Elevated expression of transforming growth factor β3 in carbon tetrachloride-treated rat liver and involvement of retinoid signaling

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Abstract. Transforming growth factor (TGF) β is a pro-fibrotic cytokine. While three isoforms (TGF-β1, 2 and 3) are known, the functional differences between them are obscure. To investigate the roles of TGF-β isoforms during liver fibrogenesis, male Wistar rats were administrated carbon tetrachloride (CCL₄) subcutaneously twice a week for two months. Livers were excised and sectioned for histochemical examinations. These livers were also used to quantitate the expression of genes associated with fibrogenesis, including TGF-β isoforms, as well as those associated with retinoid metabolism. Expression levels of Tgfβ1 and Tgfβ3 were up-regulated in CCL₄-treated rat livers while that of Tgfβ2 was not changed. The mRNAs for lecithin-retinol acyltransferase (Lrat) and retinoic acid hydroxylase, Cyp26a1, were also elevated. By immunohistochemical staining, TGF-β3 protein was found to be localized mainly in liver parenchymal cells (hepatocytes). These results indicate that retinoid mobilization likely takes place within the rat’s liver following CCL₄ treatment, and suggest the possibility that the expression of Tgfβ mRNA is regulated by retinoic acid receptors. Reporter analyses of a region of the Tgfβ3 gene were performed using the rat liver parenchymal cell line, RLC-16, and a positively responsive region was identified within its intron.

Introduction

Exposure to carbon tetrachloride (CCL₄) has hepatotoxic effects, including fatty degeneration and fibrosis. These effects are initiated by highly reactive radicals such as CCL₄• and CCL₄OO•, which can modify DNA, lipids, protein and carbohydrates (1). These radicals also damage membranous structures, such as the plasma membrane, endoplasmic reticulum, mitochondria, and the Golgi apparatus through lipid peroxidation. These modifications of cellular components and organelles lead to a loss of Ca²⁺ homeostasis, reduced protein synthesis, apoptosis, and DNA mutation (1). Many factors, including cytokines, chemokines, NO, and bile acids are proposed to mediate the hepatotoxic effects of CCL₄ (2).

Among those factors mediating CCL₄-induced fibrogenesis, TGF-β is the most important and well-studied cytokine (3-5). TGF-β was originally identified as a protein inducing anchorage-independent growth of non-neoplastic cells (6,7). It was later revealed to be involved in many cellular processes including cell proliferation, differentiation, motility, adhesion, and death. Mammalian TGF-β has three isoforms which are encoded by different genes and these three isoforms show similarities both structurally and functionally. TGF-β binds to specific receptors on the cell surface, and binding signals are transduced into the nucleus by Smad proteins. There, collagen gene expression is up-regulated, leading to the production and deposition of collagen proteins within the liver (5).

It is known that hepatic stellate cells, which reside within the perisinusoidal space between hepatic cords and liver sinusoidal endothelial cells, are involved in the fibrotic process (8,9). Another remarkable feature of hepatic stellate cells upon cirrhosis is the loss of vitamin A. However, the implication of this decrease in vitamin A within the liver is totally unknown. Vitamin A is obtained from foods in the form of the retinyl ester or β-carotene, and once converted to retinol in the intestine, it is carried in the bloodstream as retinyl ester to the liver, where it is stored for future usage (10,11). In target cells, vitamin A is converted to active metabolites (retinooids) which bind to nuclear transcription factors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), to activate transcription of their target genes (12,13) through retinoic acid response elements (RAREs) within gene promoters.

In this study of CCL₄-induced fibrosis of rat livers, we analyzed changes in mRNAs related not only to fibrogenesis,
but also to retinoid metabolism. A possible link between TGF-β1 signaling and retinoic acid signaling was also analyzed in this study.

Materials and methods

Materials. CCl₄, liquid paraffin, and all-trans retinoic acid (RA) were from Wako Pure Chemical (Osaka, Japan). Retinol (ROL) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA, USA) and Vitromex (Vilshofen, Germany), respectively. Oligonucleotides were synthesized by Invitrogen. The mouse embryonal teratocarcinoma cell line, F9 (RCB1555), and the rat liver parenchymal cell line, RLC-16 (RCB1474) were provided by the RIKEN Cell Bank (Tsukuba, Japan).

