Effects of retinoic acid on the development of liver fibrosis produced by carbon tetrachloride in mice

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Abstract

The role of retinoic acid (RA) in liver fibrogenesis was previously studied in cultured hepatic stellate cells (HSCs). RA suppresses the expression of α2(I) collagen by means of the activities of specific nuclear receptors RARα, RXRβ and their coregulators. In this study, the effects of RA in fibrogenesis were examined in carbon tetrachloride (CCl4) induced liver fibrosis in mice. Mice were treated with CCl4 or RA and CCl4, along side control groups, for 12 weeks. RA reduced the amount of histologically detectable fibrosis produced by CCl4. This was accompanied by an attenuation of the CCl4 induced increase in α2(I) collagen mRNA and a lower (2-fold versus 3-fold) increase in liver hydroxyproline. Furthermore, RA reduced the levels of 3-nitrotyrosine (3-NT) protein adducts and thiobarbituric acid (TBA) reactive substance (TBARS) in the liver, which are formed as results of oxidative stress induced by CCl4 treatment. These in vivo findings support our previous in vitro studies in cultured HSC of the inhibitory effect of RA on type I collagen expression. The data also provide evidence that RA reduces CCl4 induced oxidative stress in liver, suggesting that the anti-fibrotic role of RA is not limited to the inhibition of type I collagen expression.

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1. Introduction

Many factors cause liver injury that often progresses to fibrosis. Type I collagen overexpression and deposition feature the primary pathology of liver fibrosis leading to cirrhosis [1,2]. Type I collagen is composed of two α1 I and one α2 I collagen polypeptide chains [3]. Hepatic stellate cells are the principal sources of type I collagen during liver fibrogenesis [4,5]. In the quiescent state, stellate cells store vitamin A [6,7]. Transformation of quiescent HSC to activated HSC occurs upon liver injury of different causes. Concomitantly vitamin A storage is diminished in activated hepatic stellate cells, as well as the signal transduction induced by retinoic acid (RA), a potent metabolite of vitamin A. These changes are the foundation for investigation of the role of RA in liver fibrosis.

Retinoic acid reduces human liver stellate cell proliferation and collagen production in culture [8,9]. Retinoic acid was shown to down regulate type I collagen production in activated HSCs by suppressing the expression of α2(I) collagen [10,11]. This downregulatory effect of RA is mediated by specific nuclear receptors RARβ and RXRα, and through their interactions with their coregulators [11,12]. In addition, RA was shown to be protective to oxidative stress mediated tissue injury [13,14].

Carbon tetrachloride (CCl4) induced liver injury and fibrosis [15–18] has been widely used as a rodent model of fibrosis. The mechanism underlying the hepatotoxicity of CCl4 involves oxidative stress initiated by CCl4-derived reactive free radical metabolite trichloromethyl (CCl3) radicals (CCl3·) [19]. CCl3· is biotransformed into peroxynitrite, a strong oxidant able of disrupting physiological cellular structure and function through peroxidation of cellular lipids, protein and DNA [20–23]. Peroxynitrite interacts with tyrosine residues to form 3-nitrotyrosine (3-NT) protein adducts [24,25], which reflects the peroxynitrite-mediated damage and is used as a biomarker of peroxynitrite formation [24]. Elevation of 3-NT occurs in chronic hepatitis and cirrhosis [26–28]. Lipid peroxidation by peroxynitrite, on the other hand, generates thiobarbituric acid.
(TBA) reactive substance (TBARS) in tissue [29–32], which is also an indicator of oxidative stress induced tissue injury.

In this study, we examined the effect of RA on CCl₄ induced liver fibrosis in mice, to further determine the role of RA in suppressing liver fibrosis in vivo.

2. Materials and methods

2.1. Animals and materials

Male wild type C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, ME. Sirius Red was obtained from Polysciences, Inc, Warrington, PA. Carbon tetrachloride, All-trans retinoic acid and thiobarbituric acid were from Sigma Chemical Co., St. Louis, MO. Polyclonal rabbit-anti-3-NT antibody was obtained from Upstate USA Inc., Lake Placid, NY.

