

Attenuated acute liver injury in mice by naked hepatocyte growth factor gene transfer into skeletal muscle with electroporation

F Xue, T Takahara, Y Yata, M Minemura, C Y Morioka, S Takahara, E Yamato, K Dono, A Watanabe

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Background: Hepatocyte growth factor (HGF) plays an essential role in hepatic development and regeneration, and shows proliferative and antiapoptotic activity in hepatocytes.

Aims: To establish an effective new method for HGF gene transfer in vivo and to investigate its effects in acute liver injury.

Animals: Eight week old female mice were used.

Methods: Rat HGF gene in a modified pKSCX plasmid was transferred to the tibialis anterior muscle by electroporation using a pulse generator. Four days later, plasma HGF concentrations were determined by enzyme linked immunosorbent assay every two days for three weeks. To confirm the efficacy of electroporation, a plasmid bearing green fluorescence protein (GFP) was transferred similarly. Four days after electroporation, carbon tetrachloride (CCl₄) was administered to mice to induce acute liver injury. Plasma alanine aminotransferase (ALT) activity was measured. Hepatic apoptosis was assessed by Hoechst 33258 staining and the TUNEL method.

Results: Fluorescence microscopy showed strong green fluorescence where the GFP gene had been transferred into muscle. In mice given the HGF gene, HGF in plasma was increased up to fourfold from pretreatment amounts, peaking 6–9 days after electroporation and quickly decreasing within three weeks. Compared with the group without HGF transfer, the percentage of apoptotic hepatocytes after CCl₄ intoxication was significantly lower, as was ALT activity. In addition, ALT activity normalised more rapidly in the HGF gene transfer group.

Conclusions: Naked DNA injection and transfer by electroporation efficiently brings about HGF expression in vivo, which can attenuate acute liver injury.

See end of article for authors' affiliations

Correspondence to:
Dr T Takahara, Third
Department of Internal
Medicine, Toyama Medical
and Pharmaceutical
University, 2630 Sugitani,
Toyama, 930-0194 Japan;
terutaka-ty@umin.ac.jp

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Hepatocyte growth factor (HGF), first identified as a potent mitogen for mature hepatocytes,^{1,2} has been reported to have multiple biological properties, including mitogenic, morphogenic,^{3–5} and antiapoptotic activity in a wide variety of cells.^{6,7} HGF plays an important role in liver regeneration.^{8–10} Several trials of HGF given to minimise experimental hepatocellular injury in vivo and in vitro^{11–13} have shown an effect against hepatic inflammation although the exact underlying mechanism remains unknown. Recently, HGF gene therapy was tested in a rat model of liver fibrosis where it attenuated liver fibrosis.^{14–16}

A number of transfection methods and vector systems have been used for various in vivo studies such as those above but all have their respective limitations. For example, while retroviral vectors have been used to transfect the liver in rats with partial hepatectomy,¹⁷ transfection efficiency remains relatively low.^{18–20} Adenoviral vectors appear more efficient for transfection of hepatocytes in vivo but these vectors can cause cytotoxicity because of the high immunogenicity of adenoviral proteins.^{21–23} On the other hand, naked DNA injection appears attractive from a clinical point of view, the procedure is simple, and no viral vector is necessary. Moreover, stable expression of a transfected gene can be attained in skeletal muscle cells due to their postmitotic nature and longevity, although the transfected DNA does not usually undergo chromosomal integration. Unfortunately, relatively low expression limits the application of naked DNA injection. Recently however naked plasmid DNA injected into the murine tail vein achieved high levels of foreign gene expression in vivo but rapid injection of a large volume of physiological saline as vehicle was required.²⁴ In experimental interleukin 5 gene transfer into

muscle by in vivo electroporation, we have achieved high expression in vivo.²⁵ Among non-viral techniques for gene transfer, our method is simple, inexpensive, safe, and effective. In the present study, we used this method to transfer the HGF gene into skeletal muscle in vivo and then determined its protective effect against carbon tetrachloride (CCl₄) induced acute liver injury in mice.

MATERIALS AND METHODS

Plasmid DNA

Plasmid pKSCX-HGF was constructed by inserting the full length cDNA of rat HGF, representing about 2.6 kb between the XbaI and BamHI sites, including a chicken β-actin promoter and a 3'-rabbit β-globin poly A sequence. Plasmids were grown in *Escherichia coli* JM109 strain and extracted by an Endofree Plasmid Maxi kit (Qiagen, Germany). DNA was dissolved in Endofree TE buffer, and its quantity and quality were assessed by measuring optical density at 260 and 280 nm.

