp) was used as an internal control (Ambion, Austin, TX, U.S.A.). The first exon (1–206) of the rat α1(I) collagen gene was subcloned in pGEM-3Zf(+) (Promega) [17]. The T_7 promoter in plasmids was used to generate single-strand antisense RNA probes. The template for rat cyclophilin was obtained from Ambion and yielded a 103 bp protected fragment. The antisense probes were synthesized and ^{32}P-labelled by MAXIscript™ (Ambion). RPA was carried out with RPA II™ kits (Ambion). The radioactivity in each band was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) [26,34].

Western blotting analysis

Using standard techniques, SDS/PAGE (10%) resolving gel was used to separate proteins (20 μg/lane). A standard rTβ-RII (68 kDa) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The separated proteins were detected by anti-Tβ-RII polyclonal antibody (Santa Cruz Biotechnology) or anti-BTEB antibodies, and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). Protein bands were visualized using chemiluminescence reagent (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A.).

Inhibition of AP-1 or BTEB expression by antisense oligonucleotides

The procedures were described previously [17,34]. Briefly, phosphorothioate-modified sense and antisense oligonucleotides were synthesized by Life Technology. Antisense c-Jun or BTEB oligonucleotides at 50 μg/ml were previously found to be the optimal concentration for blocking the expression of c-Jun or BTEB protein respectively, as determined by Western blotting analyses [17,34]. This optimal concentration of antisense c-Jun oligonucleotides for inhibition of the AP-1 *trans*-activating activity was confirmed in HSC transfected with an AP-1 reporter plasmid, as described previously [34]. Sense or antisense c-Jun or BTEB oligonucleotides at 50 μg/ml were added to cultured HSC 4 h prior to the addition of acetaldehyde. The media containing oligonucleotides and acetaldehyde was replaced every 24 h.

The antisense c-Jun oligonucleotide was 5′-TTC CAT CTT TGC AGT CAT-3′ [35] and the antisense BTEB oligonucleotides was 5′-ATG TCC GCG GCC GCC TAC ATG-3′ [17].

Statistical analysis

Differences between means were evaluated using an unpaired two-sided Student's t test, where $P < 0.05$ was considered to be significant. Where appropriate, comparisons of multiple treatment conditions with controls were analysed by ANOVA with the Dunnett's test for *post hoc* analysis.

RESULTS

Acetaldehyde activates TGF-β signalling in cultured HSC

To evaluate effects of acetaldehyde on TGF-β signalling, HSC were transfected with p3TP-Lux, a TGF-β-inducible luciferase reporter plasmid [36,37], and treated with or without acetaldehyde at the indicated concentrations. As shown in Figure 1(A), acetaldehyde caused a dose-dependent increase in luciferase activities in these cells. Pretreatment of cells with PHMB, which inhibited the formation of aldehyde–protein adducts, eliminated the increase in luciferase activities. Acetaldehyde at 200 μM was chosen for the following experiments [16,18,19]. Pretreatment of cells with anti-TGF-β1 antibodies (30 μg/ml) for 30 min prior to the addition of acetaldehyde abrogated the acetaldehyde effect (Figure 1A), suggesting an involvement of autocrine TGF-β1 in activation of TGF-β signalling by acetaldehyde in cultured HSC. To evaluate the effect of acetaldehyde on cultured HSC responding to TGF-β1 stimulation, HSC, transfected with p3TP-Lux, were treated with acetaldehyde (200 μM) for 24 h prior to the addition of exogenous active TGF-β1 (0–3 ng/ml) for an additional 24 h (Figure 1B). The results demonstrated that exogenous TGF-β1 increased luciferase activities, in a dose-dependent manner, in both groups of cells with or without acetaldehyde treatment (Figure 1B). However, acetaldehyde increased the half-maximal responding concentration of cells to exogenous TGF-β1 from 0.6 ± 0.15 to 0.93 ± 0.13 ng/ml. In addition, acetaldehyde significantly enhanced the maximal response of HSC to TGF-β1 stimulation (Figure 1B).

As mentioned earlier, TGF-β1 is synthesized and secreted in a latent, biologically inactive form, which must be activated before binding to Tβ-RII and initiating signalling. To evaluate the effect of acetaldehyde on TGF-β1 production in cultured HSC, HSC were serum-starved for 48 h prior to the treatment with or without acetaldehyde for an additional 24 h. Conditioned medium was analysed for the amount of both total TGF-β1 and the active form of this cytokine by immunoassays (ELISA), as described in the Materials and methods section. Results indicated that, compared with the control, acetaldehyde increased the amount of total TGF-β1 in the conditioned media by 55%, and enhanced the amount of active TGF-β1 by 275% (Figure 1C). These results suggested that acetaldehyde stimulated TGF-β1 signalling by increasing both the secretion and activation of latent TGF-β1.

Acetaldehyde increases ^{125}I-TGF-β1 binding to cultured HSC and up-regulates the expression of Tβ-RII gene

To further explore the mechanism by which acetaldehyde enhanced the maximal response of cultured HSC to TGF-β1 stimulation, it was hypothesized that in addition to increasing the secretion and activation of latent TGF-β1, acetaldehyde might induce the expression of elements in the TGF-β signal transduction pathway, including Tβ-RII. ^{125}I-TGF-β1 binding assays were carried out to elucidate the effect of acetaldehyde on TGF-β1 binding to cultured HSC (Figure 2). Serum-starved HSC, treated with or without acetaldehyde for 24 h, were exposed to ^{125}I-TGF-β1 (0.1–3.5 ng/ml) in the absence or presence of a 50-fold excess of unlabelled TGF-β1 to measure total or non-specific binding respectively. A specific binding curve was obtained by subtraction of non-specific binding from total binding (Figure 2A). Compared with the control, acetaldehyde treatment of serum-starved HSC significantly increased ^{125}I-TGF-β1-specific binding. These data were further plotted for Scatchard analysis (Figure 2B), which indicated that acetaldehyde did not alter the binding affinity of receptor(s) to TGF-β1. These results collectively indicated that instead of changing the affinity of receptor(s) to exogenous TGF-β1, acetaldehyde increased the number of TGF-β1-binding receptor(s) in serum-starved HSC. Results from further experiments supported the observation. As