2.2. Carbon tetrachloride and retinoic acid administrations in mice and liver harvesting

The animal experiments were approved and conducted in accordance with the institutional guidelines of the Animal Care and Use Committee of the Johns Hopkins University. Male WT C57BL/6 mice 4–6 weeks of age weighing 20–25 g were kept in a temperature-controlled room with an alternating 12-h dark and light cycle. Four groups of mice were studied. One group of 8 mice was treated with CCl₄ alone, while another group of 8 mice was treated with CCl₄ and RA. The other two groups of 4 mice each were treated with olive oil (control) or RA alone. CCl₄ was given biweekly as intraperitoneal injections in a dose of 0.5 μl/gram body weight (BW) as a 20% solution. RA was also injected intraperitoneally in a dose of 1 μg/gram BW as a 0.5 μg/μl solution, three times per week. CCl₄ was dissolved in olive oil and the retinoic acid was sonicated into olive oil. The mice were sacrificed 12 weeks after the start of the injections.

The livers were removed, rinsed with PBS, and divided into portions: (a) fixed in 10% buffered formaldehyde formalin and embedded in paraffin; (b) snap frozen at −70 °C for hydroxyproline assay; (c) homogenized in appropriate buffer and aliquots frozen at −70 °C for biochemical assays; and (d) used immediately for total RNA isolation.

2.3. Morphometric collagen determination

The liver sections imbedded in paraffin were cut (5 μ) and stained with hematoxylin–eosin (H&E), Masson’s trichrome, or Sirius red [33]. Fibrosis was determined histologically by measuring the intensity of fibrosis in 4–6 (×100) digital images captured from Sirius red stained slides of each mouse liver using MetaMorph 6.0 imaging software (Molecular Devices Co., Sunnyvale, CA). A total fibrosis density score was determined by multiplying the integrated image intensity with the area from which the integrated intensity was calculated. Intensity exclusion parameters were identical for each of the images captured.

2.4. Liver hydroxyproline

Liver slices were homogenized in buffer and hydrolyzed in 12M HCl at 100 °C for 12 h. After hydrolysis the pH was adjusted to pH 7.0 with 10N NaOH and the samples were centrifuged at 750×g for 15 min. The hydroxyproline content was measured by the spectrophotometric method of Bergman and Loxley [34].

2.5. Determination of messenger RNA by real time quantitative polymerase chain reaction (RT-qPCR)

Total cellular RNA was isolated from a portion of liver using the Stat-60 reagent (AMS Biotechnology Ltd., Milton Abingdon, Oxon, UK), followed by further cleaning with the use of an RNeasy kit (Qiagen, Valencia, CA). The concentration of the isolated RNA was determined from the optical density at 260 nm and its purity from the 260 nm/280 nm OD ratio. The isolated RNA is stored at −80 °C.

To determine the quantity of mRNAs, the reverse transcription was performed with the use of Superscript First strand synthesis kit (Invitrogen, Carlsbad, CA) to synthesize cDNA. Gene-transcript levels of α₂ (I) collagen

![Fig. 1. Histology with sirius red staining of collagen of the livers of mice treated with (A) olive oil (control); (B) retinoic acid (RA); (C) CCl₄ and (D) CCl₄ and RA both. (original magnification ×100).](image-url)
and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by RT-qPCR with the use of respective Taqman gene expression assay kit (Applied Biosystems, Foster City, CA), on an ABI 7900 (Applied Biosystems, Foster City, CA). Variation in the amount of transcript in different samples was corrected for by GAPDH expression. Sequences of the primer pairs used for the PCR to identify the transcripts were proprietary.

2.6. Immunohistochemical detection of 3-nitrotyrosine

Liver sections (5 μ) were immunostained for 3-nitrotyrosine protein adducts with rabbit polyclonal anti-nitrotyrosine antibody (1:1000 dilution, Chemicon-Upstate, Temecula, CA) followed by anti-rabbit secondary antibody (Rabbit ImmunoCruz Staining system kit, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The sections were visualized by light microscopy.