Green fluorescent protein (GFP) plasmids were obtained commercially (Wako, Japan).

Abbreviations: HGF, hepatocyte growth factor; GFP, green fluorescence protein; ALT, alanine aminotransferase; ELISA, enzyme linked immunosorbent assay; CCl₄, carbon tetrachloride; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT mediated dUTP-X nick end labelling; ABC, avidin-biotin complex; DAB, diaminobenzidine; PBS, phosphate buffered saline; PCNA, proliferative cell nuclear antigen.

Animals

Eight week old female C57BL/6J mice free of specific pathogens were used throughout the experiments. Mice were maintained in the animal centre at the Toyama Medical and Pharmaceutical University according to animal care guidelines. The HGF gene was transferred into muscle by electroporation according to our previous procedure.²⁵ Briefly, 50 µg of plasmid DNA were injected into the tibialis anterior muscle of mice. The muscle was held by an electrode (a pair of stainless steel pincers of 10 mm in diameter), and electric pulses were delivered to the muscle by pulse generator (Square Electroporator CUY 21; Tokiwascience, Japan). The pulse was a square wave at 25 V throughout its duration. Three pulses of the indicated voltage followed by three more pulses of opposite polarity were administered at each injection site at a rate of one pulse/second, with a pulse duration of 100 ms.

To determine the efficacy of HGF gene transfer, sequential plasma samples were obtained from 10 mice on days 4, 6, 9, 14, and 21 after electroporation. GFP plasmid was also transferred to the tibialis anterior muscle in three mice as a positive control for successful electroporation. These muscles and several HGF electroporated muscles were removed and frozen at -80°C.

To induce acute liver injury, 15 mice were given a single dose (25 ml/kg) of a 5% (v/v) mixture of CCl₄ in olive oil through an intragastric tube at four days after HGF gene electroporation. Mice were killed on days 1, 2, 3, and 5 after CCl₄ intoxication, and plasma and livers were collected. As controls, another 15 mice electroporated with only phosphate buffered saline (PBS) were similarly exposed to CCl₄ intoxication.

Efficacy of electroporation

To confirm the efficacy of gene transfer by electroporation, we cut fresh frozen muscle treated with GFP plasmid into 10 µm thick sections with a cryostat, and then directly examined the cryosections by fluorescence microscopy.

Measurement of HGF in plasma

To determine the concentration of HGF at various time points after electroporation, we used an enzyme linked immunosorbent assay (ELISA) kit for rat HGF (Institute of Immunology, Tokyo, Japan) including an antibody that cross reacts with mouse HGF. Values are expressed in ng/ml.

Histological examination

Formalin fixed tissue samples were embedded in paraffin, and 3 µm thick sections were used. The sections were stained with haematoxylin and eosin for routine histological study, Hoechst 33258 for confirming chromatin condensation, terminal deoxynucleotidyl transferase (TdT) mediated dUTP-X nick end labelling (TUNEL) assay kit (see later), and anti-proliferative cell nuclear antigen (PCNA) antibody.

For inspection of chromatin condensation, sections were stained with 8 µg/ml Hoechst 33258 in H₂O, mounted in glycerol, and observed by fluorescence microscopy. Stained nuclei were scored according to the condensation and staining characteristics of the chromatin. Cells with condensed or fragmented chromatin were considered apoptotic.^{26, 27}

TUNEL assay

Apoptotic hepatocytes were labelled in situ using a TUNEL peroxidase apoptosis detection kit (ApopTag Plus; Intergen Inc., USA) according to the manufacturer's protocol. Briefly, 3 µm thick deparaffinised sections were pretreated with proteinase K (20 µg/ml) for 15 minutes at room temperature. After washing in PBS, the endogenous peroxidase was inactivated in 3% H₂O₂ for five minutes, followed by incubation with TdT enzyme at 37°C for one hour. After this incubation, sections were incubated with antidigoxigenin conjugate at room temperature for 30 minutes followed by incubation with