Animals. Protocols for animal experimentation were approved by the Animal Research Committee, Akita University Graduate School of Medicine. All animal experiments adhered to the ‘Guidelines for Animal Experimentation’ of the University. Male Wistar rats, 6 weeks of age, and weighing between 165-175 g, were injected subcutaneously with 0.3 ml of CCl₄ per kg of body weight, in liquid paraffin (1:9 mixture), twice a week for two months (a total of 17 times). Rats were then anaesthetized, and livers were excised and immersed in 10% formalin for fixation and then embedded in paraffin for sectioning. For RNA preparation, small pieces of livers were quick-frozen in liquid nitrogen and kept frozen at -85˚C. At the time of sacrifice, rats weighed 360-430 g.

RNA extraction and quantitative RT-PCR. Frozen rat livers were lysed in TRIzol reagent (Invitrogen), and total-RNA was isolated according to the manufacturer's protocols. Then, cDNAs were synthesized from 5 µg of total-RNA using SuperScript III Reverse Transcriptase (Invitrogen) with an oligo(dT)₁₈ primer. Quantification of cDNA was conducted with the LightCycler 480 SYBR-Green I Master (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. The primer sequences used are listed in Table I. PCR reactions were performed in triplicate, and standard deviations were calculated. Transcript amounts were normalized against the Gapdh transcript, and transcription levels in the normal liver were set to 1.0 for each gene.

The mouse embryonal teratocarcinoma cell line, F9, was cultured in RPMI-1640 medium supplemented with 10% FBS. RA in ethanol or ethanol alone was added to 10 cm culture dishes to a final concentration of 0.1 µM. After 24 h cultivation, cells were treated as above with 2 ml of TRIzol reagent for quantification of mRNA levels for Tgf/b1, Tgf/b2, Tgf/b3 and Rarb.

Immunohistochemistry. Paraffin-embedded livers were sectioned at 3 µm intervals and de-paraffinized by immersion in xylene. Buffers were substituted sequentially with 100, 90, 80, 70 and 50% ethanol. Specimens were treated in boiled citrate buffer (0.01 M) in order to unmask the antigens and then cooled to room temperature. For inactivation of endogenous peroxidase activity, slides were immersed in 1% H₂O₂ for 20 min. Slides were treated with 2% bovine serum albumin (BSA) in phosphate-buffered saline containing 0.1% Tween-20 (PBST) to block non-specific binding of antibodies. Slides were then incubated with rabbit polyclonal anti-TGF-β1 antibody (1:50 dilution, sc-146, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal anti-TGF-β3 antibody (1:50 dilution, sc-82, Santa Cruz Biotechnology) in PBST plus 2% BSA for 2 h at room temperature. After washing, slides were treated with a 1:500 dilution of peroxidase-conjugated AffiniPure goat anti-rabbit IgG. After washing, slides were incubated with DAB substrate (Roche Diagnostics) for 30 min. Nuclei were counterstained with Mayer’s hematoxylin solution (Wako Pure Chemical). Slides were washed with water and then immersed with 20, 40, 80 and 100% ethanol and finally, 100% xylene. Dried slides were sealed with Entellan new (Merck, Darmstadt, Germany) and recorded digitally using NanoZoomer Digital Pathology (Hamamatsu Photonics, Hamamatsu, Japan).

Plasmids. The genomic sequence of rat Tgf/b3 was obtained from the Ensembl database (ENSNOT00000013516). The intron sequence connecting exons 3 and 4 (4001-5390, numbers designate nucleotide positions within the intron) was amplified by PCR using a RoboCycler (Stratagene, La Jolla, CA, USA) with primers containing Km and XhoI overhangs and inserted in the pGL4.26 vector (Promega, Madison, WI, USA). The cloned fragment was verified by sequencing. There was an insertion of TGTGTG after 4087 and substitutions from A→G at 4156 and A→C at 5078. Though there is an insertion within the cloned fragments, we employed nucleotide numbering based on the database sequence. The reporter plasmid containing retinoic acid responsive element was used as a positive control as described elsewhere (14).

Mutagenesis of the cloned intron sequence (putativeRARE) was performed as described (15) using the primer pairs CACCTCCTGTTAAAGAGGCCCCACTATTGTACACCCCAACC/GGTGGGTTCAATAGTGCCGGTCCTTTACAGGAGG with the reporter plasmid in the same well. Three independent cultures were used for each luciferase assay, and the firefly luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) with a luminescence reader (BLR-201, Aloka, Tokyo, Japan). Luciferase activity from the experimental plasmid was normalized against luciferase activity from the control pRL-CMV plasmid in the same well. Three independent cultures were used for each luciferase assay, and the calculated standard deviations are indicated in the figures.

