Hepatoprotective action of adenovirus-transferred HNF-3γ gene in acute liver injury caused by CCl₄

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Abstract Hepatocyte nuclear factor-3γ (HNF-3γ) is an important regulator of liver-specific genes and the expression of this factor is reduced in the liver injured by carbon tetrachloride (CCl₄). Wistar rats were infected with a recombinant adenovirus carrying the cDNA for HNF-3γ (AxCAHNF3γ) via the tail vein and were treated with CCl₄ by intraperitoneal injection. Liver damage, such as swelling of the hepatocytes and increases in serum marker enzymes were markedly alleviated by AxCAHNF3γ infection. Interestingly, hepatocyte growth factor (HGF) was strongly induced in the AxCAHNF3γ-infected liver. Likewise, HNF-1α and HNF-1β levels were increased, but HNF-3α and HNF-3β levels were depressed in the liver. Our results suggest that the transduced HNF-3γ gene leads to a hepatoprotective effect via the induction of HGF by the combined actions of liver-enriched transcription factors.

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Key words: Acute liver injury; Adenovirus;
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Carbon tetrachloride; Gene therapy;
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1. Introduction

The mammalian liver is the largest solid organ and plays a central role in the metabolism of nutrients, waste matter and poisonous chemicals, among other things, by producing proteins and liver-specific enzymes such as albumin, transthyretin, aldolase, alcohol dehydrogenase and many other hepatic enzymes. The expression of these proteins is controlled by the combined actions of liver-enriched transcription factors such as hepatocyte nuclear factor (HNF)-1α, 1β, HNF-3α, 3β, 3γ, HNF-4 and C/EBPα, β, δ [1].

Mature hepatocytes express at least three HNF-3 isoforms (α, β and γ) that interact to regulate liver-specific phenotypes [1–3]. Since these different HNF-3 isoforms have overlapping functions, the specific physiological role of each isoform in hepatocytes is not well understood. The targeted disruption of the HNF-3γ gene decreases the expression of liver-specific genes, indicating that HNF-3γ is an important activator for some hepatic genes in vivo [2]. In addition, the expression of HNF-3γ, compared with that of HNF-3α and HNF-3β, may be necessary for hepatocytes to maintain highly differentiated functions [4]. In our previous study, to identify the role of HNF-3γ in the regulation of liver-specific gene expression

and the maintenance of differentiated liver functions, replication-defective adenovirus vectors were used to efficiently overexpress the rat HNF-3γ gene in primary hepatocytes. As a result, the transgene was shown to conserve some liver functions in the hepatocytes [5].

Previously, endogenous HNF-3γ gene expression has been revealed to be down-regulated in rats with carbon tetrachloride (CCl₄)-induced hepatic injury, compared with the upregulated expression of HNF-3α and 3β. It is of interest to define the involvement of HNF-3γ in the molecular pathophysiology of acute liver damage. Since the damage by CCl₄ occurs broadly in the liver, we analyzed the role of HNF-3γ by means of adenovirus-mediated gene transfer, which achieves efficient overexpression in almost 100% of hepatocytes through the blood circulation from the portal vein, peripheral vein or abdominal cavity in the normal rat [6,7].

2. Materials and methods

2.1. Recombinant adenovirus carrying the rat HNF-3γ gene

The recombinant adenovirus harboring the rat HNF-3γ gene was constructed as described previously [5,8]. Briefly, cDNA encoding HNF-3γ was inserted into the *Swa*I site of cosmid vector pAxCAwt, carrying the cytomegalovirus immediate early enhancer and modified chicken β-actin promoter (CAG promoter) [9] and rabbit β-globin polyadenylation signal in a cassette. The construct was co-transfected with *Eco*T221-digested Ad5-dlX DNA tagging viral terminal protein into the 293 cells, which were a human embryonic kidney cell line transformed by *E1A* and *E1B* genes. The recombinant adenovirus (AxCAHNF3γ) was generated by genetic recombination and was propagated within the 293 cells. Virion was purified by CsCl-equilibrium centrifugation and titrated with the 293 cells by a conventional procedure [10]. A recombinant adenovirus vector AxCALacZ bearing the *Escherichia coli LacZ* gene was provided by Dr Izumu Saito and used as a control [10].

