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Augmenter of liver regeneration (ALR) gene therapy attenuates CCl_4-induced liver injury and fibrosis in rats

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ABSTRACT

Liver fibrosis represents a process of healing and scarring in response to chronic liver injury. Augmenter of liver regeneration (ALR) has been shown to protect hepatocytes from various toxins. The aim of this study was to investigate the effects of ALR gene therapy on liver injury and fibrosis induced by CCl_4 in rats and further explore the underlying mechanisms. Human ALR expression plasmid was delivered via the tail vein. ALR gene therapy might protect the liver from CCl_4-induced injury and fibrogenesis by attenuating the mitochondrial dysfunction, suppressing oxidative stress, and inhibiting activation of HSCs. This report demonstrated that ALR gene therapy protected against the ATP loss, increased the activity of ATPase, decreased intrahepatic reactive oxygen species level, and down-regulated transforming growth factor-β1, platelet-derived growth factor-BB, and α-smooth muscle actin expression. Following gene transfer liver function tests were significantly improved. In brief, ALR gene therapy might be an effective therapeutic reagent for liver fibrosis with potential clinical applications.

1. Introduction

Liver fibrosis represents the consequences of a wound healing response to chronic liver injury from a variety of causes, including viral, autoimmune, drug-related, cholestatic, and metabolic diseases, which if persistent leads to cirrhosis and liver failure [1,2]. It is characterized by excessive production and deposition of extracellular matrix (ECM) [1–5]. The activated hepatic stellate cells (HSCs) are the main ECM-producing cells in liver fibrosis. HSCs activation, characterized by enhanced cell proliferation and overproduction of ECM, is triggered by the release of mitogenic platelet-derived growth factor (PDGF) from activated HSCs and fibrogenic transforming growth factor (TGF)-β1, mostly from Kupffer cells [2,5,6]. Inflammation is a key event in the stimulation of HSCs activation and liver fibrosis, which are induced by oxidative stress [7]. Cell death induces mitochondrial reactive oxygen species (ROS) production, impairs mitochondrial function, and depresses ATP levels which can lead to apoptosis and inflammation. Moreover, mitochondria are not only the source of oxidative stress but are also a target of ROS [8].

The hepatotrophic factor designated augmenter of liver regeneration is thought to be one of the factors responsible for the extraordinary regenerative capacity of mammalian liver. It has also been called hepatic regenerative stimulation substance (HSS) or hepatopoietin (HPO) [9]. Besides well-known growth factors like hepatocyte growth factor (HGF) and epidermal growth factor (EGF), ALR is another cytokine of vital importance [10,11]. It belongs to a novel group of so called cytozymes as it acts as a growth factor and a sulfhydryl oxidase enzyme, that binds FAD containing a redox-active CxxC disulfide proximal to a flavin ring [12]. ALR is heavily expressed in the liver and stimulates hepatocytes but not other cell types as it may have its specific receptors on the surface of liver cells only [13]. Exogenous ALR administration ameliorated hepatic damage, enhanced hepatic proliferative capacity and improved survival of animals following toxin-induced liver injury [14–16]. Its activity is essential for the survival of hepatocytes for the biogenesis of mitochondria [17]. ALR can decrease cytochrome *c* release from mitochondria to protect human hepatocyte against apoptosis induced by ethanol [11]. ALR has also been shown to mitigate hepatocyte injury through suppression of the mitochondrial permeability transition in vitro [18]. A recent study shows that ALR may have clinical potential, as exogenous ALR administration to rats with thioacetamide-induced liver fibrosis is able to remarkably decrease fibrosis [19]. In the present study, we had investigated ALR gene therapy against CCl_4-induced liver injury and fibrosis in rats and explored its underlying mechanism.

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2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats, weighing 120 ± 10 g, were obtained from Medical Laboratory Animal Center of Guangdong (China). All animals were housed in the animal facilities of 458 Hospital with free access to food and water. They were kept in an air-conditioned room at 23 ± 2 °C with a 12/12-h light and dark cycle and allowed to acclimate for 1 week prior to use. All procedures were performed under the approval of the Ethics Review Committee for Animal Experimentation of Institute of Clinical Pharmacology, 458 Hospital.

