Induced hypothyroidism accelerates the regression of liver fibrosis in rats

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Abstract

Background and Aim: It has been shown in previous studies that hypothyroidism prevents the development of liver fibrosis in bile duct ligated rats and in rats chronically treated with thioacetamide (TAA). In recent years, regression of liver fibrosis (occurring spontaneously or during treatment) has been demonstrated in rodent models such as bile duct ligation and CCl4 administration. Therefore, in the present study, the potential therapeutic effect of hypothyroidism on liver fibrosis was investigated.

Methods: Liver fibrosis was induced in rats by administration of TAA (200 mg/kg, i.p., twice weekly) for 12 weeks. Hypothyroidism was then induced by either methimazole (0.04%) or propylthiouracil (0.05%) administered in drinking water for 8 weeks. Control euthyroid rats received normal drinking water. Hypothyroidism was confirmed by a significant elevation of serum thyroid-stimulating hormone levels.

Results: Eight weeks after the cessation of TAA administration, spleen weight, histological score of liver fibrosis, and hepatic hydroxyproline content were significantly lower in both groups of hypothyroid rats as compared to euthyroid controls (P < 0.001).

In vitro studies using the rat hepatic stellate cell line HSC-T6 using northern blot analysis and zymography, respectively, showed that high concentrations of triiodothyronine (T3) enhanced transforming growth factor (TGF)-β-induced collagen I gene expression, and reduced metalloproteinase (MMP)-2 secretion, implying that reducing the levels of T3 may contribute to resolution of fibrosis. Additionally, low T3 concentration inhibited HSC-T6 proliferation.

Conclusion: Pharmacologically induced hypothyroidism accelerates the resolution of liver fibrosis in rats. This beneficial effect may in part be due to prevention of T3-induced stimulation of collagen synthesis and reduction of MMP-2 secretion.

Key words

collagen, fibrosis, hypothyroidism.

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Introduction

Liver cirrhosis is the net result of increased formation and reduced degradation of extracellular matrix in the liver. Hepatic cirrhosis represents a dynamic biphasic process determined by progressive fibrosis and severe distortion of normal lobular architecture due to regenerative nodules or pseudolobules.1 To date, there is no satisfactory medical treatment for preventing or reversing the fibrotic process.3

Examples have been described in both acute and chronic liver injury in which spontaneous resolution occurs after successful treatment of the underlying disease. These include hemochromatosis responding to phlebotomy,4 successful treatment of viral hepatitis with interferon and other antiviral agents,5,7 treatment of autoimmune hepatitis with steroids and immunosuppression,8 and biliary decompression for secondary biliary cirrhosis.9 Resolution, which in some cases may take many years, is associated with reduction of the extracellular matrix and a restoration of normal or near-normal liver architecture. In human studies, it is impossible to clearly define the fate of activated stellate cells during resolution of fibrosis. In contrast, rodent models such as bile duct ligation and CCl4 administration provide an excellent means to determine the mechanisms underlying the recovery from fibrosis and cirrhosis.10,11 The proposed mechanism that enables fibrosis regression is hepatic stellate cell (HSC) apoptosis associated with decreased hepatic expression of tissue inhibitors of metalloproteinase (TIMP)-1 and collagen I, associated with persistent degradation of collagen by matrix metalloproteinase (MMP) activity.10,11

We have shown in several experimental studies that induced hypothyroidism proved beneficial for various liver insults such as portal vein ligation,12 thioacetamide (TAA)-induced fulminant liver failure13 and acetaminophen intoxication.14 In accordance
with these animal studies, we have shown in a human study that the clinical course of cirrhotic patients with hypothyroidism was more favorable than those with euthyroidism.15

Although these studies concentrated on the prevention of liver damage by hypothyroidism, we conducted the present study in order to find out whether hypothyroidism could contribute to the regression of established hepatic fibrosis in rats. Herein, we showed that pharmacologically induced hypothyroidism accelerates the regression of liver fibrosis in rats, and that this effect of hypothyroidism may in part be due to prevention of the T3-induced stimulation of collagen I synthesis and reduction of MMP-2 secretion.

**Methods**

**Animals**

Male Wistar rats (200–250 g), obtained from Tel-Aviv University Animal Breeding Center, received humane care in compliance with institution’s guidelines. They were kept in the animal breeding house of the Wolfson Medical Center and fed a Purina rodent chow ad libitum.