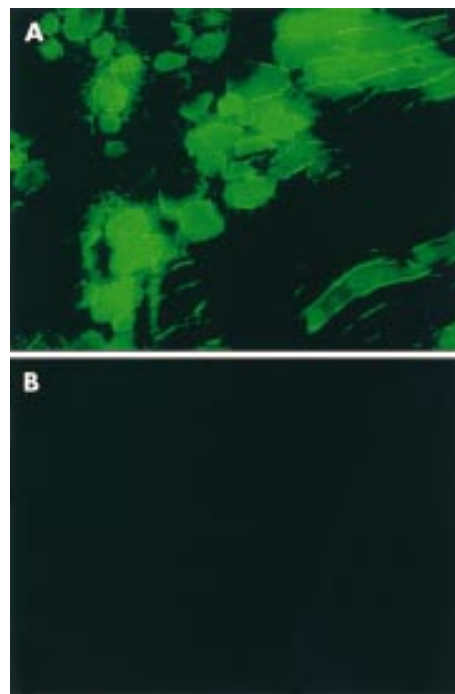


Figure 1 Efficacy of gene transfer and expression by electroporation. Mice tibialis anterior muscle section was obtained seven days after electroporation with green fluorescence protein (GFP) plasmid. Homogeneous fluorescence was seen in the treated muscular fibres (A) but not in the control treated with phosphate buffered saline alone (B) (fluorescence microscopy, original magnification $\times 200$).

diaminobenzidine (DAB) solution. Apoptotic cells were analysed using NIH Image software version 6.1 (NIH Research Service Branch), excluding vascular areas. For each slide, we randomly counted three sections, each with 4.4×10^5 pixels of area, using a light microscope at a magnification of $\times 100$. Hepatocytes showing sharply condensed chromatin masses, crescents in the nucleus, or nuclear fragments were counted as apoptotic cells.

Immunohistochemistry of PCNA

Briefly, 3 µm thick deparaffinised sections were first inactivated by endogenous peroxidase in 3% H₂O₂ for five minutes followed by incubation with normal sera. The sections were incubated with polyclonal anti-rabbit PCNA antibody (Dako, Denmark; 1:1000 final dilution) at 4°C overnight. After washing with PBS, the sections were treated according to the avidin-biotin complex (ABC) method using Vectastain ABC kit (Vector Laboratories, Burlingame, California, USA). Peroxidase activity was determined by DAB solution. PCNA positive nuclei in hepatocytes were counted and expressed as the percentage of the total number of hepatocyte nuclei. The index was analysed by NIH Image 1.61 software (NIH Research Service Branch). For each slide, three areas were randomly counted at a magnification of $\times 100$.

Measurement of ALT

To evaluate the degree of liver injury after CCl₄ intoxication, plasma activity of alanine aminotransferase (ALT), a representative cytosolic enzyme of hepatocytes, was determined by a dry chemical method (Reflotron, Roche Inc.) according to the manufacturer's instructions using a reading machine (Reflotron, Roche Inc.). Normal values in this assay are less than 40 U/l.

Statistical evaluation

Data are expressed as mean (SD). The significance of differences between groups was evaluated by the Student's *t*

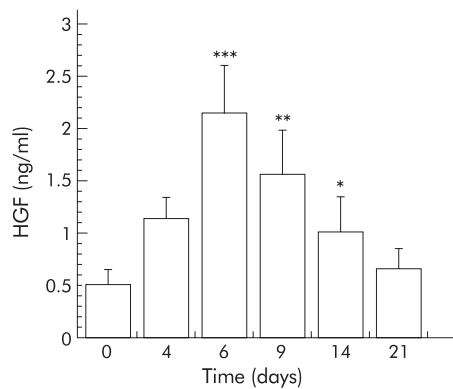


Figure 2 Plasma levels of hepatocyte growth factor (HGF) at various time points. Blood samples were collected on days 4, 6, 9, 14, and 21 after electroporation with pKSCX-HGF plasmid and were detected by polyclonal anti-rat HGF ELISA. Data are expressed as mean (SD), $n=10$. * $p<0.01$, ** $p<0.005$, *** $p<0.001$ versus control.