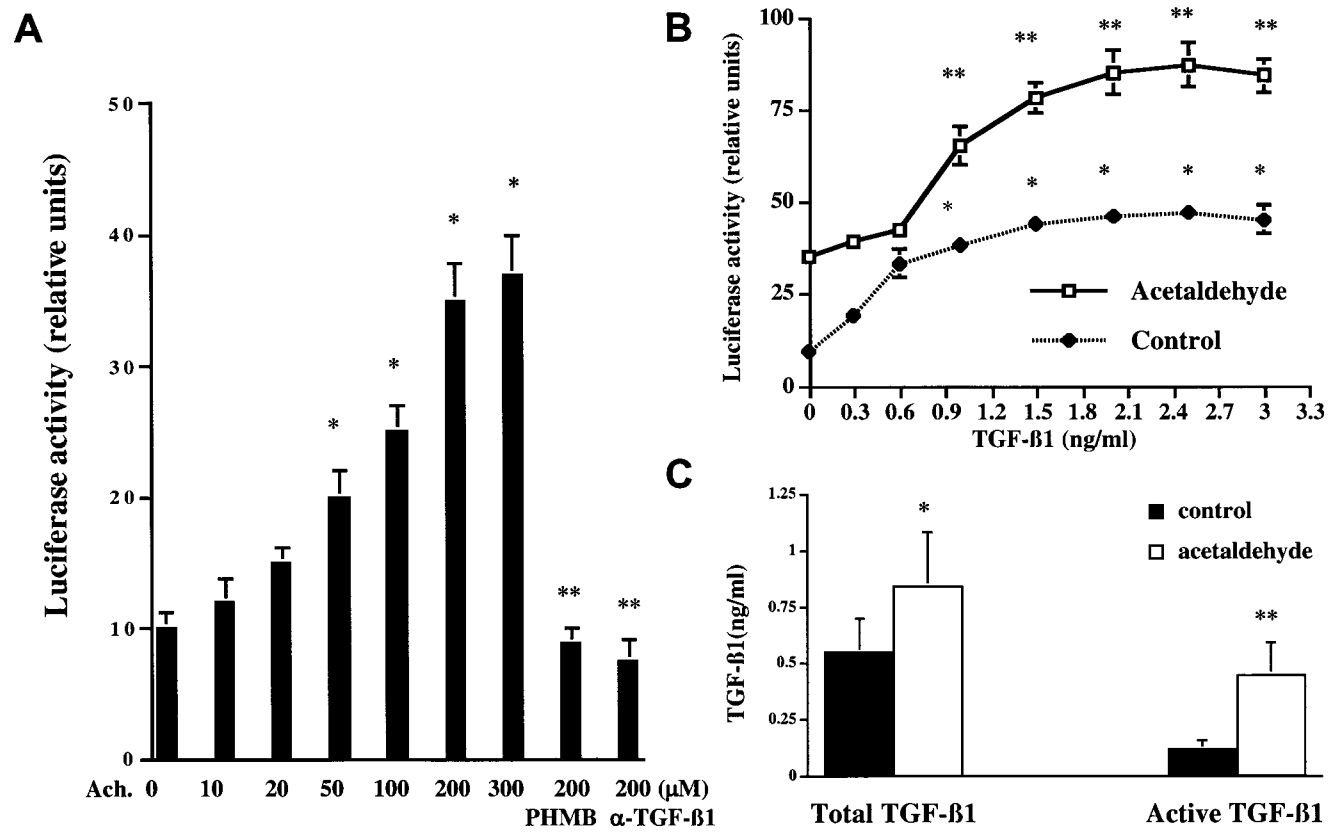


Figure 1 Acetaldehyde induces TGF-β signalling and activates latent TGF-β1 in cultured HSC

(**A**) Acetaldehyde induces TGF-β signalling. HSC, transfected with p3TP-Lux, a TGF-β-inducible luciferase reporter, were treated with acetaldehyde at the indicated concentrations (0–300 μM). In some experiments, neutralizing anti-TGF-β1 antibodies (30 μg/ml) or PHMB (4 μM), an acetaldehyde–protein adduct-formation inhibitor, were added 30 min prior to the addition of acetaldehyde (Ach.; 200 μM). The media with acetaldehyde was replaced once every 12 h. Luciferase activities were determined after β-galactosidase normalization. Values were means $\pm$ S.D. ($n = 6$). $^{*}P < 0.05$ versus cells without acetaldehyde. $^{**}P < 0.01$ versus cells treated with 200 μM acetaldehyde. (**B**) Acetaldehyde increases the response of HSC to exogenous TGF-β1. HSC, transfected with p3TP-Lux, were treated with (□) or without (◆) acetaldehyde (200 μM) for 24 h prior to the addition of exogenous active TGF-β1 (0–3 ng/ml) for an additional 24 h. Luciferase activities were determined after β-galactosidase normalization. Values were expressed as means $\pm$ S.D. ($n = 6$). $^{*}P < 0.05$ versus cells without acetaldehyde and TGF-β1; $^{**}P < 0.05$ versus cells treated with acetaldehyde only, without exogenous TGF-β1. (**C**) Acetaldehyde increases the secretion and activation of latent TGF-β1. Aliquots of conditioned media from serum-starved HSC, treated with or without acetaldehyde (200 μM) for 24 h, were analysed for total and active TGF-β1 by ELISA ($n = 6$; see the Materials and methods section for details). $^{*}P < 0.05$ versus total TGF-β1 in cells without acetaldehyde; $^{**}P < 0.05$ versus active TGF-β1 in cells without acetaldehyde.

shown by RPA in Figures 3(A) and 3(B), compared with the control (0 h), acetaldehyde increased the abundance of endogenous Tβ-RII mRNA in serum-starved HSC by 95, 152 and 168 % after 8, 16 and 24 h treatment respectively. In agreement with this result, acetaldehyde significantly increased the amount of Tβ-RII protein in these cells by approx. 16, 140 and 275 % after 3, 6 and 24 h treatment respectively (Figures 3C and 3D). PHMB completely abrogated the increase induced by acetaldehyde. A commercial standard, rTβ-RII (68 kDa; ctr in Figure 3C), was used to locate the endogenous Tβ-RII band (70 kDa) in Western blotting analyses. 28 S rRNA and β-actin was used as an internal control for mRNA and protein quantification, respectively (Figure 3). The control levels did not change within the time span of the experiments. Taken together, these results demonstrated that acetaldehyde increased ^{125}I-TGF-β1 binding to serum-starved HSC by inducing Tβ-RII gene expression.

Localization of acetaldehyde responsive elements in the Tβ-RII gene promoter

To begin elucidating the mechanism by which acetaldehyde induced Tβ-RII gene expression, HSC were transfected with plasmids pTβ-RII-N, in which various sizes of the Tβ-RII gene promoter were subcloned into the luciferase reporter vector pGL2 [30]. Luciferase activities were analysed in cells treated with or without acetaldehyde (200 μM). As shown in Figure 4(A), two major acetaldehyde response enhancers were located within -219 to -172 and -47 to -2 in the Tβ-RII gene promoter. Deletion of these two regions from the Tβ-RII gene promoter led to failures in response to acetaldehyde stimulation (Figure 4A). Removal of the fragment from -100 to -47 resulted in a marked increase in luciferase activity (Figure 4A), suggesting a repressor in this fragment. The repressor and its binding protein are under investigation and will be addressed in another report. The region between -47 and -2 was essential for basal transcription of this gene (Figure 4A).