2.2. ALR expression plasmid preparation

The human ALR expression plasmid (pCDNA3.1-ALR vector) was conserved by Liver Disease Key Laboratory, 458 Hospital. In brief, the full length of human ALR cDNA was subcloned into a pCDNA3.1 expression vector designed for high-level stable and transient expression in mammalian hosts with a human cytomegalovirus immediate-early (CMV) promoter. pCDNA3.1 was used as a vehicle control. These plasmids were purified using the E.N.Z.A. Endo-free Plasmid Maxi Kit (Omega Biotek, USA).

2.3. Liver fibrosis induction and gene therapy

Liver fibrosis was induced by weekly intragastric administration of carbon tetrachloride (CCl_4) for 8 weeks [20]. At the beginning of injection of CCl_4, plasmid (pCDNA3.1-ALR or pCDNA3.1) was administrated into rats with a dose of 500 μg/kg through the tail vein every other week, with some modifications, as described previously [21,22]. Four experimental groups of animals were analyzed: healthy rats (group I, $n = 10$), fibrotic rats (group II, $n = 10$), fibrotic rats injected with either of pCDNA3.1 (group III, $n = 5$) or pCDNA3.1-ALR (group IV, $n = 10$). In group II, four rats were sacrificed at the end of 7 weeks for confirmation of liver fibrosis. Another rats were sacrificed at 12 h after the last pCNA3.1-ALR injection after an 8-week treatment. All animals were performed under anesthesia by diethyl ether. Selection of fibrotic animals for treatment with pCDNA3.1-ALR or pCDNA3.1 was completely randomized.

2.4. Serum biochemical measurements

Blood was collected from each rat by inferior vena cava puncture when sacrificed. After coagulation, sera were collected and stored at −20 °C for further analyses. Serum alanine aminotransferase (ALT) levels were analyzed by standard spectrophotomeric methods using commercial test reagents (Jiancheng Biotechnology, China).

2.5. Analysis of liver tissue oxidative stress levels

Liver tissues were homogenized in 0.05 M ice-cold PBS and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was taken for the assays of malondialdehyde (MDA) and antioxidant enzymes activities. MDA was assayed by the measurement of thiobarbituric acid-reactive substances (TBARS) levels at 532 nm. Glutathione peroxidase (GPx) activity was tested by measuring the reduction of glutathione (GSH) per min on the base of its catalysis. All above measurements were performed according to the protocols of kits (Jiancheng Biotechnology, China).

2.6. Histopathology and immunohistochemistry

A portion of the liver was removed and fixed in 10% formalin, processed by routine histology procedures, embedded in paraffin. The 5 μm sections were stained with 0.1% sirius-red in picric acid (Sigma–Aldrich) for 1 h at room temperature. For immunohistochemistry, sections were treated sequentially with normal goat serum for 20 min and incubated with mouse anti-α-SMA antibody 1A4 (Dako, Denmark; diluted 1:100) for 2 h at room temperature. The sections were washed three times with PBS. Counterstaining was performed with hematoxylin and using DAB as a substrate.

2.7. Enzyme-linked immunosorbent assay

Levels of hepatic ALR were determined by enzyme-linked immunosorbent assay (ELISA) methods in accordance with standard ELISA procedures. Mouse anti-ALR antibody was prepared by Liver Disease Key Laboratory of 458 Hospital as described previously [23]. Supplemental ELISA products were purchased from Whiga Technology (China). Briefly, supernatants or standards were added to 96-well plates coated with coating antibody, and plates then incubated at room temperature for 2 h. Plates were washed five times and a detecting antibody was added to each well. Plates were incubated at room temperature for 2 h and 100 μL substrate solution was added to each well. After incubation for 30 min, 100 μL stop solution was added to each well. The absorbance was measured in an ELISA reader at 450 nm.

2.8. RNA preparation and analysis

The expressions of PDGF-BB and TGF-β1 mRNA were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) technique. Total RNA was isolated from liver tissue using Trizol Reagent according to the manufacturer's instructions (Invitrogen, USA). The same quantity of total RNA was reverse-transcribed into complementary DNA (cDNA) using M-MLV Transcriptase in the presence of oligo-dT primers (Invitrogen, USA). Quantitative PCR (qPCR) was performed using SYBR Green I (Takara, Japan) for 40 cycles at 30 s at 93 °C and 45 s at 55 °C with ABI 9700 (Applied Biosystems) according to the manufacturer's instructions. The primer sequence and expected product size are described in Supporting Table 1. Results were analyzed by $^{\Delta\Delta}$Ct method.