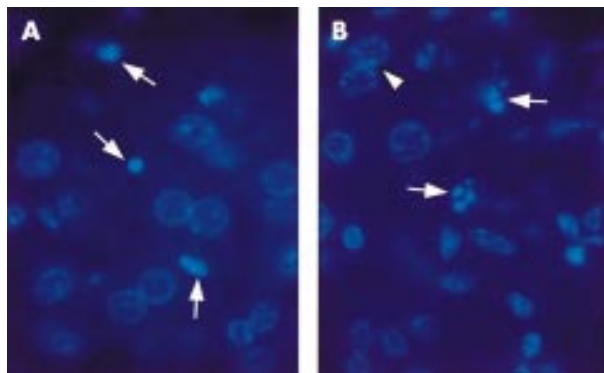


Figure 3 Nuclear morphology of hepatocytes two days after carbon tetrachloride intoxication. Deparaffinised section was stained with 8 $\mu\text{g/ml}$ Hoechst 33258. Apoptotic nuclei were detected as condensed nuclei (A), DNA breaks (B, arrows), or marginally located at the nuclear membrane (B, arrowhead) (fluorescence microscopy, original magnification $\times 1000$).

test. A level of $p<0.05$ was considered to indicate statistical significance.

RESULTS

Green fluorescent protein (GFP) expression in vivo

To confirm the efficacy of gene expression by electroporation, we used a GFP plasmid as a positive control. Strong green fluorescence was detected under the fluorescence microscope in muscle fibres to which GFP plasmid had been transferred seven days before. Negative control muscle fibres into which only PBS was electroporated showed no fluorescence (fig 1).

Sequential HGF concentrations in plasma

After a single electroporation of pKSCX-HGF plasmid, a substantial increase in HGF was detected by ELISA in the plasma of mice for three weeks. Figure 2 illustrates plasma concentrations of HGF, which were 0.52 ng/ml before electroporation. These began to increase on day 4 and peaked on days 6–9 at 2.1 ng/ml, four times the baseline amount. HGF then began to decrease, decreasing to essentially normal concentrations by three weeks.

Morphological changes in CCl_4 intoxicated livers

For inspection of chromatin condensation and fragmentation, liver sections were stained with Hoechst 33258 and observed under fluorescence microscopy. Figure 3 shows fluorescent morphology of hepatic nuclei. In normal hepatocytes, nuclear

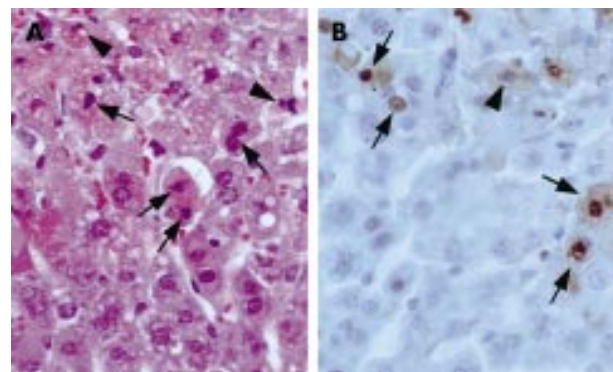


Figure 4 Apoptotic hepatocytes two days after carbon tetrachloride intoxication. Continuous liver sections were stained with haematoxylin and eosin (A) and TUNEL assay (B). (A) Apoptotic hepatocytes were shown adjacent to the central vein and were characterised by chromatin condensation (arrows) or apoptotic bodies (arrowheads). (B) The TUNEL positive nucleus was shown as condensed or DNA breaks (arrows). Necrotic cells exhibited a weakly non-specifically stained nucleus and cytoplasm (arrowhead) (microscopy, original magnification $\times 500$).

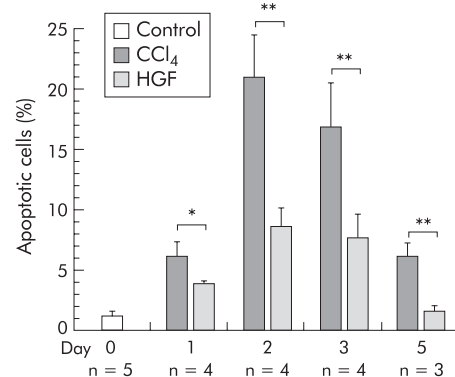


Figure 5 TUNEL positive hepatocytes were evaluated on days 1, 2, 3, and 5 after administration of carbon tetrachloride (CCl_4) in both hepatocyte growth factor (HGF) treated and control groups. TUNEL positive nuclei were counted in three random areas for each section, each with 4.4×10^5 pixels of area. Results are expressed as per cent apoptotic cells of the total number of hepatocytes in these fields. Data are means (SD). * $p<0.01$, ** $p<0.005$ versus control.

chromatin was faintly stained and homogeneously dispersed throughout the whole nucleus. On the other hand, apoptotic nuclei were clearly stained with typical condensed nuclei; condensed chromatin marginally located at the nuclear membrane or nuclear fragments consisting of condensed masses of chromatin were observed (fig 3A, B).