**Induction of liver cirrhosis and hypothyroidism**

Liver cirrhosis was induced in rats by administration of TAA (200 mg/kg, i.p., twice weekly; Sigma, St Louis, MO, USA) for 12 weeks, as previously described.16 Such long-term administration of TAA resulted in characteristic lesions of mixed macro and micronodular cirrhosis with regenerative nodules formation in the animals’ livers. These lesions were shown to persist for at least 2 months after withdrawal of TAA.

Hypothyroidism was induced by the administration of either 0.04% methimazole (MMI; Taro, Israel) or 0.05% propylthiouracil (PTU; Teva, Israel) in drinking water for 8 weeks following TAA administration.

**Experimental design**

Four groups of 8 rats each were studied, of which two groups were euthyroid controls and two were hypothyroid. All rats received TAA for 12 weeks then as follows: group I, nothing else; group II, a further 8 weeks of normal drinking water; group III, a further 8 weeks of PTU in the drinking water; group IV, a further 8 weeks of MMI in the drinking water.

The animals in group I were killed after 12 weeks to confirm the induction of liver cirrhosis, and those in groups II–IV after 20 weeks. Their livers were removed immediately and the spleen weights were measured.

**Analysis of liver histopathology**

For semiquantitative analysis, the midsections of the left lobes of the excised livers were processed for light microscopy. This processing consisted of fixing the specimens in a 5% neutral formal solution, embedding the specimens in paraffin, slicing sections 5 μm in thickness, and staining the sections with hematoxylin and eosin (HE). The tissue slices were scanned and scored semiquantitatively by an expert pathologist who was not aware of sample assignment to experimental groups. The degrees of inflammation and fibrosis were expressed as the mean of 10 different fields within each slide that had been classified on a scale of 0–3 according to Muller et al.17

**Cell culture conditions**

We used the immortalized rat hepatic stellate cell line HSC-T6, kindly provided by Dr S.L. Friedman (Mount Sinai Medical Center, NY, USA). Cells were grown in Dulbecco’s modified Eagle medium (DMEM; Biological Industries, Bet-Haemek, Israel) supplemented with 10% fetal calf serum (FCS) (v/v), 2 mmol/L L-glutamine, and 100 mg/mL of penicillin-streptomycin.

For experimental studies, the medium was changed 24 h after plating the cells to hormonally defined medium (HDM). HDM consisted of DMEM supplemented with 100 mg/mL penicillin-streptomycin, 2 mg/mL bovine serum albumin, 610 mg/mL nico- tinamide, 740 mg/mL ZnSO4 × H2O, 20 mg/mL CuSO4 × 5H2O, 5 mmol/L glutamine, 5 mg/mL insulin, 5 mg/mL iron-saturated transferrin, 5 ng/mL sodium selenite and 10−7 mol/L dexamethasone (all materials from Sigma). The media was supplemented with or without 10 ng/mL PDGF BB (PeproTech Asia/CytoLab, Rehovot, Israel) and/or different concentrations of T3.

**Northern blot analysis**

Cultured HSC-T6 cells were washed with cold phosphate buffered saline (PBS) and total RNA isolated using an EZ RNA kit (Biological Industries). RNA samples were resolved by electrophoresis through 1% agarose, denaturing formaldehyde gels in MOPS (3-N-morpholinopropanesulfonic acid) buffer and transferred to Hybond N paper (Amersham). The blots were hybridized with cDNA probes labeled with 32PdCTP by primer extension. The cDNA probe used was α1(I) Pro-collagen, whose expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Gelatin zymography assay**

The rat hepatic stellate cell line HSC-T6 was plated in 24 wells (80 000 cells/well). After 24 h in DMEM supplemented with 10% FCS, the media was replaced with HDM supplemented with or without 10 ng/mL transforming growth factor (TGF)-β and 10−7 mol/L T3. After 24 h, the supernatant was collected and cell number was evaluated using a hemacytometer. The supernatant was mixed with non-reducing sample buffer (0.4 mol/L Tris, pH 6.8, 5% sodium dodecyl sulfate (SDS), 20% glycerol, 0.03% bromophenol blue). Proteins were separated by electrophoresis through an 8% polyacrylamide gel containing gelatin (25 mg/mL). Following electrophoresis, gels were washed in 2.5% Triton X-100 with gentle shaking for 30 min, and then incubated for 30 min in developing buffer (40 mmol/L Tris-HCl, 0.2 mmol/L NaCl, 0.67 mmol/L CaCl2, 0.1% Triton X-100, pH 7.8). The developing buffer was replaced with fresh buffer and the gel incubated overnight at 37°C. The gels were stained with 0.5% Coomassie Blue G-250 in 10% acetic acid and 40% methanol, and de-stained in water. MMP activity was visualized as bands of lysis, which appear white on a dark background.
**Proliferation assay**