2.9. ATP content and ATPase activity assay

Adenosine triphosphate (ATP) levels were measured using the ATP Bioluminescence Assay Kit CLS II from Roche (Germany), which uses the ATP dependency of the light emitting luciferase-catalyzed oxidation of luciferin. The activities of ATPase were estimated by quantifying the release of inorganic phosphorus from ATP according to the manufacturers' instructions of detection kits (Jiancheng Biotechnology, China). One activity unit of ATPase is expressed as 1 μmol phosphate per milligram protein per hour.

2.10. Statistical analysis

The results were expressed as mean values with their corresponding standard errors. One-way analysis of variance (ANOVA) with least significant difference test (LSD) was used to compare experimental groups. A calculated $P < 0.05$ was considered statistically significant. All analyses were performed by SPSS for Windows Software version 13.0.

3. Results

3.1. ALR gene transfer to the fibrotic liver

As expected, protein levels of ALR were significantly increased in the ALR-treated rats as compared to healthy rats and fibrotic rats (Fig. 1A). We also determined protein expression of ALR in lung, spleen and kidney from ALR-treated rats. ALR was almost absent in lung, spleen and kidney (data not shown). Taken together, these data indicate that ALR vector was able to transduce the fibrotic liver and to express functional ALR protein. Moreover, ALR was specifically expressed in liver.

3.2. ALR ameliorates chronic liver injury in CCl_4-induced rats

As shown in Fig. 1B, compared with those in healthy controls, the activities of serum ALT were significantly higher in fibrotic rats, indicating that liver injury, as an important disease indicator of chronic liver fibrosis, spontaneously occurred in rats treated with CCl_4. The levels of serum ALT were markedly reduced by administration of ALR.

Changes in liver volume and spleen volume are the morphological index of chronic liver disease. Liver and spleen indices in fibrotic rats were significantly higher than that in healthy rats. After ALR administration, hepatomegaly was almost absent, while splenomegaly was incompletely reversed (Fig. 1C).

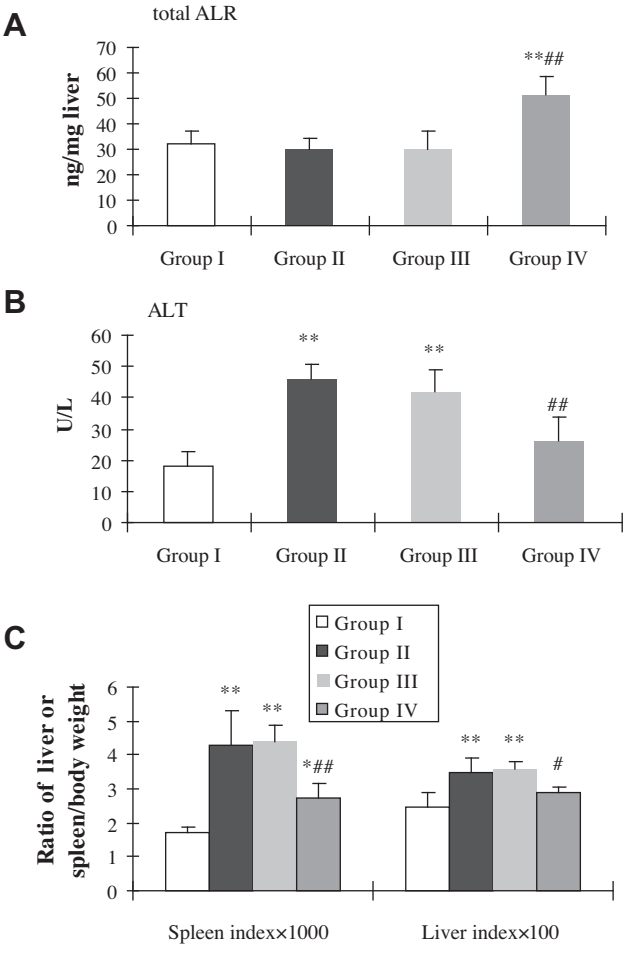

Fig. 1. Analysis of ALR expression and liver function parameters. ALR protein was evaluated by ELISA (A). ALT (B), liver index and spleen index (C) were evaluated from healthy rats (group I) or from fibrotic rats (group II) or from fibrotic rats treated with empty plasmid (group III) or ALR (group IV). ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. group I. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. group II or III).