Routine haematoxylin and eosin staining showed both apoptotic and necrotic cells surrounding the centrilobular zone after CCl_4 administration. Morphological criteria were applied to identify apoptotic cells: cell shrinkage, nuclear condensation/crescent formation, and apoptotic bodies (fig 4A).

In addition, the TUNEL assay was used to support the morphological findings (fig 4B). A clearly stained nucleus was detected around the centrilobular zone. Some necrotic cells were noted with non-specific staining of the nucleus and cytoplasm. TUNEL positive cells were detected on day 1 after CCl_4 intoxication, peaking on day 2, and then decreased nearly to normal by day 5. By image analysis, the percentage of apoptotic hepatocytes was much lower in the HGF group, peaking on day 2 at 8.7%, compared with a peak of 21% on day 2 in the control group. On day 5, TUNEL positivity in the HGF group was about 1.7%, essentially the same as in controls without intoxication (1.2%) (fig 5).

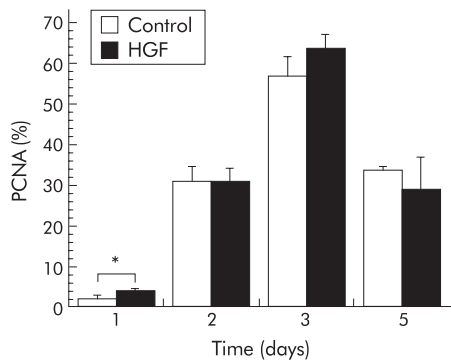


Figure 6 Serial changes in proliferative cell nuclear antigen (PCNA) positive hepatocytes in hepatocyte growth factor (HGF) treated and control groups. PCNA positive nuclei were counted and expressed as a percentage of the total number of hepatocyte nuclei. A significant difference was observed only on day 1 after liver intoxication (* $p < 0.05$).

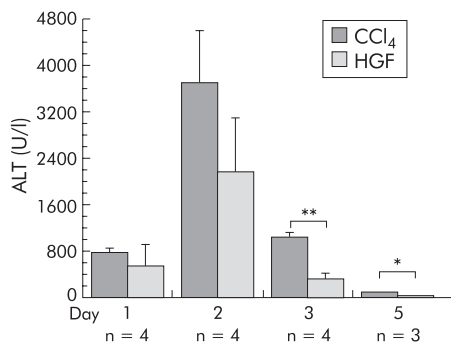


Figure 7 Plasma alanine aminotransferase (ALT) activities at various times after intoxication with carbon tetrachloride (CCl₄). Blood samples were detected by the dry chemical method using test paper. Data are expressed as mean (SD). * $p < 0.01$, ** $p < 0.005$ versus the control group.

Serial changes in PCNA labelling index

To determine hepatic proliferation in response to liver intoxication, immunostaining of PCNA was performed and the labelling index was counted. Figure 6 shows that the PCNA labelling index began to increase in both HGF treated and control groups on day 1 after intoxication (4.1 (0.41)% *v* 2.43 (0.56)%; $p < 0.05$), peaked on day 3 (63.42 (3.59)% *v* 56.7 (4.78)%) and then decreased. These differences were not significant, except for those observed on day 1 ($p < 0.05$).

ALT in plasma

ALT activity was examined sequentially after CCl₄ intoxication. The highest activity was detected on day 2 after intoxication in both HGF transfer and PBS transfer groups, but ALT activity was lower in the HGF group. On days 3 and 5, ALT had decreased markedly, rapidly declining to the normal range (fig 7).

DISCUSSION

HGF is a growth factor which has multiple biological properties with potential therapeutic use in various types of liver injury. Because it has a very short half life ($T_{1/2}$ 3–5 minutes),²⁸ HGF is most likely to be used in the treatment of liver diseases as gene therapy. It is therefore essential to study different gene therapy approaches for delivery of this molecule *in vivo*. Until now, almost all previous studies have used the damaged liver as the target organ to produce substantial HGF.^{12–14} However, from a clinical standpoint, it is theoretically inadvisable to place damaged hepatocytes at risk as targets of

transfection. Thus we investigated skeletal muscle for expression and delivery of the protein into the systemic circulation and to show its actions on the target organ.