2.2. Animal treatments

Male adult Wistar rats weighing 150 g were purchased from the Shizuoka Laboratory Animal Center Japan. Each three rats were infected with 1.5×10^8, 5×10^8 and 1.5×10^9 plaque forming units (pfu) of AxCAHNF3γ or 1.5×10^9 pfu of AxCALacZ via the tail vein. Mock-infected control rats were intravenously given an equal volume of saline. At 4 days post-infection, these rats were intraperitoneally injected with 2 ml of CCl₄:olive oil (1:1 mixture) per kg of body weight. All rats were fed ad libitum and received human care in compliance with the institution's guidelines for the care and use of laboratory animals in research.

2.3. Histological procedures and biochemical measurements

Livers from the rats were fixed in 10% formaldehyde and embedded in paraffin. The paraffin blocks were sectioned at 5 μm and counterstained with hematoxylin and eosin. The liver-specific cytosolic enzyme activities of alanine transaminase (ALT) and aspartate transaminase (AST) in blood of the rats were determined according to the manufacturer's manual (Wako Pure Chemicals, Tokyo, Japan).

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2.4. RNA extraction and Northern blot analysis

Total RNA was extracted from the livers of rats infected with adenovirus vectors as described elsewhere [11]. Total RNA (10 µg) was electrophoresed in a 7% formaldehyde agarose gel and transferred to a nylon membrane. The membrane was hybridized with specific cDNA probes for 24 h at 42°C in 50% formaldehyde with sodium phosphate-EDTA solution. Probes were radiolabelled by the random primer method. Intensities of the hybridization signals were determined by densitometric scanning of the autoradiograms and by a Molecular Imager (Bio-Rad Laboratories, CA, USA).

3. Results

3.1. Transferred HNF-3γ alleviates acute hepatic injury

We have previously shown that adenovirus-transferred HNF-3γ conserves some liver function in primary cultured hepatocytes of the adult rat [5]. On the basis of these observations, it is of interest to determine whether HNF-3γ would be of potential benefit in rats with CCl_4-induced acute liver injury. Hence, Wistar rats were infected with 100 µl (1.5×10^9 pfu) of AxCAHNF3γ, or AxCALacZ as a control, via the tail vein. At 4 days post-infection, liver damage was induced in the infected rats by CCl_4 and the rat livers were then analyzed in terms of their biochemical and morphological features.

ALT and AST activity in the sera of mock- and AxCA-LacZ-infected rats were increased 5–7-fold over that of the controls in normal rats, whereas in the AxCAHNF3γ-infected rats, there was an approximately 60% increase in the activity of these enzymes (Fig. 1). These data show that the transferred HNF-3γ gene can alleviate hepatic injury induced by CCl_4.

A histological analysis was therefore carried out with the livers from these rats. As shown in Fig. 2, numerous hepatocytes were found to exhibit severe centrilobular necrosis (indicated by arrows) with the infiltration of leukocytes (indicated by arrowheads) in mock- (Fig. 2B) and AxCALacZ-
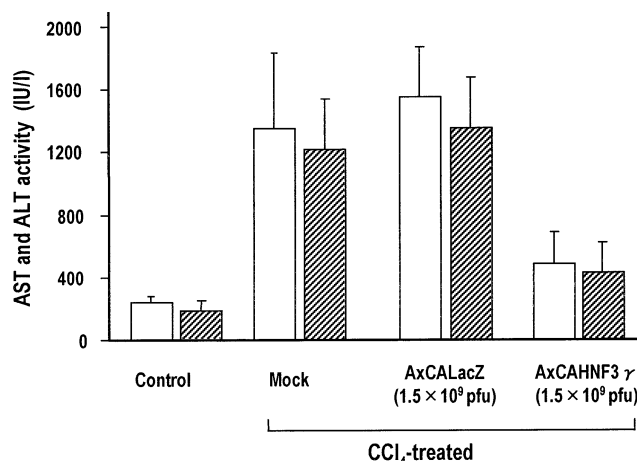