Transfection and luciferase assay. Transfection of plasmids into the rat liver parenchymal cell line RLC-16 was conducted with Lipofectamine 2000 reagent (Invitrogen). Two hundred and 50 ng of each reporter plasmid, pCDNA3.1-rRARα and pCDNA3.1-rRXRα, along with 12.5 ng of pRL-CMV (Promega) as an internal control, were transfected in 12-well formats. Three hours after transfection, the medium was changed and ligands were added. Twenty-four hours after ligand addition, cells were lysed and luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) with a luminescence reader (BLR-201, Aloka, Tokyo, Japan). Luciferase activity from the experimental reporter plasmid was normalized against luciferase activity from the control pRL-CMV plasmid in the same well. Three independent cultures were used for each luciferase assay, and the calculated standard deviations are indicated in the figures.

Statistical analysis. Statistical analyses were performed using the Hulinks KaleidaGraph software. Data are expressed as
means ± standard deviation (SD). The statistical significance of the differences was evaluated by the unpaired Student's t-test for data in Figs. 2 and 5 and by one-way analysis of variance (ANOVA) combined with the Tukey test for data in Fig. 4. P-values <0.05 were considered to be statistically significant.

Results

CCl₄-induced liver fibrosis. Liver fibrogenesis induced by chronic administration of CCl₄ was confirmed by Azan staining (Fig. 1). As was previously demonstrated, fibrotic areas were mainly distributed around central veins (Fig. 1C and D) and steatosis of parenchymal cells was also observed.

Expression of various genes involved in fibrogenesis as well as retinoid metabolism in CCl₄-treated rat liver. We quantified mRNA levels in the livers of normal and CCl₄-injected rats. We focused on genes associated with fibrogenesis, as well as those associated with retinoid metabolism, because it is known that retinoid loss is a frequently observed symptom in liver cirrhosis (8,9). As shown in Fig. 2, types I (Col1a1 and Col1a2) and type III (Col3a1) collagen, which constitute a major portion of liver fibrosis, were up-regulated in CCl₄-treated rat livers. Type IV collagen (Col4a1), which is a main component of basal lamina, was also up-regulated. Among the TGF-βs, the most important cytokines for hepatic fibrogenesis, Tgfb1 and Tgfb3 were up-regulated 2.4- and 7.1-fold, respectively, while Tgfb2 was not significantly altered. Rxra, whose ligand is 9-cis retinoic acid, and Rara, Rarb and Rarg, whose ligand is all-trans retinoic acid, were all up-regulated in CCl₄-treated rat livers, though the increase in Rara was not significant. Peroxisome proliferator-activated receptors (Ppara, Pparb/d, Pparγ), which are transcription factors responsible for lipid metabolism and energy consumption, were also up-regulated. Cyp26a1, the gene for a P450 enzyme, CYP26A1, which catalyzes hydroxylation of retinoic acid for its elimination, was greatly up-regulated. The gene for lecithin-retinol acyltransferase (Lrat), which esterifies retinol to make retinylesters, was also up-regulated. The genes encoding RBP (Rbp4), CRBP-I (Rbp1), and CRABP-II (Crabp2), which are serum retinol, cellular retinol, and cellular retinoic acid binding proteins, respectively, were slightly up-regulated in CCl₄-treated rat livers.

Immunohistochemical localization of TGF-βs in CCl₄-treated rat liver. Quantitative RT-PCR analyses revealed that TGF-β isomers were differentially regulated in CCl₄-treated rat livers (Fig. 2). We thus used immunohistochemical methods to assess the localization of the TGF-β isomers. Mature TGF-β isomers are very similar to one another; TGF-β3 is ~80% similar to TGF-β1 and TGF-β2, while TGF-β2 is 72% similar to TGF-β1 (16). Therefore, cross-reactivity of antibodies against TGF-β

Table I. Primers for RT-PCR.

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*Species Gene Forward Reverse Length (bp)*

Figure 1. Carbon tetrachloride (CCl₄)-induced liver fibrosis. Male Wistar rats were administered CCl₄ in liquid paraffin (C and D) or liquid paraffin alone (A and B) subcutaneously twice a week for two months. Livers were excised and sectioned for Azan staining. (B and D) Magnifications of central vein regions of panels (A) and (C), respectively. PV, portal vein; CV, central vein. Scale bars, 100 µm.

