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Adenovirus-mediated transfer of siRNA against PAI-1 mRNA ameliorates hepatic fibrosis in rats☆

Ping-Fang Hu[1], Han Chen[2], Wei Zhong[1], Yong Lin[1], Xin Zhang[3], Yue-Xiang Chen[1], Wei-Fen Xie[1,*]

[1]*Department of Gastroenterology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China*
[2]*Department of General Surgery, Changzheng Hospital, Second Military Medical University, Shanghai, China*
[3]*Chinese National Human Genome Center at Shanghai, Shanghai, China*

***Background/Aims*: Plasminogen activator inhibitor-1 (PAI-1) is a potential profibrotic molecule. The aim of this study was to evaluate the therapeutic effect of PAI-1 small interfering RNA (siRNA) on experimental hepatic fibrosis and investigate the intrinsic mechanisms.**

***Methods*: Hepatic fibrosis in rats was induced by dimethylnitrosamine (DMN) administration or bile duct ligation (BDL). An adenovirus carrying PAI-1 shRNA (AdshPAI) was generated and administered via tail vein injection. The expression of PAI-1 was confirmed by real-time RT-PCR and immunohistochemistry. The effect of AdshPAI on fibrosis was evaluated by histological and immunohistochemical examination.**

***Results*: We found that PAI-1 was downregulated after AdshPAI administration. Liver fibrosis was significantly improved after AdshPAI administration in both DMN and BDL models. AdshPAI treatment facilitated matrix degradation by correcting the levels of matrix metalloproteinases (MMPs) and its inhibitors (TIMPs) through upregulation of MMP9, MMP13 and downregulation of TIMP-1. Moreover, AdshPAI treatment stimulated hepatocellular proliferation and inhibited cellular apoptosis.**

***Conclusions*: This study suggests that AdshPAI treatment has a protective effect on hepatocytes and ameliorates liver fibrogenesis. Inhibiting the upregulation of PAI-1 during liver fibrosis may be an antifibrotic pathway worth exploiting.**
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***Keywords*: Plasminogen activator inhibitor-1 (PAI-1); RNA interference (RNAi); Adenovirus; Hepatic fibrosis**

1. Introduction

Hepatic fibrosis is a major medical problem associated with significant morbidity and mortality. It is characterized by the accumulation of excess extracellular matrix (ECM), regardless of the underlying etiology [1]. The amount of matrix deposition depends on the balance between its synthesis and degradation. When synthesis of ECM exceeds its degradation, the pathological accumulation of ECM leads to liver fibrosis.

The reversible nature of experimental hepatic fibrosis [2] and the striking decrease in collagenolytic activity observed in liver fibrosis models [3,4] suggest a crucial role for impaired matrix degradation in hepatic

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* Corresponding author. Tel.: +86 21 63610109; fax: +86 21 63520020.
E-mail address: weifenxie@medmail.com.cn (W.-F. Xie).

Abbreviations: PAI-1, plasminogen activator inhibitor-1; siRNA, small interfering RNA; DMN, dimethylnitrosamine; BDL, bile duct ligation; MMPs, matrix metalloproteinases; ECM, extracellular matrix; uPA, urokinase type plasminogen activator; tPA, tissue-type plasminogen activator; PFU, plaque forming unit; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; HSCs, hepatic stellate cells; RNAi, RNA interfering.

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fibrogenesis. Moreover, the impact of ECM on liver cells has been reported [5]. Increasing deposition of abnormal ECM accelerates the activation of collagen producing cells [6]. The state of hepatic stellate cells (HSCs), the major collagen producing cells in liver, is affected by direct interaction with ECM and depends on the ECM content [7]. Furthermore, an inverse relationship between hepatocyte proliferation and ECM accumulation has been documented [8]. Therefore, enhancing matrix degradation may prove particularly valuable when the matrix deposition in response to injury is already extensive, especially in clinical events.