Previous experiments revealed an AP-1 site (-195 to -189) and a GC box (-26 to -18) within the regions between -219 to -172 and -47 to -2 respectively, in the Tβ-RII gene promoter [30]. Further experiments were performed to evaluate effects of these two potential *cis*-activating elements on acetaldehyde-induced activation of the Tβ-RII gene promoter (Figure 4B). Luciferase assays indicated that, compared with the parental plasmid p-219 [i.e. pTβ-RII ($-219/+35$)], site-directed

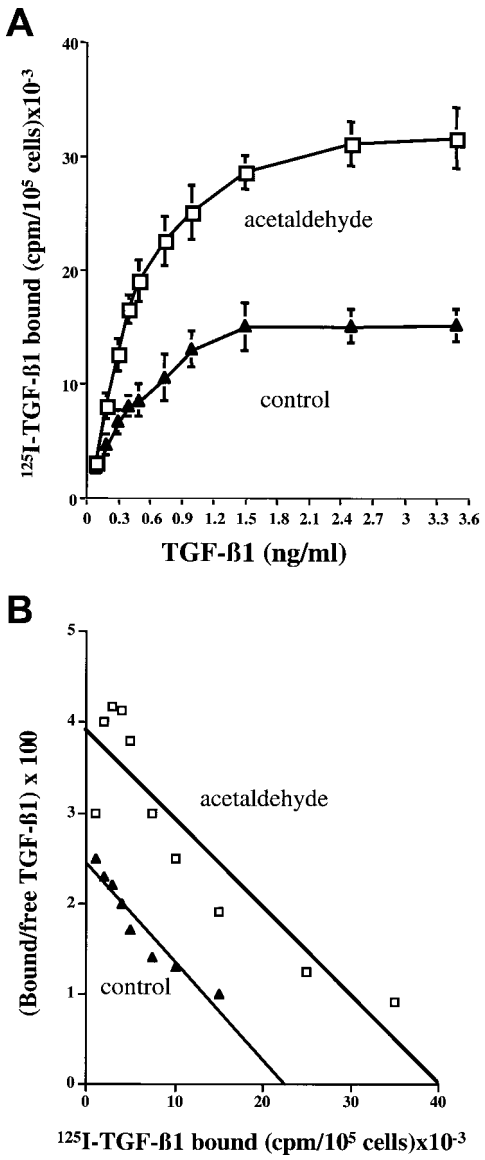


Figure 2 Acetaldehyde increases ^{125}I-TGF-β1 binding to serum-starved HSC

Serum-starved HSC were treated with (□) or without (▲) acetaldehyde (200 μM) for 24 h. Cells (10^5) with different treatments were incubated with ^{125}I-TGF-β1 at the indicated concentrations at 4 °C for 4 h in the absence or presence of a 50-fold excess of unlabelled TGF-β1 to measure total or non-specific binding respectively. Radioactivities of the aliquots of soluble fraction were measured using a gamma counter. ^{125}I-TGF-β1-specific binding was obtained by subtraction of non-specific binding from total binding and was expressed as c.p.m./10^5 cells (means ± S.D., $n = 3$). (**A**) Specific binding of ^{125}I-TGF-β1 at the indicated concentrations to serum-starved HSC treated with or without acetaldehyde. (**B**) Scatchard analysis of ^{125}I-TGF-β1-specific binding data.

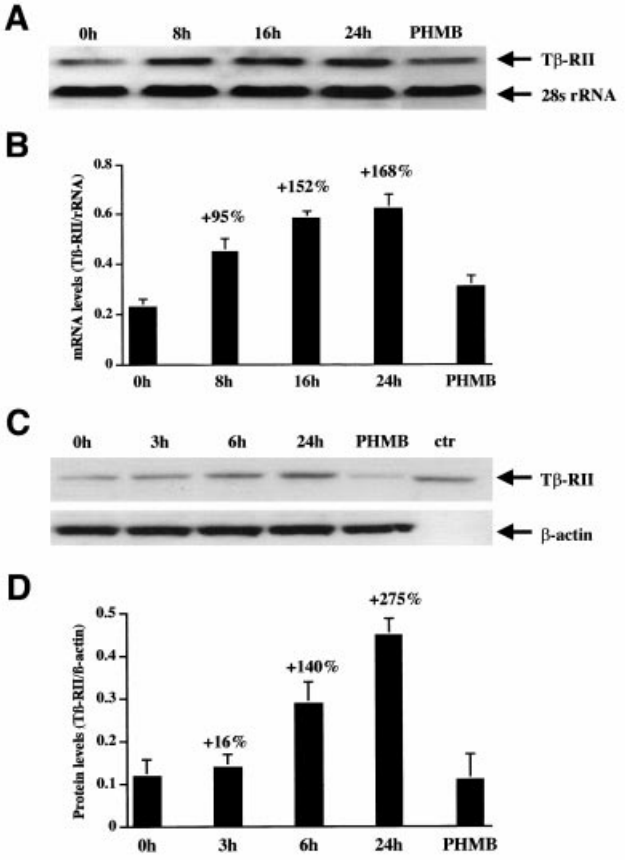


Figure 3 Acetaldehyde up-regulates Tβ-RII gene expression in HSC

Serum-starved HSC were treated with acetaldehyde (200 μM) in the presence or absence of PHMB (4 μM) for the indicated times. Total RNA (10 μg/sample) or proteins (20 μg/sample) were analysed for Tβ-RII mRNA and protein by RPA and Western blotting analyses respectively. Percentages are increases in levels of mRNA or protein compared with the control (0 h). (**A**) A representative of RPA from three independent experiments. 28 S rRNA was used as an internal control to normalize equal loading. (**B**) Quantification of Tβ-RII mRNA levels in RPA ($n = 3$). (**C**) A representative of three independent Western blots. A commercial standard, rTβ-RII (68 kDa), was used to locate the endogenous Tβ-RII band (70 kDa). β-Actin was used as an internal control to normalize equal loading. ctr, control. (**D**) Quantification of Tβ-RII protein levels in Western blots ($n = 3$).

mutations in either the AP-1 site (AP-1 mut.) or the GC box site (GC mut.) resulted in a significant reduction in luciferase activities induced by acetaldehyde by approx. 42 and 27% respectively (Figure 4B). Site-directed mutations in both sites caused a further reduction by 65% (Figure 4B). To determine what protein complex interacted with the AP-1 motif, activated HSC were co-transfected with p-219 and an expression plasmid of dominant negative c-Jun or c-Fos (Figure 4B). Luciferase assays showed that dominant negative c-Jun or c-Fos significantly reduced luciferase activities induced by acetaldehyde in these cells, suggesting that the proteins interacting with the AP-1 site might be a heterodimeric complex of c-Jun and c-Fos. Taken together, these results indicated that both the AP-1 site and the GC box, as *cis*-activating elements, positively mediated the activation of the Tβ-RII gene promoter by acetaldehyde in cultured HSC.