Figure 2. Quantification of mRNAs for genes associated with fibrogenesis and retinoid metabolism. Livers from normal and CCl₄-injected rats were analyzed for mRNA expression by quantitative RT-PCR using the primer sequences listed in Table I. Transcript levels were normalized against the Gapdh transcript, and the transcript levels of normal liver were set to 1.0 for each gene. *p<0.05 vs. control.
isoforms is very common. The commercially available anti-TGF-β1 antibody sc-146 (Fig. 3C and D) actually cross-reacts with TGF-β2, and antibody sc-82 (Fig. 3A and B), nominally an anti-TGF-β3 antibody, cross-reacts with both TGF-β1 and -β2, per the manufacturer. In spite of the cross-reactivity of the sc-146 antibody (which detects TGF-β1 and -β2), it stained very few cell types in CCl₄-treated rat liver sections (Fig. 3D) and almost none in normal rat liver sections (Fig. 3C). In CCl₄-treated rat livers, some non-parenchymal mesenchymal cells within pseudolobes or septa were positive for sc-146 staining (red arrows in Fig. 3D). Positive cells were more abundant in the central region than in the portal region, which is consistent with the fibrogenic tendency induced by CCl₄ (Fig. 1). Parenchymal cells (Fig. 3, open arrows), hepatic stellate cells (Fig. 3, solid arrows), Kupffer cells (Fig. 3, open arrowheads), and endothelial cells (Fig. 3, solid arrowheads) were generally negative for sc-146 staining both in normal and degenerated rat livers.

On the other hand, immunohistochemical staining by sc-82 antibody (which detects all three isoforms of TGF-β) showed strong staining of hepatocytes both in normal (Fig. 3A) and degenerated livers (Fig. 3B). Staining of non-parenchymal mesenchymal cells within the septa (Fig. 3B) was generally weak compared to that of hepatocytes. Taking into account the antibody specificity described above, strong staining of parenchymal cells in normal and CCl₄-treated rat livers is due to the presence of TGF-β3 in these cells. Parenchymal staining in CCl₄-injected livers was more intense than that in normal livers, which may reflect the induction of Tgfβ3 mRNA in CCl₄-treated rat livers (Fig. 2). Hepatic stellate cells (Fig. 3, solid arrows), Kupffer cells (Fig. 3, open arrowheads), endothelial cells (Fig. 3, solid arrowheads) seemed to be negative for sc-82 staining, though it was difficult to discriminate the cytoplasm of these cells from that of the parenchymal cells adjacent to these cells.

Retinoic acid-responsive elements were defined by either DR5 (direct repeat of (A/G)(G/T)TCA intervened by any five nucleotides) or DR2 (direct repeat of (A/G)G(T/T)TCA with any two intervening nucleotides) elements. Therefore, we searched for such elements in the Tgfb3 gene. Within an intron, which intercalates between exons 3 and 4 of Tgfβ3, a DR2 type of RARE 4322-GGGTCAatAGTTCA-4309 (numbers represent nucleotide positions within the intron and lower case letters indicate intervening nucleotides between two direct repeat sequences) was discovered (Fig. 4). A fragment containing this putative RARE was cloned and incorporated in a pGL-4.26 vector (Fig. 4), which contained a minimal promoter between multiple cloning site and the luciferase gene. A defined sequence of the DR5 type of RARE was used as a positive control. As shown in Fig. 4, the region within intron 3-4 of the Tgfb3 gene, which contains a putative DR2 type of RARE, was positively regulated by RA. This sequence was then mutated to GGGTCAatAGTGGC (putativeRAREm1), or GGGAGCattAGTCCA (putativeRAREm2), or GGGAGCattAGTCCG (putativeRAREm12), and these constructs were analyzed by the luciferase assay (Fig. 4). None of these mutants responded to RA, confirming that this specific element is responsible for the retinoid responsiveness within this DNA fragment.