The plasminogen activator (PA)/plasmin system, which is located upstream of the fibrolysis system, is capable of directly degrading matrix components, and indirectly inhibits the deposition of ECM by activating latent matrix metalloproteinases (MMPs). There is increasing evidence that supports a pivotal role for the PA/plasmin system in regulating the balance between ECM degradation and deposition [9–11]. Our previous study indicated that delivery of exogenous urokinase type plasminogen activator (uPA) gene into HSC-T6 decreased the amount of collagen and was accompanied by an increased expression of MMP-2, while treatment with uPA significantly ameliorated ECM deposition in experimental hepatic fibrosis [12]. In addition, a study by Bueno et al. also revealed that uPA gene administration could induce cirrhosis regression and ameliorate hepatic dysfunction in experimental liver cirrhosis via upregulation of collagen-degrading enzymes such as MMP-13 and MMP-2 [13]. Plasminogen activator inhibitor-1 (PAI-1), the major physiological inhibitor of both uPA and tissue-type plasminogen activator (tPA), is a powerful fibrosis-promoting molecule and is a promising therapeutic target for fibrotic diseases. Our previous studies have suggested that PAI-1 is upregulated during liver fibrosis, and its expression was closely correlated with the deposition of collagens. Meanwhile, downregulation of PAI-1 expression by siRNA in HSC-T6, could downregulate the expression of pro-fibrogenic molecules and inhibit HSC proliferation and collagen secretion [14]. Additionally, there is substantial evidence indicating that PAI-1 deficiency has a protective effect against organ fibrosis, including the liver, renal and lung [9–11], whereas PAI-1 overexpression exacerbates fibrosis. Therefore, we postulated that downregulation of PAI-1 expression will protect against hepatic fibrogenesis *in vivo*.

RNA interfering (RNAi) has opened new avenues in gene therapy. The major bottleneck in the development of siRNA therapies is their low transfection efficiency *in vivo*. In the present study, by combining a well-defined H1-RNA promoter with the conventional AdEasy™ vector system [15], which are widely used in gene therapy for their transduction efficiency, we first constructed an adenovirus vector carrying PAI-1 shRNA (AdshPAI). Hepatic fibrosis was induced by two mechanistically distinct models, either by dimethylnitrosamine (DMN) administration or by bile duct ligation (BDL). We found that AdshPAI administration could prevent the progression of hepatic fibrogenesis by inhibiting HSC activation and promoting hepatocellular proliferation. Meanwhile, AdshPAI also regulated the balance between MMPs and TIMPs, which would, in turn, facilitate ECM degradation.

2. Materials and methods

2.1. Adenovirus vectors

AdshPAI, which expresses the PAI-1 shRNA under the H1 promoter, was prepared as previously described (Supplementary reference 1). The control adenovirus AdNC which expresses the negative control (NC) shRNA, was prepared as well.

2.2. Animal models

Two distinct models of experimental hepatic fibrosis were established in rats, either by DMN administration or by BDL (Supplementary Fig. 1). In DMN-induced fibrosis, rats were injected intraperitoneally with 1% DMN (1 ml/kg body weight) for three consecutive days per week for up to 4w. At the end of the second week, rats were infused with a single dose of 4×10^9 plaque forming unit (PFU) AdNC or AdshPAI via the tail vein, respectively. Rats were sacrificed 4w after DMN. BDL induced fibrosis was performed as previously described [16]. Rats were injected with the same doses of adenovirus via tail vein 2d before or 3d after BDL.

2.3. Histology and immunohistochemistry

Masson's trichrome and Sirius Red staining were used to determine collagen deposition. Immunohistochemical examination was carried out to detect the expression of PAI-1 (America Diagnostica, Stamford, CT), α-SMA, TGF-β1, MMP-9, TIMP-1 (Boster, Wuhan, China) and PCNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in liver tissues. Immunohistochemistry was used to detect the coexpression of PAI-1 with α-SMA using serial sections.

2.4. 5′-Bromo-2′-deoxyuridine (BrdU) immunohistochemical staining

One hour before sacrifice, 100 mg BrdU (Sigma, St. Louis, MO) per kg body weight was injected intraperitoneally. The sections were labeled with an anti-BrdU monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the labeled antigen was visualized with an Envision™ detection kit (Gene Tech, Shanghai, China) followed by the diaminobenzidine reaction.

2.5. Immunofluorescence staining of apoptotic cells

Immunofluorescence staining of apoptotic cells was performed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) using the commercial APO-Brdu™ TUNEL Assay Kit (Invitrogen, Carlsbad, CA).

2.6. Real-time RT-PCR

The housekeeping gene β-actin was used as an internal control, and gene-specific mRNA expression was normalized against β-actin expression. Primer sequences were summarized in Supplementary Table 1.

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