In our study, we demonstrated that *in vivo* electroporation of HGF plasmid into skeletal muscle enhanced plasma concentrations of HGF, which peaked at one week to reach 2.1 ng/ml, four times basal amounts. The HGF increase was sustained for nearly three weeks. Electroporated skeletal muscle showed GFP expression or HGF expression after transfer of the respective plasmids. According to our previous reports,²⁵ the efficacy of *in vivo* electroporation was very high at about 100-fold that of simple DNA injection. Moreover, histochemical analysis showed that *in vivo* electroporation increased both the number of muscle fibres taking up plasmid DNA and the copy number of plasmids introduced into the cells. The efficiency of electroporation was equal in regenerating and normal muscle,²⁵ suggesting that the mechanism by which muscle cells take up DNA with electroporation is different from that following simple DNA injection. Unlike viral vectors, there was no significant evidence that *in vivo* electroporation activated cellular or humoral immune responses and neither had it the risk of the insertional mutagenesis that was associated with the use of viral vectors.^{29–31} This method can therefore be applied *in vivo* with the added advantages of simplicity, safety, and lack of toxicity. This may represent a new approach to human gene therapy. Furthermore, it is possible that sustained plasma concentrations may be achievable by repeated transfection.

It has been confirmed in several reports that HGF inhibits DNA fragmentation and apoptosis, which may be one mechanism underlying the cytoprotective effects of HGF *in vivo*.^{32–33} In the present study, we showed that rat HGF significantly protected mouse hepatocytes from apoptosis and necrosis after CCl₄ intoxication; on day 5 the apoptosis percentage had decreased to an essentially normal level, in agreement with previous studies.^{12–34–35} Correspondingly, plasma increases in the cytosolic enzyme ALT caused by CCl₄ intoxication were also suppressed in the HGF group, confirming that rat HGF has a cytoprotective effect on acute liver injury in mice. We first hypothesised that the cytoprotective effects of HGF may be partially due to additional expressed HGF by gene transfer and its strong proliferative activity, even though the CCl₄ intoxication model itself exerted temporary high expression of mouse HGF (data not shown). However, detection of PCNA labelling index in both HGF treated and control groups did not show major differences, except on day 1 when there has a small but significant difference against background of a relatively high index observed in the HGF treated group. These data suggest that other mechanisms are involved in the cytoprotective effect. Okano *et al* implied that mechanisms for HGF cytoprotection were due to prostaglandins E₁ and E₂ via downregulation of tumour necrosis factor α in activated macrophages and also via stabilisation of hepatocyte membranes.¹² Kosai *et al* further suggested that increased plasma HGF after acute liver injury does not simply correlate with biologically active HGF level. It is known that proHGF is activated by HGF activator, which is mainly produced in hepatocytes, and only active HGF has potent antiapoptotic action and can prevent acute liver failure.³⁶ In this respect, pretreatment with HGF may be very important to produce mature HGF, leading to prevention of apoptosis and liver failure.³⁶

It has been reported that overexpressed HGF/Met in transgenic mice can promote tumorigenicity, and because of its profound effects on cell motility, HGF/Met has also been implicated in invasion and metastasis of tumour cells.^{37–39} Recently, Arakaki and colleagues⁴⁰ showed that HGF had an antitumour effect resulting from induction of apoptosis. These contradictory results have been thought to be dose dependent. Transgenic mice in which HGF expression exceeds 50-fold that of normal mice have been found to develop tumours⁴¹

whereas in transgenic mice that expressed HGF at lower levels (20–30-fold), the development of tumours was inhibited.⁴² Other *in vivo* studies of HGF expressed at levels similar to ours (5–10-fold) also found that there was no development of liver tumours.¹⁴ Thus the transgenic animal studies may suggest that HGF with transient expression at levels less than 10-fold normal does not carry the risk of developing tumour. Whether HGF has a mitogenic effect on existing tumour cells *in vivo* needs further consideration in the future.

In conclusion, our study suggests that naked HGF plasmid transfer by electroporation into skeletal muscle may be a practical approach for gene therapy to treat various types of liver damage. HGF gene transfer showed cytoprotective activity in mice with acute liver injury, partially due to prevention of apoptosis.

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Authors' affiliations

F Xue, T Takahara, Y Yata, M Minemura, C Y Morioka, A Watanabe, Third Department of Internal Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan
S Takahara, Department of Urology, Osaka University Medical School, Osaka, Japan
E Yamato, Department of Nutrition and Physiological Chemistry, Osaka University Medical School, Osaka, Japan
K Dono, Department of Surgery and Clinical Oncology, Osaka University Medical School, Osaka, Japan

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