Rat hepatic stellate cells HSC-T6 were plated in 24-well plates (30,000 cells/well). The cells grew in DMEM supplemented with 10% FCS until the logarithmic growth phase. Cells were incubated in serum-free HDM with decreasing concentrations of T3 \((10^{-7} \text{ mol/L}, 10^{-9} \text{ mol/L} \text{ and } 10^{-11} \text{ mol/L})\). After 24 h, cells were trypsinized and counted using a hemocytometer.

**Statistical analysis**

Data are expressed as the means of three different experiments ± SD. Statistical analysis was performed with unpaired two-tailed Student’s \(t\)-test. \(P\)-values less than 0.05 were considered significant.

**Results**

**Induction of liver cirrhosis and the effect of hypothyroidism on regression**

Administration of TAA for 12 weeks resulted in a uniform fine granulation of the surface of the rat livers. Microscopic analysis revealed cirrhosis-like structural patterns characterized by mixed-sized fibrotic nodules in these TAA-treated rats (TAA for 12 weeks: pathological score of 2.7 ± 0.1, Table 1, Fig. 1). The administration of either PTU or MMI for 8 weeks after cirrhosis had been established was followed by significant elevation of thyroid-stimulating hormone levels (Table 1, \(P < 0.01\)) and a marked decrease of the pathological score, hepatic hydroxyproline levels and the spleen weights (\(P < 0.001\), Table 1, Fig. 1).

**Enhancement of basal and TGF-β-induced collagen α1(I) gene expression by T3**

Northern hybridization analysis was carried out to evaluate the effect of \(10^{-7} \text{ mol/L} \text{ T3}\) on collagen α1(I) (Col I) gene expression. As shown in Fig. 2, T3 increased the basal expression of Col I (\(P = 0.05\) compared to normal). The effect of T3 was even more significant on TGF-β-induced Col I expression (\(P < 0.01\) compared to TGF-β only).

**Reduction of TGF-β-induced MMP-2 activity in HSC by T3**

The effect of \(10^{-7} \text{ mol/L} \text{ T3}\) on MMP-2 activity was examined using gelatin zymography assay. Cultured hepatic stellate cells were exposed to T3 in the presence or absence of 10 ng/mL TGF-β for 24 h. Although T3 \(10^{-7} \text{ mol/L}\) had no effect on the basal activity of MMP-2, the TGF-β-induced MMP-2 activity was reduced by T3 (Fig. 3).

### Table 1 Effect of induced hypothyroidism on the regression of hepatic fibrosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TSH (μU/mL)</th>
<th>Fibrosis score (0–3)</th>
<th>Spleen weight (mg)</th>
<th>Hydroxyproline (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.35 ± 0.05</td>
<td>0</td>
<td>425 ± 61</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>TAA 12 weeks</td>
<td>0.31 ± 0.05</td>
<td>2.2 ± 0.4</td>
<td>975 ± 90</td>
<td>6.5 ± 2.1</td>
</tr>
<tr>
<td>TAA 12 weeks + 8 weeks no treatment</td>
<td>0.39 ± 0.04</td>
<td>1.8 ± 0.3</td>
<td>815 ± 96</td>
<td>4.6 ± 1.1</td>
</tr>
<tr>
<td>TAA 12 weeks + 8 weeks MMI</td>
<td>7.6 ± 0.9*</td>
<td>0.8 ± 0.2*</td>
<td>472 ± 73*</td>
<td>1.4 ± 0.4*</td>
</tr>
<tr>
<td>TAA 12 weeks + 8 weeks PTU</td>
<td>8.3 ± 1.1*</td>
<td>0.7 ± 0.2*</td>
<td>405 ± 78*</td>
<td>1.5 ± 0.6*</td>
</tr>
</tbody>
</table>

Values shown as mean ± SD, \(n = 8\) in each group. *\(P < 0.01\) compared to TAA 12 weeks + no treatment 8 weeks.