Acetaldehyde induces AP-1 and BTEB DNA binding to the Tβ-RII promoter

EMSA were carried out to clarify the proteins which bound to the acetaldehyde-response elements in the Tβ-RII gene promoter, as well as to evaluate effects of acetaldehyde on DNA-binding activities of these proteins. Nuclear extracts (10 μg/each) of serum-starved HSC, treated with or without acetaldehyde (200 μM) for the indicated times, were analysed by EMSA. A DNA fragment (−219 to −172), containing the AP-1 site in the

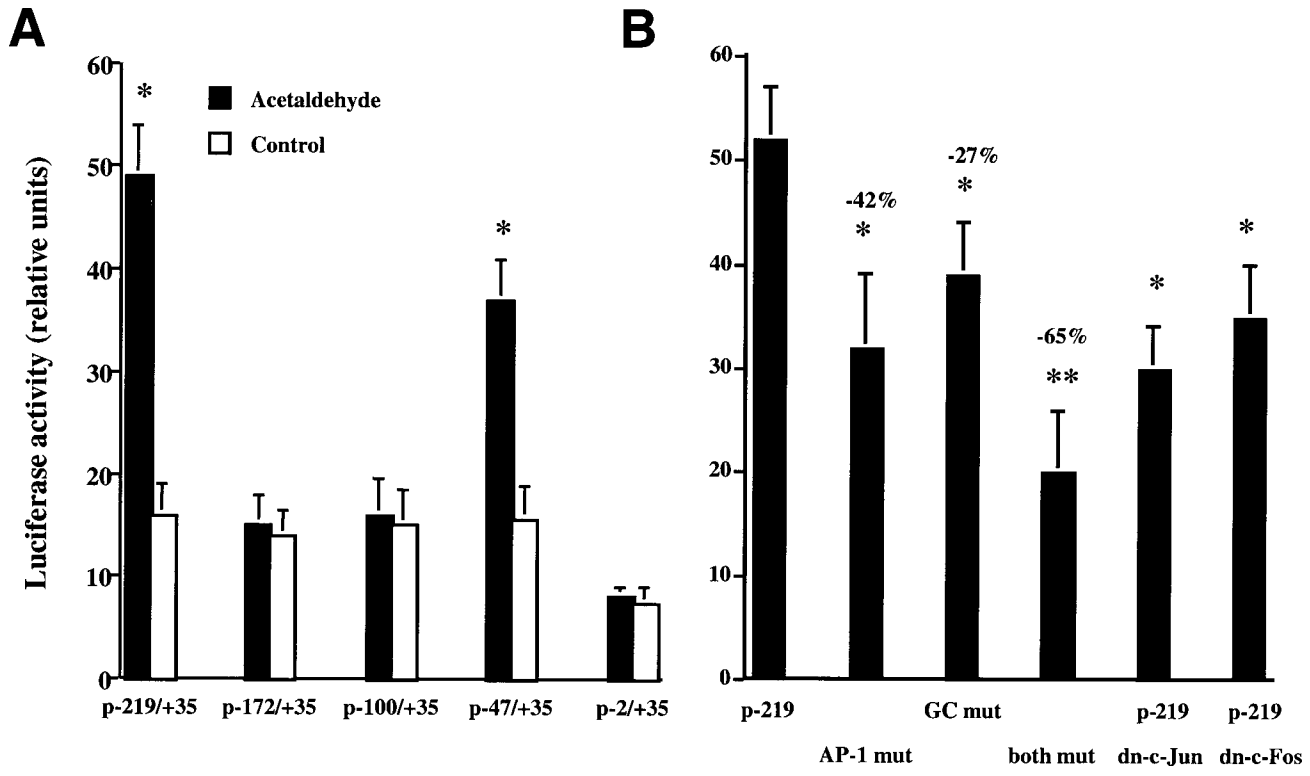


Figure 4 Localization of acetaldehyde responsive elements in the Tβ-RII gene promoter

HSC, transfected with Tβ-RII reporter plasmids, were treated with or without acetaldehyde (200 μM). Luciferase activities were analysed and normalized by β-galactosidase activities. Values were means $\pm$ S.D. ($n = 6$). (**A**) Cells were transfected with pTβ-RII-N, where N is the size of the Tβ-RII gene promoter. *$P < 0.05$ versus the same group of cells without acetaldehyde treatment. (**B**) Cells were transfected with p-219, or with p-219 (AP-1 mut), containing site-directed mutations within the AP-1 site, or with p-219 (GC mut), having site-directed mutations in the GC box, or with p-219 (both mut), with site-directed mutations within the AP-1 sites and the GC box. In some experiments, HSC were co-transfected with p-219 and an expression plasmid of dominant negative c-Jun (dn-c-Jun) or dominant negative c-Fos (dn-c-Fos). *$P < 0.05$ versus cells transfected with p-219; **$P < 0.05$ versus cells transfected with p-219 (AP-1 mut) or p-219 (GC mut). Percentages are changes in luciferase activities compared with that in cells transfected with p-219.

Tβ-RII gene promoter, was used as a probe. As shown in Figure 5(A), acetaldehyde, in a time-dependent pattern, stimulated protein binding to the ^{32}P-labelled probe (Figure 5A, lanes 1–3). An excess of the unlabelled DNA fragment (50 $\times$) competitively prevented the protein from binding to the probe (Figure 5A, lane 4), showing the binding specificity. Pretreatment with either anti-c-Jun (Figure 5A, lane 5) or anti-c-Fos (Figure 5A, lane 6) antibodies resulted in an apparent supershift and eliminated the protein-binding band, indicating that the protein was AP-1 formed by c-Jun and c-Fos. Taken together, these results demonstrated that acetaldehyde stimulated AP-1 binding to the promoter of Tβ-RII gene in serum-starved HSC.