Fig. 1. AST and ALT activities in the sera of treated rats. Sera were obtained from the normal control and mock-, AxCALacZ (1.5×10^9 pfu)- and AxCAHNF3γ (1.5×10^9 pfu)-injected and CCl_4-treated rats. AST and ALT activities are represented by an open box and a hatched box, respectively. $n = 3$, the mean ± S.D. are shown.

infected (Fig. 2C) rat liver. In contrast to the livers of rats infected with AxCAHNF3γ (Fig. 2D), the levels of injured hepatocytes were markedly reduced, similar to the levels in the normal liver (Fig. 2A). These finding indicate that the adenovirus-transferred HNF-3γ gene leads to hepatoprotective effects against CCL_4-induced acute hepatic injury.

3.2. Involvement of HNF-3γ in hepatocyte growth factor (HGF) gene expression

The expression of HGF is rapidly and dramatically induced by either partial hepatectomy or chemical agents, indicating that HGF serves as a potent hepatotrophic factor for liver regeneration [12,13]. As shown in Fig. 3, Northern blot anal-
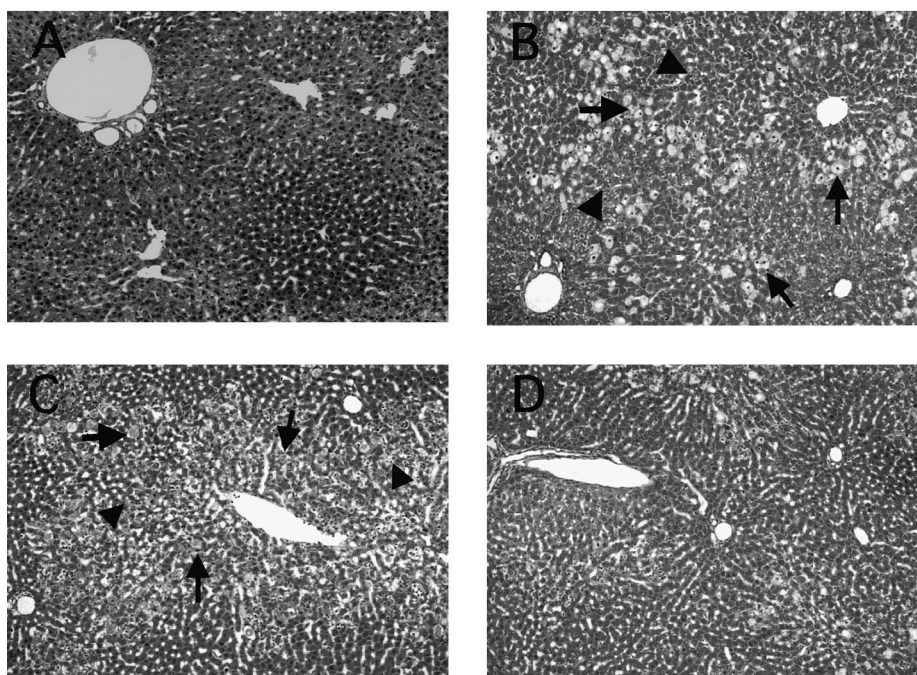

Fig. 2. Histological features of the CCl_4-injured liver. Liver sections were prepared from (A) normal rat and (B) mock-, (C) AxCALacZ (1.5×10^9 pfu)- and (D) AxCAHNF3γ (1.5×10^9 pfu)-infected rats, which were followed by CCl_4 treatment for 2 days. These sections were stained with hematoxylin-eosin and microphotographed at a magnification of ×30.

ysis showed that the HGF levels were slightly increased in the CCl_4-treated livers of the mock- and AxCALacZ-infected rats compared with those of normal rat livers. In contrast, a dramatic increase in HGF expression was observed in the rat liver infected with AxCAHNF3γ (1.5×10^9 pfu). On the other hand, expression of the *c-met* gene encoding the membrane receptor for HGF was scarcely increased or even decreased by CCl_4 treatment and AxCAHNF3γ infection (Fig. 3). Taken together with previous reports that HGF has a potent cytoprotective or anti-hepatitis action [14,15], these results support the hypothesis that HGF is activated by HNF-3γ and that HGF overexpression is responsible for the hepatoprotective effects against CCl_4-induced injury.