MMI, methimazole; PTU, propylthiouracil; TAA, thioacetamide; TSH, thyroid stimulating hormone.

**Figure 1** Effect of hypothyroidism on the regression of hepatic fibrosis induced by thioacetamide (TAA) administered for 12 weeks. (a) Control rats (TAA only): severe distortion of the liver architecture with nodules surrounded by fibrotic septa. (b) Euthyroid rats (TAA + 8 weeks no treatment): liver cirrhosis still present with regenerative nodules and fibrotic septa. (c) Hypothyroid rats (TAA + 8 weeks PTU): significant regression of liver fibrosis. (d) Hypothyroid rats (TAA + 8 weeks MMI): significant regression of liver fibrosis. (HE, original magnification x80.)
Regression of liver fibrosis by hypothyroidism

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while the low, hypothyroid T3 concentration 10⁻⁷ mol/L T3 in the presence or absence of 10 ng/mL transforming growth factor (TGF)-β for 24 h. Total RNA (10 µg) was separated on a 1% agarose gel. Hybridization was carried out with probes for pro-α(1II) collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the levels of pro-α(1II) collagen mRNA were normalized relative to GAPDH. T3 enhanced basal and TGF-β-induced Col I mRNA expression. The graph shows means ± SE of three independent experiments.

Figure 2  Col I mRNA expression in cultured hepatic stellate cells HSC-T6. Cultured stellate cells were exposed to 10⁻⁷ mol/L T3 in the presence or absence of 10 ng/mL transforming growth factor (TGF)-β for 24 h. Total RNA (10 µg) was separated on a 1% agarose gel. Hybridization was carried out with probes for pro-α(1II) collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the levels of pro-α(1II) collagen mRNA were normalized relative to GAPDH. T3 enhanced basal and TGF-β-induced Col I mRNA expression. The graph shows means ± SE of three independent experiments.

Effect of T3 on HSC proliferation

In chronic liver injury, HSC undergo a process of activation from a resting fat-storing phenotype towards a myofibroblast-like phenotype, characterized by increased cell proliferation. We examined the effect of various concentrations of T3 on the proliferation of HSC, by culturing the HSC-T6 hepatic stellate cell line in serum-free medium. The results presented are those of 24 h culture. We found that 10⁻⁷ mol/L T3 induced HSC-T6 proliferation compared to euthyroid, physiological dose of T3 (10⁻⁹ mol/L), while the low, hypothyroid T3 concentration 10⁻¹¹ mol/L, inhibited hepatic stellate cells proliferation (Fig. 4).

Discussion

Although for many years liver cirrhosis was considered an irreversible condition, there is now a substantial body of evidence in both human liver disease and animal models to indicate that liver fibrosis and cirrhosis are dynamic processes that can both progress and regress over time, depending in part on whether or not the underlying cause still persists. In humans, regression of liver cirrhosis has been associated with elimination of the cause of liver disease such as abstinence from alcohol in patients with advanced alcoholic cirrhosis or the implementation of an effective treatment, such as corticosteroid therapy for autoimmune hepatitis, phlebotomy for hemochromatosis, long-term treatment with lamivudine for chronic hepatitis B, weight loss in patients with non-alcoholic fatty liver disease, and successful treatment of hepatitis C with pegylated interferon and ribavirin.

We have shown in previous studies that hypothyroidism prevents the development of liver fibrosis in bile duct ligated rats and in rats chronically treated with TAA. The present study was conducted to examine whether in addition to prevention of liver cirrhosis, hypothyroidism would also show a therapeutic effect and reverse established liver damage in a rat model of fibrosis induced by chronic administration of TAA. Indeed, we observed a substantial beneficial therapeutic effect of hypothyroidism in TAA-induced liver characteristic with both antithyroid treatments. We used two different drugs, MMI and PTU, to exclude a direct interaction of TAA with a specific antithyroid drug, a very unlikely event in this experimental protocol, as TAA administration was stopped before treatment with antithyroid drugs was initiated.