Additional EMSA were carried out to determine the protein binding to the GC box in the Tβ-RII gene promoter, and to further evaluate the effect of acetaldehyde on the GC-box-binding activity of this protein. The GC box and flanking nucleotides in the Tβ-RII promoter (-33 to -14) was used as a probe, while a consensus SP-1 probe was used as a control (Figure 5B). The SP-1 DNA-binding activity was not altered by acetaldehyde in serum-starved HSC (Figure 5B, lanes 1 and 2). In contrast, the DNA-binding activity of the protein to the GC box was induced by acetaldehyde in a time-dependent manner (Figure 5B, lanes 3–5). Compared with SP-1, this GC-box-binding protein had a distinct mobility shift. Its binding band was competed by an excess (50 $\times$) of the unlabelled probe (Figure 5B, lane 6), but not by a probe with site-directed mutations in the GC box (Figure 5B, lane 7), or by the SP-1 probe (Figure 5B, lane 8), suggesting that this protein specifically bound to the GC box. Both SP-1 and BTEB were GC-box-binding proteins [32,38]. Previous studies have shown that the GC-box-binding activity of BTEB was induced by acetaldehyde in serum-starved HSC [17]. Supershift assays demonstrated that anti-BTEB antibodies caused a supershift and an apparent reduction, if not disappearance, of the protein-binding band (Figure 5B, lane 9), suggesting that BTEB was the protein binding to the GC box in the promoter of Tβ-RII gene. Western blot analyses using anti-BTEB antibodies further indicated that acetaldehyde caused a time-dependent increase in the abundance of BTEB protein in serum-starved HSC (Figure 6). Taken together, these results demonstrated that acetaldehyde stimulated BTEB binding to the GC box in the Tβ-RII gene promoter in cultured HSC.

AP-1 and BTEB as *trans*-activating elements are required for Tβ-RII gene expression induced by acetaldehyde in cultured HSC

Antisense oligonucleotides have been used to specifically block the expression of corresponding proteins, as reviewed recently in [39,40]. Phosphorothioate-modified antisense oligonucleotides of c-Jun or BTEB at 50 μg/ml dramatically, if not completely, suppressed c-Jun and BTEB protein expression respectively [17,34]. Antisense c-Jun oligonucleotides at 50 μg/ml blocked the AP-1 *trans*-activating activity [17,34]. To evaluate the roles of AP-1 and BTEB, as *trans*-activating elements, in regulating the

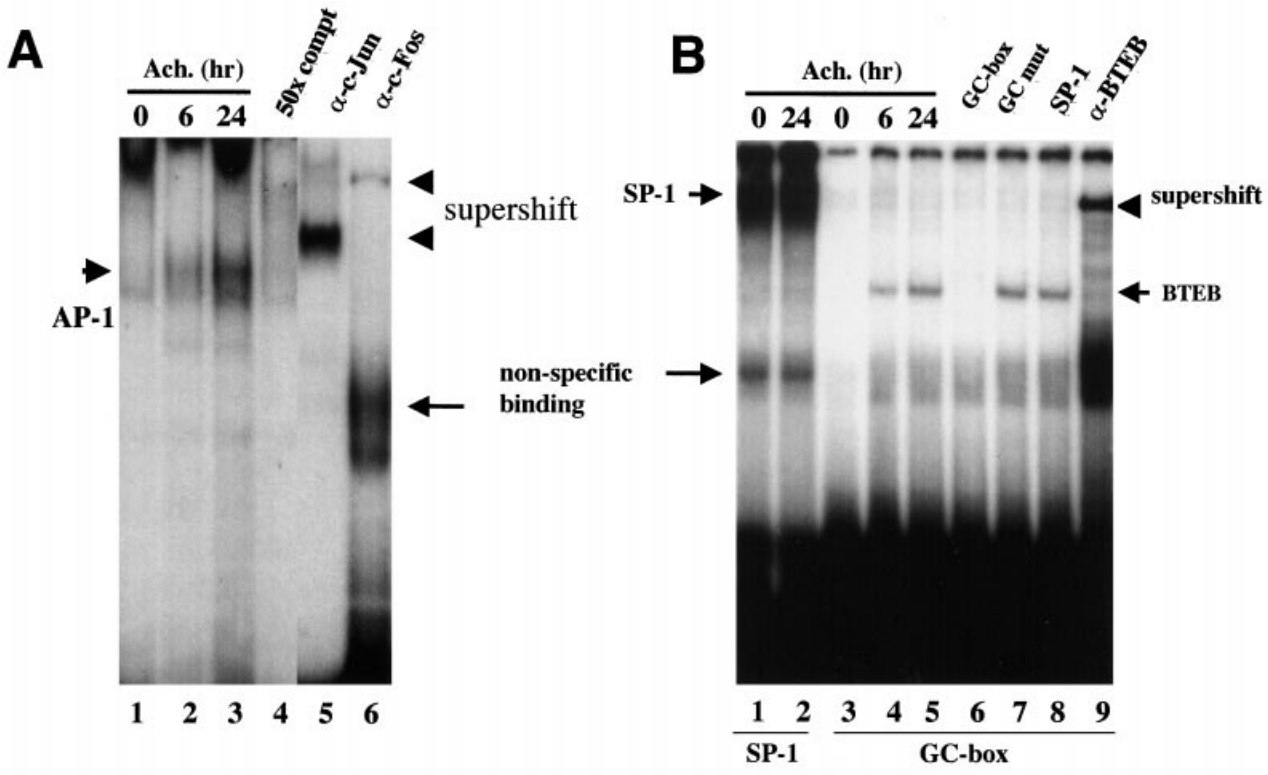


Figure 5 Acetaldehyde stimulates AP-1 and BTEB binding to the Tβ-RII gene promoter

Nuclear extracts (10 μg/each) of serum-starved HSC, treated with or without acetaldehyde (Ach.; 200 μM) for the indicated times, were analysed by EMSA. An excess of unlabelled probe (50 $\times$; a DNA fragment, GC box or SP-1) was used in competition (compt) assays. Anti-c-Jun (α-c-Jun), anti-c-Fos (α-c-Fos) or anti-BTEB (α-BTEB) antibodies were used in supershift assays. Representatives of three independent experiments are shown here. (**A**) Using a ^{32}P-labelled DNA fragment (-219 to -172), containing the AP-1 site in the Tβ-RII promoter; (**B**) utilizing a ^{32}P-labelled consensus SP-1 probe (SP-1), and a ^{32}P-labelled GC box (GC-box) located in the Tβ-RII gene promoter (-32 to -14).