To assess the impact of ALR on liver fibrosis caused by CCl_4, liver sections were stained with Sirius red for detecting the deposition of collagens. Compared with sections from the healthy controls, liver sections from fibrotic control rats showed prominent red staining in the fibrotic septa between nodules, suggesting a high level of collagen deposition. ALR treatment remarkably reduced the size stained with Sirius red in the liver (Fig. 2). Collectively, these results demonstrated that ALR protected the rat liver from CCl_4-induced injury and fibrogenesis.

3.3. ALR blocks HSCs activation

α-SMA is the unique marker for activated HSCs. To evaluate the effect of ALR on HSCs activation in vivo, α-SMA positive cells were examined in liver tissues from the rats using immunohistochemistry. In the healthy rat liver, α-SMA was exclusively and strongly expressed in vessel walls (portal vessels and centrilobular veins). The staining for α-SMA was dramatically increased in the septa surrounding nodules in fibrotic liver, whereas it was significantly decreased in the ALR-treated liver (Fig. 3A).

HSCs activation is coupled with the up-expression of TGF-β1 and PDGF. At the mRNA level, the expressions of TGF-β1 and PDGF-BB were up-regulated in fibrotic livers but markedly suppressed in those treated with ALR (Fig. 3B). These results indicated ALR therapy efficiently blocks HSCs activation in the damaged liver.

3.4. ALR improves the mitochondrial dysfunction

Because mitochondria control cell energy homeostasis, we measured ATP content in livers. Remarkably, ATP levels in healthy liver were higher than that in fibrotic livers (Fig. 4A). ALR-treated liver showed significant increased ATP content compared to fibrotic liver. In addition, ATPase was chosen to assess mitochondrial membrane permeability, because it plays important roles in maintaining the normal active transport for positive ions. In the present study, it was observed that ATPase activity was significantly decreased in fibrotic livers compared to healthy controls, whereas it was observably higher in ALR-treated rats than in fibrotic rats (Fig. 4B).

In agreement with the above data we observed reduced reactive oxygen species (ROS) levels in the liver of ALR-treated rats. The

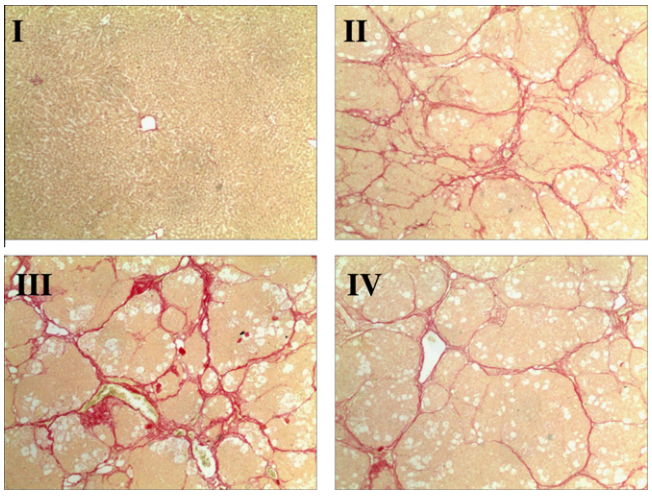

Fig. 2. Assessment of liver fibrosis in ALR-treated rats and controls. Extracellular deposition was stained with Sirius red. (Magnification 40×).