2.7. Lipid peroxidation assay

Hepatic lipid peroxidation in the mouse liver of various treatment was determined by measuring colorimetric changes resulted from the reaction between thiobarbituric acid (TBA)-reactive substance (TBARS), the products of lipid peroxidation in animal tissue, with TBA [32]. Liver tissue was homogenized in 9 vol of 50 mmol/L Tris–HCl buffer (pH 7.4) containing 180 mmol/L KCl, 10 mmol/L EDTA, and 0.02% butylated hydroxytoluene. To the tissue homogenate, equal volume of 8.1% sodium dodecyl sulfate, 7.5 vol of distilled water and 25 vol of butanol/pyridine mixture (15:1, v/v) were added and mixed by vortexing. The samples were then incubated in a water bath at 95 °C for 1h. After cooling on ice, 5 vol of distilled water and 5 vol of 0.9% TBA were added and vortexed. After centrifugation at 10,000 × g for 10 min, absorbance of the resulting lower phase was determined at 532 nm. The TBARS concentration was calculated using 1,1,5,5-tetraethoxypropane as standard.

2.8. Statistical analysis

Data were analyzed with the Student’s t test when appropriate or by two way analysis of variance (ANOVA) when comparing means of more than two groups.

3. Results

3.1. Histomorphological characterization of effects of CCl4 and RA on liver fibrosis

The morphological changes of liver injury and fibrosis caused by CCl4 were visualized in liver sections stained by H&E (not shown) and Sirius red. The changes include necrosis, inflammation with macrophages and lymphocytes, balloon cell change and bridging fibrosis (Fig. 1C). The degrees of necrosis, inflammation and balloon cell change were not significantly different in animals treated with both CCL4 and RA as compared with CCl4 alone (Table 1). Fatty infiltration was minimal in both groups. Liver fibrosis was less evident in mouse liver treated with both RA and CCl4 (Fig. 1D). RA treatment alone did not cause evident changes (Fig. 1B).

3.2. Effects of RA on increase of collagen production in liver due to CCl4 treatment

The amount of fibrosis (collagen deposition) was quantified in the liver slices stained with Sirius Red (Fig. 2). The increase of the collagen deposition caused by CCl4 (p < 0.01) was repressed by RA treatment (p < 0.01). RA alone did not cause an evident change in liver fibrosis. Collagen content was also determined by measurement of hydroxyproline content in the livers (Fig. 3). The mean hydroxyproline level was significantly higher in the CCl4 treated groups than in the RA treated groups.

### Table 1

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<tr>
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<th>CCl4</th>
<th>CCl4 + RA</th>
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<tbody>
<tr>
<td>Necrosis</td>
<td>2.2 ± 0.21</td>
<td>1.9 ± 0.26</td>
</tr>
<tr>
<td>Inflammation (zone 3)</td>
<td>1.8 ± 0.18</td>
<td>1.9 ± 0.25</td>
</tr>
<tr>
<td>Balloon cell change</td>
<td>3.6 ± 0.19</td>
<td>3.0 ± 0.26</td>
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Liver cell necrosis was evaluated on a scale of 1–4 as follows: 1, occasional; 2, mild scattered; 3, confluent zonal and 4, extensive. Scores for hepatic inflammation were: 1, scattered; 2, mild; 3, moderate and 4, marked. Balloon cell change was assessed as average number per 20X fields as follows: 1, 1; 2, 2–4; 3, 5–10 and 4, >10. The values are expressed as means±SE from H&E stained slides of 8 mice per group.
treated group ($p<0.01$). The mice treated with both RA and CCl4 had a lower 2-fold increase from the control value of liver hydroxyproline compared to the 3-fold increase in mice treated with CCl4 alone. RA alone had no significant effect on liver hydroxyproline.

CCl4 caused a marked increase in $\alpha_2$(I) collagen gene mRNA ($p<0.01$) (Fig. 4). Treatment with RA significantly attenuated the increased in the $\alpha_2$(I) collagen gene mRNA caused by CCl4 ($p<0.05$). RA alone had no effect.

3.3. Effect of RA in reducing CCl4 induced oxidative stress in liver

RA (Fig. 5D) reduced the 3-nitrotyrosine (3-NT) protein adduct immunohistochemical staining intensity in the liver sections obtained from CCl4 treated mouse (Fig. 5C). RA also prevented the increase of thiobarbituric acid (TBA) reactive substance (TBARS) in the liver homogenates of the mice treated with CCl4. The level of TBARS was $3.64\pm0.08$ for CCl4 treatment, which is significantly higher ($p<0.05$) than $3.03\pm0.26$ and $3.07\pm0.22$ for no treatment control and RA treatment alone, respectively. The RA treatment alongside CCl4 reduced the hepatic TBARS to $3.17\pm0.14$ ($p<0.05$).

