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Pituitary tumor transforming gene as a novel regulatory factor of liver fibrosis

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ABSTRACT

Aims: Pituitary tumor-transforming gene (PTTG) is involved in multiple cellular pathways. We studied the development of liver fibrosis induced by thioacetamide (TAA) in knockout (PTTG −/−) and wildtype (PTTG +/+) mice.
Main methods: Liver fibrosis in PTTG +/+ and PTTG −/− mice was induced by escalating dose TAA treatment (50–400 mg/kg, i.p.) for 12 weeks and assessed by histochemistry, immunohistochemistry, liver hydroxyproline, serum fibrosis markers and fibrosis-related mRNA expression by real-time PCR determination.
Key findings: Both PTTG +/+ and PTTG −/− mice treated with TAA developed signs of fibrosis and inflammatory cell infiltration. However, histological signs of bridging fibrosis and connective tissue square morphometry were significantly attenuated in mice lacking PTTG. α-SMA immunohistochemistry revealed that hepatic stellate cell activation was markedly reduced in PTTG −/− mice compared to wildtype controls. Hepatic hydroxyproline levels were significantly lower in fibrotic PTTG −/− group. The serum TNFα and hepatic TNFα mRNA expression were significantly lower in fibrotic PTTG −/− animals, as well as hepatic TGFβ and VEGF mRNA levels compared to TAA-treated wildtype controls. Serum hyaluronate and TGFβ levels were markedly elevated in fibrotic mice of both genotypes, but were not altered by the absence of PTTG.
Significance: TAA-induced fibrosis development is significantly ameliorated in PTTG −/− mice. These animals demonstrated diminished stellate cell activation, suppressed circulating serum markers of inflammation, fibrogenesis and angiogenesis. The presented findings suggest that PTTG is functionally required for hepatic fibrosis progression in an animal model of chronic liver injury. PTTG can be considered as a new important target for prevention and treatment of liver fibrosis/cirrhosis.

1. Introduction

The pituitary tumor-transforming factor (PTTG) was isolated from rat pituitary tumor cells not long ago and identified as an oncogene [34]. PTTG is overexpressed in a variety of tumors, including endocrine (pituitary, thyroid, ovarian) and non-endocrine related tumors such as lung cancer [19], colon cancer [16], gastric carcinoma [49], and hepatocellular carcinoma [4,10]. The PTTG expression promotes tumor progression, invasion and metastasis, whereas a decrease of PTTG expression inhibits tumor growth. Physiologic functions of PTTG include multiple cellular pathways, such as cell proliferation, DNA repair, apoptosis, cell migration, and induction of angiogenesis [41]. Angiogenesis, which is essential for the growth of solid tumors and their metastasis, was shown to be regulated by PTTG via induction of the vascular endothelial growth factor (VEGF), which plays a pivotal role during vessel formation [23].

The role of PTTG in liver preneoplastic processes is still unknown. Angiogenesis has been shown to be highly associated with development of liver fibrosis and cirrhosis [38] where VEGF has the major implication [5,39]. The effect of PTTG in tumor angiogenesis has been shown to be modulated through up-regulation of matrix metalloproteinase-2 (MMP-2) [26] characteristic of the fibrotic processes in the liver [18]. Therefore, given that PTTG is involved in angiogenesis and fibrolysis, it could be considered as a potential contributor to liver fibrosis.

Modeling of liver fibrosis desires suitable experimental situation reproducing activation of fibrogenesis and inhibition of fibrolysis. No experimental model reproduces exactly human liver fibrosis by etiology [47]. Among different models thioacetamide (TAA) is a commonly used hepatocarcinogen for induction of experimental liver fibrosis. Based on

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biochemical and morphological observations, the TAA-induced rat liver cirrhosis resembles to the human liver disease [6,30] as it shares a number of metabolic and histological alterations usually found in the livers of afflicted human beings and can be used as a suitable animal model for studying of mechanisms of liver fibrosis.

Therefore, to establish the effects of PTTG on liver fibrosis, we studied the development of TAA-induced liver fibrosis in PTTG −/− and wild-type mice.

2. Materials and methods

2.1. Animals

Six-week-old PTTG −/− and wildtype littermates, PTTG +/+ (C57/BL6 background), were a generous gift from Dr. Shlomo Melmed (Cedars-Sinai Medical Center, Los Angeles, USA). The animals were housed in a conventional facility with a 12-hour light/dark cycle and a temperature of approximately 23 °C and provided with a standard chow and water ad libitum. The care, use and procedures performed with mice used in this study were approved by the Ethics Committee of the National Academy of Sciences, Belarus, and complied with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals.

Liver fibrosis in wild-type and PTTG −/− mice was induced by intraperitoneal (i.p.) administration of thioacetamide (TAA) (Sigma, USA), three times per week for 12 weeks, gradually increasing the dose of TAA from 50 mg/kg b.w. to 400 mg/kg b.w. during the first 2 weeks of the experiment. We used the model of escalating TAA dosing regimen (50–400 mg/kg) that was optimized specifically for mice [37]. Animal weight was recorded every week from the beginning of the experiment. Each group consisted of 10 animals.

After 12 weeks of TAA treatment, the mice were killed under anesthesia with ketamine hydrochloride (30 mg/kg) and liver specimens were snap-frozen in liquid nitrogen for RNA isolation and hydroxyproline determination or collected in formalin for histological analysis. Blood samples were taken for biochemical evaluation.