During fibrosis resolution, increased collagen degradation by MMPs due to a rapid decrease in the expression of TIMP-1 is a major mechanism of fibrosis regression. Partial degradation of fibrillar collagen occurs, and the altered interaction between activated HSC and extracellular matrix favors apoptosis. Removal of activated HSC by apoptosis precedes fibrosis resolution. Stimulation of death receptors in activated HSC and a decrease in survival factors, including TIMP-1, can precipitate HSC apoptosis. In the present study, we examined the effect of thyroid hormone T3 on proliferation of the HSC-T6 cell line. We found that HSC proliferation was decreased by low concentrations of T3. Thus, in the hypothyroid state when T3 levels are very low, HSC proliferation may be suppressed.

We have shown previously that reduced circulating T3 levels were associated with amelioration of liver fibrosis in cirrhotic patients. In addition, in vivo experiments in rats have shown that induced hypothyroidism prevented the development of liver cirrhosis due to chronic TAA administration. Thyroid hormone belongs to the nuclear receptor super family of ligand-dependent
transcription factors. Its receptor (TR) is expressed in the liver and has a role in liver fibrogenesis. Thyroid hormone influences collagen biosynthesis and degradation, thus affecting various pathophysiologic phenomena, including alterations in urinary excretion of hydroxyproline and hydroxylysine, hyperthyroid acropachy, pretibial myxedema, impaired wound repair and other fibrosis-related processes.22 Liver fibrosis following HSC activation is characterized by increased production and deposition of extracellular matrix proteins, the most abundant one being type I collagen.

To further investigate the accelerated regression of liver fibrosis by hypothyroidism, we examined the effects of T3 on TGF-β-induced Col I expression and MMP-2 secretion in HSC. Our in vitro studies demonstrate that T3 enhanced basal and TGF-β-induced Col I expression, and reduced TGF-β-induced MMP-2 secretion by HSC. These results may explain to some extent the beneficial effects of hypothyroidism on the regression of hepatic fibrosis, as reduced levels of T3 (characteristic of hypothyroidism) may suppress collagen I expression and enhance MMP-2 secretion by HSC, causing a net effect of accelerated fibrosis regression. Our finding that T3 increases both basal and TGF-β-induced Col I expression in HSC is consistent with previous observation demonstrating that, during the climax period of spontaneous metamorphosis in tadpoles, both α1(I) and α2(I) collagen are dramatically up-regulated by T3.23 However, in cardiac fibroblasts, T3 decreases protein and mRNA levels of Col I and ameliorates heart fibrosis.24

The disparity in regulation of Col I by thyroid hormone in cardiac fibroblasts compared to HSC may be explained by differences in expression of coactivators and corepressors or by expression of different TRs in the two tissues. The two major thyroid hormone receptors are TRα1 and TRβ1. We have previously shown that the cell line HSC-T6 expresses TRα1, but not TRβ1, while hepatocytes express both receptors.25 Interestingly, while HSC express TRα1,25 the rat cardiac fibroblasts express only TRβ1.26 The pro-α1(I) collagen promoter contains multiple sites to which TR and thyroid hormone-induced transcription factors can bind. Sequence analysis of the rat pro-α1(I) collagen promoter revealed that the 3.6 kb upstream region includes three activator protein-1 (AP-1) binding sites and three positive T3-response elements (TRE), which may be potential binding sites for TRs. In rat cardiac fibroblasts, the proximal AP-1 response element, located in the −225/+115 region of the collagen promoter, is essential for T3 inhibition of Col I and can bind TRβ1, but not TRα1.27 The combined presence of positive and negative TREs in the promoters of many genes, including rat growth hormone and myosin heavy chain genes, have been previously demonstrated and can result in opposite effects of T3 on the same gene in a tissue-specific manner.28,29

Several MMPs may be involved in remodeling the extracellular matrix. These Zn2+- and Ca2+-dependent enzymes degrade components of the extracellular matrix, and precise regulation of their expression is crucial in many normal conditions. Nuclear receptors control MMP gene expression through a variety of seemingly redundant mechanisms assuring precise control of extracellular matrix degradation under a variety of physiological and pathologic conditions. The synthesis of MMPs can be inhibited by several agents such as retinoids, thyroid hormone and glucocorticoids, which bind to nuclear receptors.30 Our finding that T3 reduced TGF-β-induced MMP-2 secretion by HSC is consistent with these data.

In summary, our results suggest that subclinical hypothyroidism might be beneficial not only to prevent the development of hepatic damage, but also to reverse established fibrosis in the liver. Undoubtedly, caution should be exercised in extrapolating results from these animal models to patients with chronic liver disease. Possible application of these results to humans merits evaluation in clinical trials.

References


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