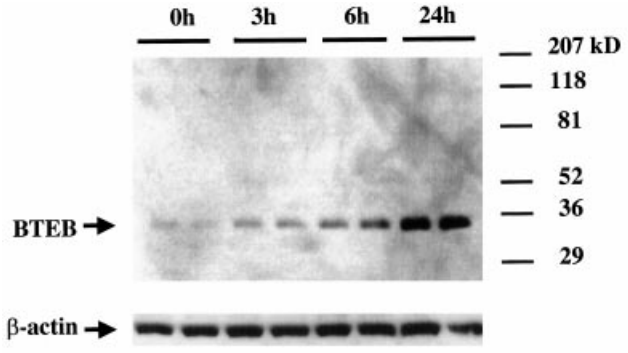


Figure 6 Acetaldehyde induces BTEB protein expression in serum-starved HSC

Whole-cell extracts (20 μg of protein/sample) of serum-starved HSC, treated with acetaldehyde for the indicated times, were assessed by Western blotting analyses using anti-BTEB antibodies. β-Actin was used as an internal control to normalize equal loading. A representative of three independent experiments is shown.

expression of Tβ-RII gene induced by acetaldehyde, sense or antisense oligonucleotides of c-Jun or BTEB at 50 μg/ml were added to HSC transfected with pTβ-RII($219/+35$) 4 h prior to the addition of acetaldehyde (200 μM). Compared with cells treated with acetaldehyde only (controls), pretreatment of cells with antisense c-Jun or BTEB oligonucleotides significantly reduced luciferase activities induced by acetaldehyde (Figure 7A). Combination of these two antisense oligonucleotides caused a further reduction in luciferase activities in these cells (Figure 7A). In contrast, neither of the sense oligonucleotides at 50 μg/ml had any effect (Figure 7A), suggesting that AP-1 and BTEB were required for activation of the Tβ-RII gene promoter by acetaldehyde. Western blotting analyses confirmed that either c-Jun or BTEB antisense at 50 μg/ml significantly reduced the abundance of endogenous Tβ-RII protein in serum-starved HSC treated with acetaldehyde (Figure 7B). Luciferase assays further demonstrated that either of the antisense oligonucleotides significantly reduced luciferase activities induced by acetaldehyde in cells transfected with p3TP-Lux (Figure 7C), indicating a role for the two transcription factors in TGF-β signalling mediated by regulation of Tβ-RII gene expression. Taken together, these data indicated that blocking the expression of AP-1 and/or BTEB resulted in a significant reduction in Tβ-RII gene expression, suggesting a requirement for AP-1 and BTEB, as *trans*-activating elements, for Tβ-RII gene expression induced by acetaldehyde in cultured HSC.

TGF-β signalling contributes to α1(I) collagen gene expression in cultured HSC treated with acetaldehyde

Acetaldehyde has been shown to stimulate the expression of the α1(I) collagen gene in cultured HSC [17,19–21]. To evaluate the effect of TGF-β signalling on α1(I) collagen gene expression

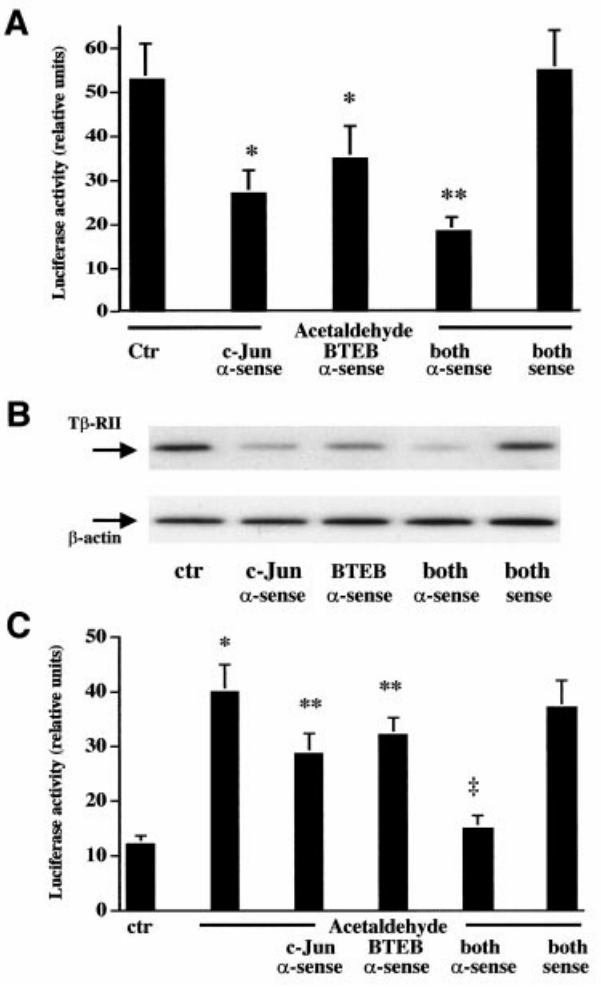


Figure 7 AP-1 and BTEB as *trans*-activating elements are required for expression of Tβ-RII gene induced by acetaldehyde in cultured HSC

Antisense (α-sense) oligonucleotides of c-Jun or BTEB, both antisense, or both sense oligonucleotides, at 50 μg/ml were added to HSC prior to the addition of acetaldehyde (200 μM). Luciferase activities were normalized by β-galactosidase activities. Values were means $\pm$ S.D. ($n = 6$). (**A**) Luciferase assays of HSC transfected with pTβ-RII ($-219/+35$). $^*P < 0.05$ versus the control (Ctr); $^{**}P < 0.05$ versus cells treated with either c-Jun or BTEB antisense oligonucleotides. (**B**) A representative of three independent Western blots of endogenous Tβ-RII in serum-starved HSC. β-Actin was an internal control to normalize equal loading; (**C**) Luciferase assays of passaged HSC transfected with p3TP-Lux. $^*P < 0.05$ versus the control; $^{**}P < 0.05$ versus cells treated with acetaldehyde only; $\ddagger P < 0.05$ versus cells treated with either c-Jun or BTEB antisense oligonucleotides.