3.3. HNF-3γ controlled expression of liver-enriched transcription factors

The liver regenerates completely when moderate damage is inflicted by partial hepatectomy or chemical agents and the expression of liver-enriched transcription factors is also concomitant with this compensatory hepatocyte proliferation [16,17]. Fig. 4 shows the results of the Northern blot analysis, which indicate that endogenous HNF-3γ mRNA (indicated with an arrow) was decreased 50% in the CCl_4-injured liver compared with the normal rat. However, the transduced HNF-3γ mRNA (indicated with an arrowhead) was increased dose-dependently by the AxCAHNF3γ infection in spite of

Fig. 4. Expression of liver-enriched transcription factors. Northern blot analyses were performed using ^{32}P-labelled cDNA as probes, shown on the left of the panels. Lanes are the same that in Fig. 3. HNF-3γ mRNA derived from the endogenous gene is indicated by an arrow and that from the transduced gene is shown by an arrowhead.
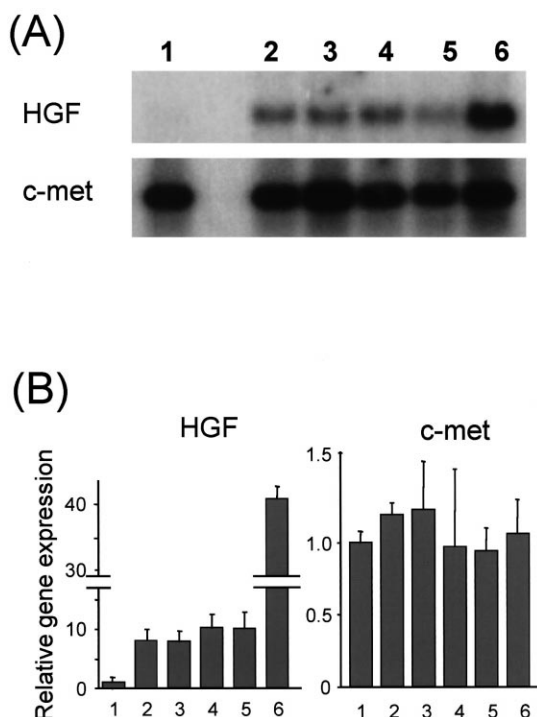

Fig. 3. Effects of HNF-3γ gene transfer on the expression of HGF and *c-met*. (A) Northern blot analysis probed HGF and *c-met* are shown by a typical pattern. Lane 1, liver RNA from the normal rat; lane 2, liver RNA form the rat injected with saline, followed by CCl_4 treatment; lane 3, liver RNA form the rat infected with Ax-CALacZ and treated with CCl_4; lanes 4–6, liver RNAs form the rats infected with AxCAHNF3γ (1.5×10^8, 5×10^8, 1.5×10^9 pfu/rat, respectively) and treated with CCl_4. (B) The hybridization signals were quantified by a Molecular Imager and normalized to those of 18S rRNA. Relative gene expression is shown by the ratio to the levels in normal liver. $n=3$, the mean with S.D. are shown.
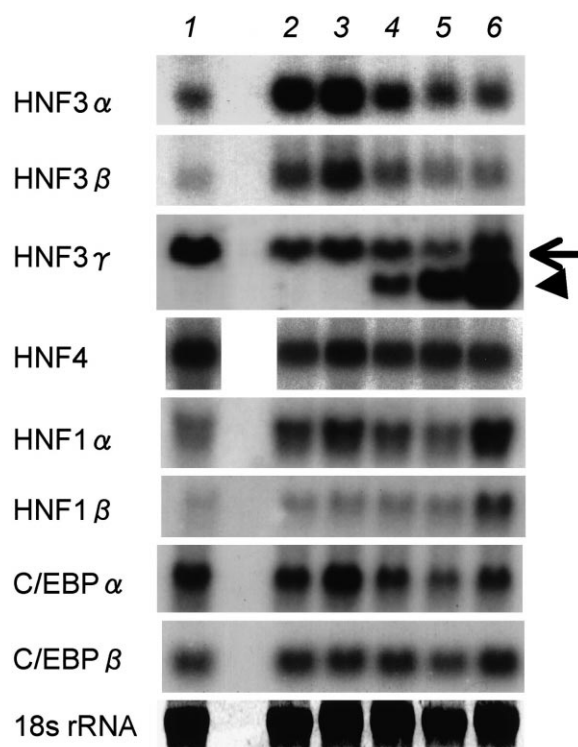
CCl_4 treatment. HNF-3α and HNF-3β were up-regulated in the CCl_4-treated livers of mock- and AxCALacZ-infected rats. Conversely, These mRNAs in the AxCAHNF3γ-infected liver were not observed to be induced by CCl_4 treatment.