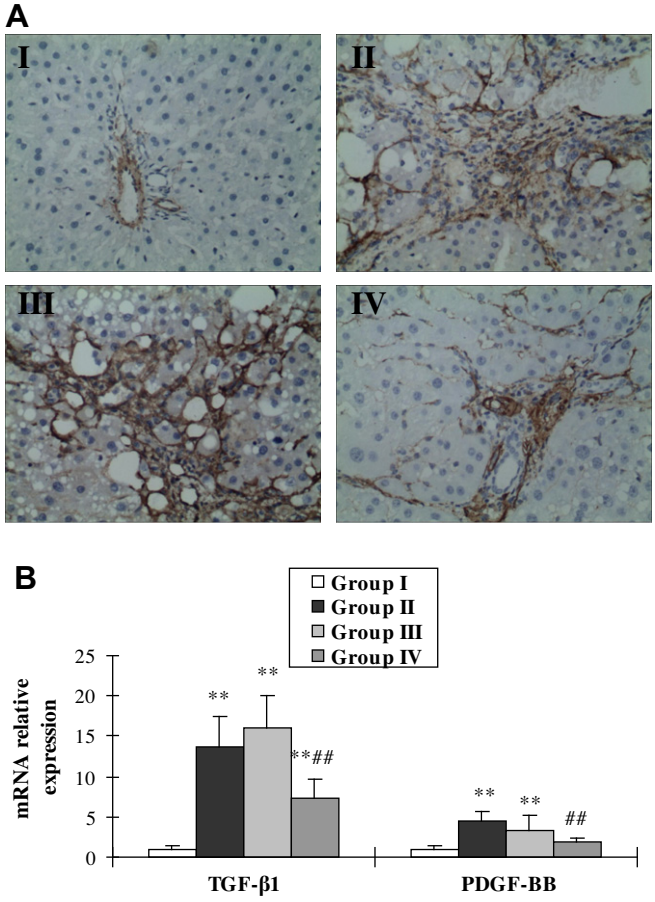

Fig. 3. Analysis of activated HSCs and profibrogenic factors. α-SMA (A) protein was detected by immunohistochemistry. TGF-β1 and PDGF-BB (B) mRNAs were quantified by real time qPCR. ($^{**}P < 0.01$ vs. group I. $^{\#\#}P < 0.01$ vs. group II or III. Magnification 200×).

concentration of MDA, as an indicator of lipid peroxides, was significantly higher in fibrotic rats than healthy rats. ALR treatment markedly reduced this elevated MDA concentration (Fig. 4C). The activity of GPx, important antioxidative enzyme in mammalian cells, was up-regulated in ALR-treated livers (Fig. 4D). It seems possible therefore that improved mitochondrial dysfunction may account for the efficient suppression of intrahepatic ROS in the fibrotic liver of ALR-treated rats.

4. Discussion

Cirrhosis is defined as an advanced stage of liver fibrosis, characterized by the disruption of liver architecture with fibrous septa and parenchymal nodules, and is associated with a high mortality [1,2,24]. Because liver transplantation can be offered to only a limited number of cirrhotic patients [25], alternative therapies for advanced cirrhosis are urgently needed.

Liver fibrosis caused by CCl_4 has been extensively used in experimental models in rats. Hepatic responses in rats to chronic CCl_4 stimulation are shown to be superficially similar to human cirrhosis [26]. CCl_4 metabolism in the liver results in the stimulation of lipid peroxidation and the production of free radicals [27,28], which causes necrosis of hepatocytes, induces inflammation, and further promotes progression of liver fibrogenesis.

ALR was considered to be an important intracellular survival factor for hepatocytes [29]. In a previous report, ALR could inhibit hepatocyte apoptosis by protecting mitochondria and reducing