4. Discussion

This study shows that RA has an inhibitory effect on the development of liver fibrosis in mice treated with CCl4. The inhibition is featured in the reduced amount of type I collagen mRNA and reduced level of type I collagen deposition in liver. The results corroborate with and reconfirm our findings from in vitro studies showing that RA suppresses the expression of type I collagen in cultured HSCs [10–12]. The correlation of the in vitro inhibitory effects of RA with the findings in vivo validates the future use of cultured HSCs in studying the role of RA in liver fibrosis.

The inhibition by RA of liver fibrosis was not accompanied by significant decrease in liver injury caused by CCl4. Prior

![Fig. 4](image1.png)

![Fig. 5](image2.png)
studies had shown that administration of retinol suppressed hepatic fibrosis produced by CCl4 and pig serum in rats [35]. In other studies also in rats, administration of an RA analog enhanced liver fibrosis produced by porcine serum [36], but suppressed liver fibrosis produced by CCl4 [37], suggesting that the suppressive effects of RA may involve inhibition of the strong necroinflammatory component that accompanies the fibrosis in the CCl4 model [38]. Liver injury produced by CCl4 treatment is mediated by reactive oxygen species (ROS) [39,40]. Through the CCl3· led cascade of peroxynitrite such as superoxide, in primary cultures of neonatal rat RA is an antioxidant and was shown to reduce enhanced ROS, adduct formation and the level of TBARS in liver homogenates.

In this study, RA treatment reduced both the 3-NT protein adduct formation and the level of TBARS in liver homogenates. RA is an antioxidant and was shown to reduce enhanced ROS, such as superoxide, in primary cultures of neonatal rat liver hepatocytes treated with staurosporin [43]. RA also decreased ROS in neutrophils of patients with promyelocytic leukemia during treatment with N-formyl-methionyl-leucyl-phenylalanine [44]. RA attenuated TNF-α induced increase of iNOS in cultured 3T3-L1 adipocytes and the accumulation of nitrite in the culture medium [45]. Our findings and those of others suggest that the anti-fibrogenic effects of RA are not limited to down regulation of the type I collagen expression during repair. Rather, the antifibrotic effects of RA begin during liver injury and inflammation, the very initial stage of fibrogenesis. Considering the vast spectrum of biological functions of RA, one should not be surprised that RA is involved in the many stages of the development of fibrosis. This finding indicates another potential target in studying the molecular mechanism of RA in suppressing fibrogenesis, namely the generation of free radical and oxidative stress in liver by hepatoxic factors.

There are similarities in the mechanisms of fibrogenesis induced by CCl4 and ethanol. Type I collagen expression in liver is upregulated by both agents [1,11,16]. ROS formation also mediates the liver injury caused by alcohol. Chronic alcohol consumption is associated with sustained oxidative stress [46,47]. However, chronic ethanol administration causes liver injury and fibrosis only in combination with a high fat diet [48]. Hence, knowledge of pathways of CCl4 induced liver injury will provide information on mechanism that may also apply to liver fibrosis in alcoholism. Indeed, oxidative stress appears to play a important role in other etiology of liver fibrosis, such as chronic viral hepatitis [26,46], indicating that it is a merging pathway for various etiological forms of liver injury and fibrogenesis.

In this study, the effects of RA in liver fibrosis were examined in chronic liver injury caused by 12-week CCl4 administration. In studies of acute liver injury, where CCl4 was administrated for 48 h or for 2 weeks [49,50], greater changes were detected in the level of indicators of oxidative stress immediately after the cessation of the treatment. This may help to explain the lesser changes in 3-NT protein adducts and the TBARS content in our findings, compared to the 150% or more increase of those observed at earlier onset of liver fibrogenesis. Nonetheless, the reduction of TBARS content caused by RA treatment in our study was statistically significant.

In conclusion, this study shows that RA suppresses liver fibrogenesis through inhibitory effect on type I collagen production in liver and through reducing liver injury caused by oxidative stress.

Acknowledgements

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