Moreover, HNF-1α and 1β mRNAs were little influenced by CCl_4 treatment, while these mRNAs were strongly induced in the HNF-3γ-transduced liver (1.5×10^9 pfu of the Ax-CAHNF3γ). The expression of C/EBPα in rat livers with acute liver injury was decreased by approximately 50% compared to that in mock- and AxCALacZ-infected rats. Furthermore, the mRNA levels of C/EBPβ were slightly increased by CCl_4-induced liver injury, independently of AxCAHNF3γ infection. On the other hand, the expression of HNF-4 was merely influenced by CCl_4 treatment or AxCAHNF3γ infection. These results suggest that the expression patterns of liver-enriched transcription factors are reflected by the levels of HNF-3γ overexpression. Notably, the up-regulation of both HNF-1α and HNF-1β is related to the alleviation of CCl_4-induced acute hepatitis.

4. Discussion

In our previous study, the endogenous HNF-3γ gene, as well as other hepatic genes, was extremely down-regulated in the CCl_4-treated liver. Furthermore, in this study, the adenovirus-transferred HNF-3γ gene was demonstrated to lead to hepatoprotective effects against CCl_4-induced acute hepatic injury.

Significantly, our results show that HGF is remarkably ac-

tivated by HNF-3γ derived from a high titer of AxCAHNF3γ. Previous studies show that following the onset of various types of hepatic injuries, HGF mRNA expression is rapidly up-regulated in the livers of experimental animals [12,13]. In addition, serum HGF levels are elevated in patients with various hepatic disorders [18]. Extensive studies have revealed that HGF strongly stimulates the DNA synthesis of hepatocytes in livers injured by partial hepatectomy or by CCl_4 administration [19]. Likewise, HGF has protective and therapeutic effects on experimental liver fibrosis/cirrhosis caused by chemical compounds, immunological responses or metabolic disorders, indicating that the efficacy of HGF does not seem to be related to the degradation of hepatotoxins, but that it induces potent cytoprotective or anti-hepatitis actions [14]. These observations suggest that HGF activated by the overexpression of HNF-3γ may be responsible for the hepatoprotective effects described in this study. However, the binding site for the HNF-3γ transcription factor has not been located in the 5′-flanking region of the rat HGF gene, as far as the sequence has been analyzed [20]. Therefore, based on the results of this study, we cannot explain the direct or indirect interaction between the transcriptional regulation of HNF-3γ and HGF expression.

The mammalian liver undergoes complete regeneration after damage from partial hepatectomy or chemical agents via proliferation of the remaining liver [21]. This compensatory hepatocyte proliferation induces the expression of some liver-enriched transcription factors as well as many of the immediate early genes such as *c-jun*, *c-fos* and *c-myc* [22]. D-galactosamine-induced liver injury activates C/EBPδ, HNF-3α, β, γ and finally C/EBPβ in proliferating liver epithelial cells [23]. Oval cells, which are induced by the administration of 2-acetylaminofluorene and partial hepatectomy, express HNF-1α, β, HNF-3γ and C/EBP [17,24]. Further study is needed to elucidate why HGF is activated by HNF-3γ overexpression in the CCl_4-injured liver.

In conclusion, the hepatoprotective effects of HNF-3γ overexpression may result from the up-regulation of HGF through the combined actions of liver-enriched transcription factors.

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