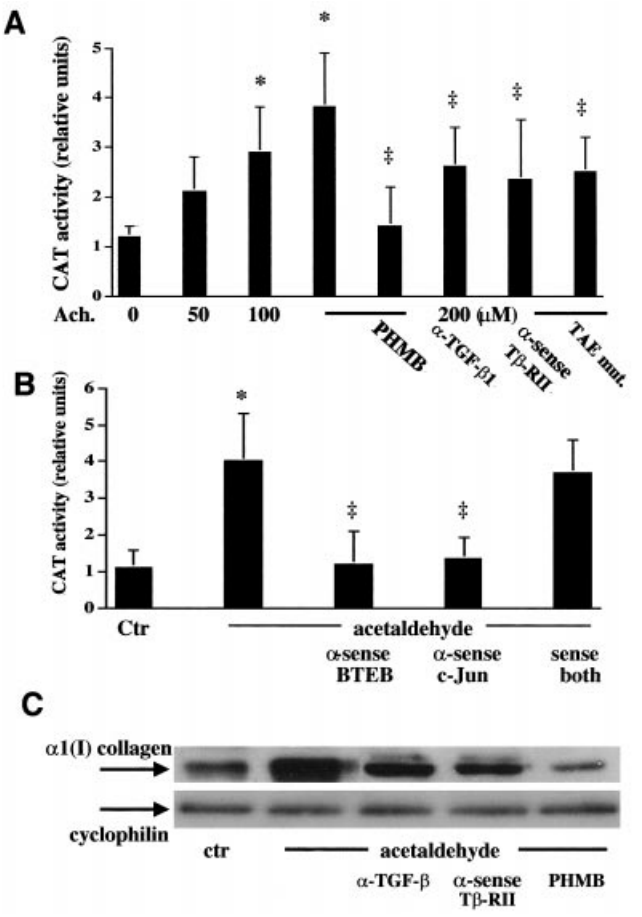


Figure 8 TGF-β signalling participates in α1(I) collagen gene expression in HSC treated with acetaldehyde

(**A**) HSC were transfected with pcolCAT 1.7 or pcolCAT 1.7 (TAE mut). PHMB, anti-TGF-β1 antibodies or antisense Tβ-RII oligonucleotides, were added prior to the addition of acetaldehyde (Ach.) at the indicated concentrations. CAT activities were normalized by β-galactosidase activities ($n = 6$). $^*P < 0.05$ versus cells without acetaldehyde (Ach. 0). $\ddagger P < 0.05$ versus cells treated with acetaldehyde only (200 μM). (**B**) Antisense c-Jun or BTEB, or both sense oligonucleotides, were added to HSC transfected with pcolCAT 1.7 prior to the addition of acetaldehyde (200 μM). CAT activities were analysed ($n = 6$). $^*P < 0.05$ versus the control (Ctr). $\ddagger P < 0.05$ versus cells treated with acetaldehyde alone. (**C**) RPA of α1(I) collagen mRNA in serum-starved HSC treated with anti-TGF-β1 antibodies, antisense Tβ-RII oligonucleotides or PHMB prior to the addition of acetaldehyde (200 μM). Cyclophilin was used as an internal control for equal loading.

induced by acetaldehyde, HSC were transfected with the α1(I) reporter plasmid pcolCAT 1.7. Cells were treated with acetaldehyde (0–200 μM). Compared with cells without acetaldehyde treatment, acetaldehyde induced a dose-dependent increase in CAT activities, which was completely blocked by PHMB (4 μM; Figure 8A). Interestingly, blocking TGF-β signalling by neutralizing anti-TGF-β antibodies (30 μg/ml), or by antisense Tβ-RII oligonucleotides (50 μg/ml), resulted in a partial, but significant, reduction in CAT activities (Figure 8A). A TAE was previously located within the α1(I) collagen gene promoter (-1625 to -1615 bp) [41,42]. HSC were transfected with pcolCAT 1.7 (TAE mut.) containing site-directed mutations in TAE. Luciferase assays revealed that mutations in TAE resulted in a reduction in response to acetaldehyde (Figure 8A). These results collectively indicated an involvement of TGF-β signalling in the activation of the α1(I) collagen gene promoter by acetaldehyde. These results also suggested that in addition to TAE, other acetaldehyde response elements might contribute to activation of the α1(I) collagen gene promoter by acetaldehyde. Further studies demonstrated that inhibition of BTEB or c-Jun protein expression by respective antisense oligonucleotides completely blocked the activation of the α1(I) collagen gene promoter (Figure 8B), suggesting that AP-1 and BTEB might play a critical role in the expression of this gene in HSC. RPA revealed that acetaldehyde increased the abundance of endogenous α1(I) collagen mRNA (Figure 8C), which was completely abrogated by PHMB. Pretreatment of these cells with either neutralizing anti-TGF-β1 antibodies or antisense Tβ-RII oligonucleotides resulted in a partial, but marked, reduction in endogenous α1(I)

collagen mRNA (Figure 8C), which confirmed the results from transfection assays (Figure 8A). In summary, these results collectively demonstrated that TGF-β signalling made a significant contribution to α1(I) collagen gene expression in cultured HSC treated with acetaldehyde.

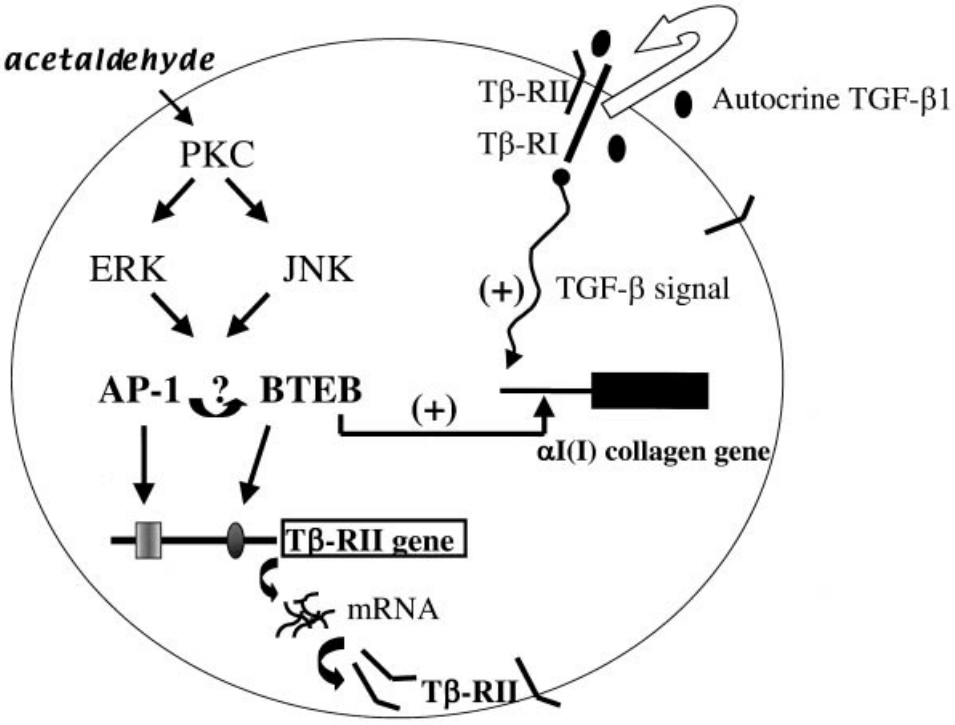
DISCUSSION

Although alcohol abuse causes a number of deaths due to cirrhosis, the underlying mechanisms remain largely unknown. It is widely accepted that TGF-β1 plays important roles in hepatic fibrogenesis [14]. It is therefore of interest to evaluate the relationship between acetaldehyde, the major active metabolite of alcohol, and TGF-β signalling. This report demonstrates that acetaldehyde activated TGF-β signalling by increasing the secretion and activation of latent TGF-β1 as well as by inducing the expression of Tβ-RII in cultured HSC. Activation of TGF-β signalling by acetaldehyde made a significant contribution to α1(I) collagen gene expression in these cells.