2.2. Histopathological and immunohistochemical analysis

Liver specimens were fixed in 10% formalin and embedded in paraffin.

5-μm thick sections were processed routinely for hematoxylin & eosin and Azan-Mallory staining. The degree of focal inflammation was expressed as the mean of 10 different fields within each slide that had been classified on a scale of 0–3 (no foci — 0, mild, <2 foci per high power field (hpf) — 1, moderate, 2–4 foci per hpf — 2, severe, >4 foci per hpf — 3). The slides stained with Azan-Mallory were used for morphometric analysis to determine the percentage of liver tissue affected by fibrosis using a computer-assisted image analyzer (BIOSCAN, Minsk, Belarus). Results from 15 random fields per slide (fibrosis index) were calculated as a ratio of the Azan-Mallory positive area to the total area examined and expressed as a relative square of connective tissue (% to the total slide square) as described before [24].

Immunohistochemistry for α-SMA was performed in formalin-fixed paraffin-embedded liver sections (5 μm). Briefly, the sections (5 μm) were deparaffinized and then incubated in phosphate buffered saline solution (PBS) containing 3% H_2O_2 for 10 min to block the endogenous peroxidase activity. After antigen retrieval (20 min boiling in citrate buffer), sections were incubated overnight at 4 °C with anti-α-SMA antibody (ab5694; Abcam, Cambridge, UK; 1:400) and subsequently incubated for 30 min with HRP-coupled secondary antibodies. The reaction was visualized by the addition of diaminobenzidine substrate solution (Dako, Denmark) and lightly counterstained with hematoxylin.

2.3. Biochemical assays

Blood was drawn from all the animals at the moment of sacrifice and the contents of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase were determined using standardized test kits (LACHEMA, Brno, Czech Republic). Serum fibrosis markers were analyzed by ELISA technique using commercial kits from R&D Systems GmbH (Wiesbaden, Germany) for transforming growth factor β_1 (TGFβ_1) and tumor necrosis factor α (TNFα). Assay kits for measurement of hyaluronic acid, collagen type III and procollagen III were from Wuhan USCN Sciences Co., Ltd. (Wuhan, China).

Hepatic collagen content was determined as relative liver hydroxyproline (μg/g liver) by a modification of Jamall's method [36]. Liver specimens (250 mg each) were hydrolyzed in 5 ml of 6 N HCl at 110 °C for 16 h, followed by incubation with chloramine T (2.5 mmol/l) for 5 min and Erlich's reagent (410 mmol/l) for 30 min at 60 °C. Absorption was measured at — 558 nm and results are expressed as μg/g of the wet tissue.

2.4. Isolation of mRNA, reverse transcription and real-time PCR

Total RNA from liver samples was extracted using a Qiagen RNeasy kit (Qiagen GmbH, Germany) following manufacturer's recommendation. cDNA was obtained by reverse transcription of 1 μg of total RNA using Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany), applying a 50-pmol random hexamer and 100-pmol oligo-dT primers (Promega, Mannheim, Germany).

Hepatic PTTG, vascular endothelial growth factor (VEGF), transforming growth factor β_1 (TGFβ_1), tumor necrosis factor α (TNFα), collagen type I and tissue inhibitor of metalloproteinase-1(TIMP-1) mRNA levels were assessed quantitatively by real-time PCR method as described before [17] using a Light Cycler Fast Start DNA Master SYBR Green I kit (Roche, Penzberg, Germany). For normalization, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified in a parallel reaction.

3. Results

The macroscopic observation of livers from PTTG +/+ mouse treated with TAA revealed a rough surface of the liver and formation of small nodules. On the contrary, PTTG −/− mice had a smooth liver surface which did not virtually differ from the livers of the control group.

Both TAA-administered groups showed bridging and septal fibrosis connecting portal veins with attendant inflammatory cell infiltration. Fig. 1 illustrates the histological analysis of liver samples from four groups. Both TAA-treated groups displayed degeneration of hepatocytes and increased inflammatory cell infiltration in the centrilobular necrotic areas. However, the inflammatory cell infiltration along portal tracts as evaluated by the inflammation scoring was lower in the liver of PTTG −/− TAA-treated animals as compared to the corresponding PTTG +/+ group (Table 1).

The development of liver fibrosis was confirmed by the liver histology stained with Azan-Mallory (Fig. 2). As anticipated, staining of collagen with Azan-Mallory revealed typical non-significant collagen deposition and portal blood vessels in both groups of untreated control mice. In contrast, TAA-treated mice showed development of liver fibrosis with significant distortion of the lobular architecture. The fibrotic changes were characterized by portal–portal septa surrounding the hepatic lobule. However, in PTTG −/− animal liver, fibrosis, as determined by stained slides morphometry, was less pronounced than in PTTG +/+ mice (4.0 ± 0.34 vs 2.3 ± 0.29, $P < 0.001$) (Table 1).

The activated hepatic stellate cells (HSC) were detected by immunohistochemistry for α-SMA. HSC activation marker α-SMA was significantly expressed around the portal tracts and along fibrous septae in the liver of the PTTG +/+ group treated with TAA (Fig. 3A). However, α-SMA expression is notably reduced in livers of PTTG −/− mice

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