Retinoic acid-mediated up-regulation of mRNAs for TGF-β isoforms in cultured cells. We also asked whether the DR2 element in the intronic region was present in the mouse Tgfβ3 gene and found that this element was conserved in rat and mouse (4392-GGGTCAggAGTTCA-4379), though this element was
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not conserved in the human Tgfβ3 gene (data not shown). We then examined a mouse teratocarcinoma cell line F9 (positive for RARα, β and γ), to determine whether the expression of TGF-β isoforms was altered in response to RA (Fig. 5). The mRNA levels for the Rarb gene, which has a definitive RARE in its promoter region, increased in RA-treated F9 cells, confirming the presence of retinoid-responsiveness in F9 cells used in this experiment. As shown in Fig. 5, minor increases in Tgfb1 (1.2-fold) and Tgfb3 isoforms (1.3-fold) were observed in RA-treated F9 cells, indicating that the expression of TGF-β isoforms was up-regulated to some extent in response to RA.

Figure 5. The effects of retinoid on gene expression of TGF-β isoforms in cultured cells. A mouse embryonal teratocarcinoma cell line F9, was treated with 0.1 µM all-trans retinoic acid (RA). After 24 h cultivation, cells were collected and assessed for mRNA expression by quantitative RT-PCR. Transcript levels were normalized against Gapdh transcript, and the transcript levels of control cells were set to 1.0 for each gene. As a representative RA-responsive gene, Rarb mRNA was quantified. *p<0.05 vs. control.

Discussion

Among TGF-β isoforms, TGF-β1 protein was the first to be purified from human platelets (7), and the first to be cloned from human placenta (17). In the initial report describing Tgfb1 cDNA cloning, several tissues and cell lines were assessed by Northern blotting. The liver was reported to be negative for Tgfb1 mRNA (17). However, the same group later reported moderate expression of Tgfb1 in rat liver (16). Another report using Northern blotting techniques stated that TGF-β1 was the most abundant isoform in both normal and CCl4-treated rat livers (18). The lowest level of Tgfb1 mRNA was found in normal adult mouse liver, while neonatal mouse liver Tgfb1 mRNA expression was 2-fold higher than in adult liver (19). By protein detection techniques such as immunohistochemistry, normal human livers were reported to be negative for TGF-β1 staining, though fibrotic areas with chronic active hepatitis were positive for TGF-β1 (20). Biliary ductular cells and hepatocytes of mouse liver were reported to be weakly positive for TGF-β1 staining (19). From these earlier reports and our present data (Figs. 2 and 3), we speculate that in normal liver, very limited numbers of cells are positive for TGF-β1, and in cirrhotic conditions, more cells become TGF-β1-positive, though it is not certain if these TGF-β1-positive cells develop within the liver or are derived from the circulation.

Using immunohistochemistry in this report, we showed that TGF-β3 protein is widely distributed in hepatocytes (Fig. 3). We also showed increases in Tgfb3 mRNA upon CCl4 treatment, which was more prominent than the increase in Tgfb1 mRNA (Fig. 2). The up-regulation of Tgfb1 and Tgfb3 mRNAs was also observed (though less prominently) following administration of RA in cultured cells (Fig. 5). By reporter analyses of an intronic region of Tgfb3 gene, we found that the DNA fragment containing RARE responded positively to RA, suggesting that the up-regulation of Tgfb3 mRNA was
partly mediated by retinoid signaling. The mechanism of up-regulation of Tgf/b1 mRNA upon CC1 treatment was not revealed in this report. It was reported that RARα, RARβ, and RXRα down-regulate the Tgf/b1 promoter by binding to AP-1 transcription factors, thus inhibiting their activities (21). It should also be noted that retinoid signaling can modify TGF-β activity indirectly. For example, retinoid signaling up-regulates TGF-β signaling by inducing the plasminogen activator, which proteolytically converts the latent form of TGF-β to an active form (22).

Several former reports stated the importance of TGF-β3 in the pathogenesis of liver cirrhosis (23,24). Cirrhotic liver tissue samples from human patients showed increased expressions of Tgf/b1, Tgf/b2 and Tgf/b3 mRNAs, of which Tgf/b3 showed the highest level of expression in human liver cirrhosis (23). The up-regulation of Tgf/b3 mRNA was also observed in livers of patients with primary biliary cirrhosis but not in livers of patients with viral cirrhosis (24). These earlier reports as well as our recent report (25) pointed out the possibility of the involvement of biliary epithelial cells in liver cirrhosis. Our present study showed the possibility that the hepatocytes are also involved in the pathogenesis of liver cirrhosis.

In summary, we show that TGF-β3 is distributed in parenchymal cells in the liver and up-regulated in CCl4-treated rat livers. We propose that the increase of Tgf/b3 mRNA is partly mediated by retinoid signaling.

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References