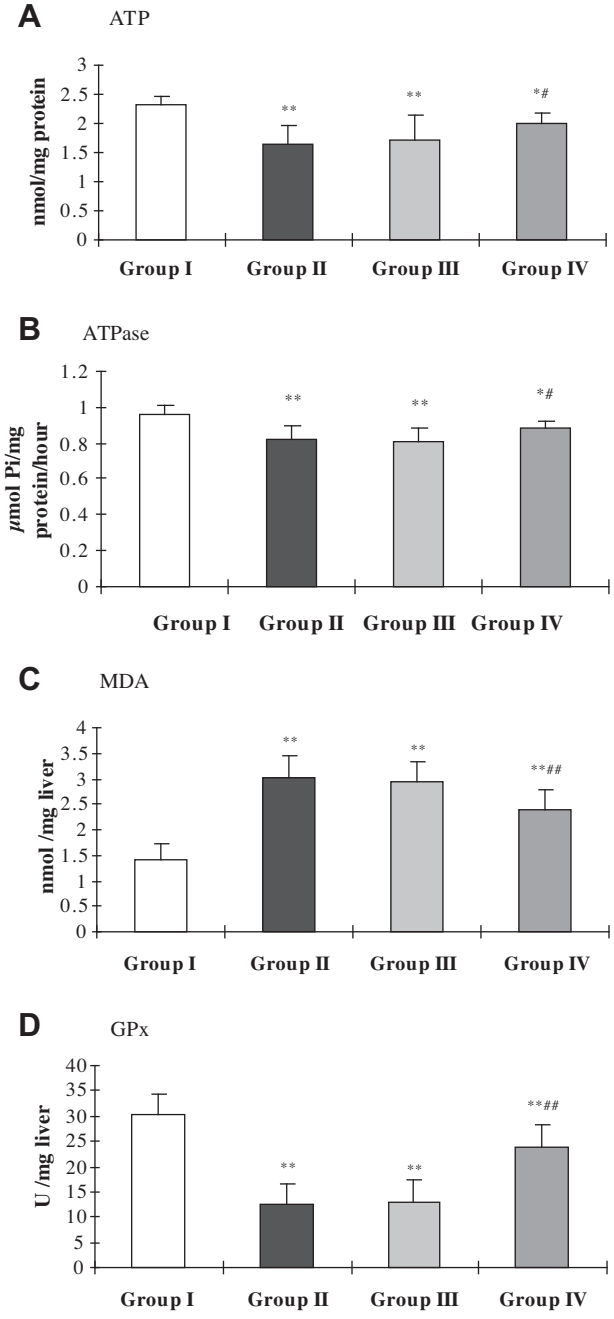

Fig. 4. Protection of hepatocyte mitochondrial function. ALR prevented ATP loss (A) and increased ATPase activities (B). Rats treated with ALR showed a lower oxidative status (MDA decrease and GPx increase) than fibrotic control rats (C and D). ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. group I. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. group II or III).

cytochrome *c* release and caspase-3 activity in vitro [30]. Current information continues to imply a direct link between hepatocyte apoptosis and liver fibrosis, thereby suggesting that therapeutic options stimulating anti-apoptotic mechanisms in hepatocytes would be highly welcome [11,31]. Chronic liver injury induces damage and subsequent loss of hepatic parenchymal cells. This cell death is a triggering event of fibrosis [24]. After oral administration of CCl_4, massive necrosis of hepatocytes occurred in the central to intermediate zones of the liver lobule in rats. Hepatic cells undergoing apoptosis actively secrete cytokines and chemokines, which activate a signal transduction pathway in the adjacent cells and release profibrogenic and inflammatory cytokines, such as TGF-β1, PDGF and TNF-α [2,24].

In our study, ALR therapy reduced the severity of CCl_4 induced mitochondrial damage in rats. It could partially prevent ATP loss and increase the activity of ATPase, suggesting a possible association of mitochondria with a protective role of ALR against CCl_4 caused oxidative damage. According to our data, intrahepatic ROS production is inversely related to the levels of ALR.

TGF-β1 is the most important cytokine, promoting the development of fibrosis in liver [32]. Under pathological conditions, TGF-β1 orchestrates a cross talk between parenchymal, inflammatory, and collagen-expressing cells. The level of TGF-β1 is remarkably elevated in the course of fibrosis [2]. In damaged liver, TGF-β1 is synthesized by different cell types such as apoptotic hepatocytes and Kupffer cells, particularly the latter. TGF-β1 is critical for activation of HSCs in fibrotic liver. Once activated, HSCs will up-regulate expression of ECM components including collagen, TGF-β, PDGF and TIMPs, which all contribute to liver fibrosis [20]. Our analysis of TGF-β1, PDGF-BB and α-SMA expression in liver tissue indicated that the effect of ALR may provide an advantage to liver to escape the promoting fibrosis growth signals of TGF-β1.

Inflammation and oxidative stress are commonly associated with hepatic fibrogenesis during chronic liver diseases [4,27,33]. Apoptotic or necrotic cells secrete TGF-β1 to activate Kupffer cells. TGF-β1 released by Kupffer cells triggers recruitment of neutrophils, monocytes and macrophages, which in turn secrete TNF-α and accelerate inflammation [33,34]. Oxidative damage is thought to be a primary cause of cell death which lead to mitochondrial dysfunction and then increased ROS production [30]. The present study demonstrated that ALR therapy significantly reduced the levels of ROS in the liver of rats with treated CCl_4.

In conclusion, our results provided evidence that ALR may be essential for hepatocyte mitochondria function in vivo. ALR gene therapy protected the liver from CCl_4-induced injury and fibrogenesis by attenuating the mitochondrial dysfunction, suppressing oxidative stress, and inhibiting activation of HSCs. Although the cirrhosis in human more difficult to revert than in experimental animals, still ALR-based gene therapy may prove useful in resolving liver diseases as it may prevent the progression of liver fibrosis and partially revert liver cirrhosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.10.039.

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