To study the mechanisms by which acetaldehyde activated TGF-β signalling, it was hypothesized that acetaldehyde might induce the expression of elements in the TGF-β signal transduction pathway. ELISA results revealed that acetaldehyde not only increased the secretion of latent TGF-β1, but also increased the amount of the active form of TGF-β1. Latent TGF-β1 must be activated before binding to TGF-β receptors and initiating TGF-β signalling [5,7]. It is as yet poorly defined how acetaldehyde stimulates the latent TGF-β1 activation. ^{125}I-TGF-β1-binding assays suggested that, instead of changing the binding affinity of surface receptor(s) to TGF-β1, acetaldehyde might increase the expression of TGF-β1-binding receptor(s), including Tβ-RII. Additional experiments demonstrated that acetaldehyde caused a time-dependent increase in ^{125}I-TGF-β1 binding to cultured HSC (results not shown). Further experiments demonstrated that acetaldehyde induced the expression of the Tβ-RII gene in cultured HSC. Substantial evidence has indicated an important role for Tβ-RII in activation of HSC *in vitro* and *in vivo*. A rapid increase in the abundance of Tβ-RII mRNA was observed in HSC *in vivo* during liver injury [4]. Disturbing Tβ-RII functions by dominant negative Tβ-RII or soluble TGF-β receptors resulted in a marked reduction in the development of hepatic fibrosis and an enhancement of hepatocyte regeneration in animals [11–13]. It must be emphasized that these results do not exclude a possible involvement of other signal elements, such as type I TGF-β receptor and Smad proteins, in TGF-β signalling induced by acetaldehyde in activated HSC.

To elucidate molecular mechanisms by which acetaldehyde induced Tβ-RII gene expression in HSC, two *cis*-activating elements, an AP-1 site and a GC box, were located in the promoter region of the Tβ-RII gene. The same AP-1 site was recently reported to be critical for Tβ-RII gene expression in human pancreatic cancer cells [43]. AP-1 and BTEB, as *trans*-activating elements, played crucial roles in regulating the expression of Tβ-RII gene induced by acetaldehyde in HSC. Previous data have shown that AP-1 and BTEB were activated by acetaldehyde in cultured HSC [17]. A GC box in a gene promoter can potentially bind to SP-1 [32,38], BTEB [26] or Zf9 [44], largely depending on the number, position and flanking nucleotide sequence of the GC box [32,38]. EMSAs in this report suggested that BTEB might be the main, if not the only, protein binding to the GC box in the Tβ-RII gene promoter.

Previous studies have shown that acetaldehyde stimulated the expression of the α1(I) collagen gene in HSC [17,19–21]. In this report, blocking TGF-β signalling resulted in a significant but



Scheme 1 Scheme of acetaldehyde's effects on Tβ-RII and α1(I) collagen gene expression in serum-starved HSC

PKC, protein kinase C; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-related protein kinase.

partial reduction in the expression of α1(I) collagen gene induced by acetaldehyde in cultured HSC, indicating an involvement of TGF-β signalling in the expression of this gene. This result was in agreement with a previous report that antisense TAE oligonucleotides significantly reduce TGF-β-induced expression of the α1(I) collagen gene in fibroblasts [45]. Furthermore, this result suggested that, in addition to TAE, other acetaldehyde response elements might be located in the α1(I) collagen gene promoter. An earlier observation supported this suggestion, showing that acetaldehyde stimulated BTEB binding to a GC box in the α1(I) collagen gene promoter and up-regulated the expression of this gene in HSC [17]. The intracellular signal transduction mechanisms are more complicated than the initially striking simplicity of the core TGF-β/Smad signalling pathway [5]. Additional studies are required to dissect the downstream mediators from Tβ-RII to α1(I) collagen gene.

Based on these and other observations, a model was proposed to explain the mechanisms by which acetaldehyde induces the expression of α1(I) collagen in HSC (Scheme 1). Acetaldehyde activates signal transduction pathways, including protein kinase C, extracellular signal-regulated kinase and Jun N-terminal kinase, leading to activation of AP-1 and to an increase in BTEB gene expression. BTEB binds to the GC box in the α1(I) collagen gene promoter and thereby directly regulates the expression of this gene. AP-1 is proposed to regulate the expression of BTEB by binding to AP-1 sites in the promoter region of the BTEB gene and, therefore, indirectly regulates α1(I) collagen gene expression in HSC. Furthermore, AP-1 and BTEB, as *trans*-activating elements, up-regulate Tβ-RII gene expression in HSC. By stimulating latent TGF-β1 secretion and activation, as well as by up-regulating the expression of Tβ-RII, acetaldehyde activates TGF-β signalling, which further enhances expression of the α1(I) collagen gene via the TAE in the promoter of this gene. This model does not exclude other mechanisms possibly involved in α1(I) collagen gene expression induced by acetaldehyde in HSC. Further studies are necessary to clarify details in this model.

In summary, the present study has demonstrated that acetaldehyde activated TGF-β signalling by stimulating the secretion and activation of latent TGF-β1, as well as by inducing the expression of the Tβ-RII gene in cultured HSC. AP-1 and BTEB, as *trans*-activating elements, were required for Tβ-RII gene expression induced by acetaldehyde. Activating TGF-β signalling by acetaldehyde made a notable contribution to α1(I) collagen gene expression in HSC. It is noteworthy that these results, collected from serum-starved or activated HSC *in vitro*, may not comprehensively reflect the condition in quiescent HSC *in vivo*, although it is believed that the process of serum starvation renders cultured HSC quiescent [18,27]. The results in this report provide a novel insight into mechanisms by which acetaldehyde stimulates expression of the α1(I) collagen gene in cultured HSC, and a better understanding of the effects of alcohol (or acetaldehyde) on hepatic fibrogenesis. These results also offer implications for studying the effects of TGF-β signalling on the stimulation of the over-expression of the ECM, including α1(I) collagen, in fibrosis found in other organs.

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