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Abstract

Resection is the only curative treatment for liver metastasis of colorectal cancers. Despite the supreme regenerative potential of the liver, major hepatectomy sometimes leads to liver failure, and the limitation of resectable liver volumes makes advanced tumors inoperable. This study was attempted to promote liver regeneration using hepatocyte growth factor (HGF) gene transfection by venous-administered adenovirus and to improve the survival of rats after massive hepatectomy. The adenovirus that encodes HGF was administered to rats before 85%-hepatectomy. The administration of HGF gene improved the survival of rats after massive hepatectomy, while the administration of control adenovirus deteriorated their survival. Gene transfection of HGF showed up-regulation of serum HGF, stimulation of hepatocellular proliferation and rapid liver regeneration. Moreover, HGF administration reduced apoptosis of hepatocytes. The administration of HGF gene prevented liver dysfunction after major hepatectomy and may be a new assist for surgery.

KEYWORDS: gene therapy, hepatectomy, HGF, adenoviral vector

Effects of Adenoviral-mediated *Hepatocyte Growth Factor* on Liver Regeneration after Massive Hepatectomy in Rats

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Resection is the only curative treatment for liver metastasis of colorectal cancers. Despite the supreme regenerative potential of the liver, major hepatectomy sometimes leads to liver failure, and the limitation of resectable liver volumes makes advanced tumors inoperable. This study was attempted to promote liver regeneration using *hepatocyte growth factor (HGF)* gene transfection by venous-administered adenovirus and to improve the survival of rats after massive hepatectomy. The adenovirus that encodes *HGF* was administered to rats before 85%-hepatectomy. The administration of *HGF* gene improved the survival of rats after massive hepatectomy, while the administration of control adenovirus deteriorated their survival. Gene transfection of *HGF* showed up-regulation of serum HGF, stimulation of hepatocellular proliferation and rapid liver regeneration. Moreover, *HGF* administration reduced apoptosis of hepatocytes. The administration of *HGF* gene prevented liver dysfunction after major hepatectomy and may be a new assist for surgery.

Key words: gene therapy, hepatectomy, HGF, adenoviral vector

Resection is the mainstay of treatments for malignant liver tumors, which depends on the extent of the disease and underlying liver function. But extended hepatectomy has been associated with a high risk of complications, especially postoperative liver failure. Preoperative embolism of the portal vein has been undertaken to increase the remnant liver volumes and reduce complications [1, 2]. Liver transplantation has also been performed for nonresectable tumors, but the procedure has many limitations [3]. The use of a bio-artificial liver as a bridge is one promising experimental approach. And the

promotion of liver regeneration using hepatocyte growth factor (HGF) or other growth factors is another approach that has been tested in several experimental models.

HGF is a well-known growth factor with mitogenic, motogenic, and morphogenic activities for a wide variety of cells and has proven to be the most potent stimulator of cellular growth and DNA synthesis of hepatocytes *in vitro* and *in vivo* [4-7]. HGF is also thought to initiate hepatic regeneration after hepatectomy, to protect cells against acute hepatic injury, and to have an anti-apoptotic effect [8-10]. Due to the rapid clearance of HGF, frequent administration is necessary to maintain effective serum levels. Thus, gene transfection has recently been examined to achieve consistent production of HGF. The

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gene transfection of *HGF* by adenoviral vector or retroviral vector has already been proved to stimulate hepatocyte regeneration after hepatectomy and to protect against liver failure [10–12].

In this study, we examined whether *HGF* gene transfection by adenoviral vector could support liver regeneration and survival of rats after massive hepatectomy.

Materials and Methods

Animal model. The 7-week old Sprague Dawley rats were purchased from Charles River Japan Inc. (Yokohama, Japan) and kept at the Animal Center for Medical Research, Okayama University. Under ether anesthesia, the median, left, right lobes, and caudate process of the caudate lobes were resected as an 85%-partial hepatectomy model. A surgeon was blinded regarding the previous treatments rats had received before the surgical procedure. All experiments were carried out in accordance with the guidelines for Animal Experiments at Okayama University.

Viral production. E1-deleted recombinant adenoviral vector, which encodes rat *HGF* cDNA under chicken beta-actin promoter (Adex1CA rat *HGF*), was supplied by the RIKEN Bio resource Center (Tsukuba, Japan). Large-scale Adex1CA rat *HGF* preparation on 293 cells and purification by two-step ultra-centrifugation on cesium chloride gradients were carried out. Serial dilutions of Adex1CA rat *HGF* were plated on 293 cells, and the titer was evaluated with a plaque assay. The E1/E3-deleted AdLacZ virus, which prepared in the same manner, was used as a negative control. Whole viral experiments were admitted by committee for recombinant DNA experiments at Okayama University and were carried out in P2 facilities in the Department of Radiation Research, Shikata Laboratory, Advanced Science Research Center, Okayama University.

Viral administration. A 1×10^9 pfu (plaque forming units) of Adex1CA rat *HGF* or AdLacZ were administered to rat with 100 μ l of PBS via penile vein (referred to as the *HGF* and LacZ groups, respectively). Control rats were administered 100 μ l of PBS alone (referred to as the Control group). After 48 h, the massive hepatectomy

was performed because adenoviral *HGF* expression started from 12 h and reached its peak at 48 h after viral administration with previous examination *in vitro* (data not shown).

Liver regeneration. Rats were sacrificed at 24, 48, 72, and 168 h following hepatectomy, and liver weight was evaluated. Based on the resected liver weights at the time of surgery and when sacrificed, the % liver weight was calculated as follows. % liver weight (%) = {Actual liver weight (g) \times 0.85 / the resected liver weight (g)} \times 100 (%).

Measurement of serum HGF. Serum *HGF* was quantified with enzyme-linked immunosorbent assay (ELISA) before and at 1, 2, 6, 12, 24, 48, 72, and 168 h following the hepatectomy. The procedures were carried out according to the manufacturer's instructions using anti-rat *HGF* monoclonal antibody Kit (Institute of Immunology, Tokyo, Japan).

Evaluation of liver function. Blood samples were analyzed for total protein (TP), albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), direct bilirubin (D-Bil), ALP, γ -GTP, and ammonia.

Proliferative activity of hepatocytes. Rats were intraperitoneally injected with 0.5 mg/body of 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich-Japan, Tokyo, Japan) diluted in PBS at a concentration of 2 mg/ml and sacrificed 1 h after BrdU administration. The liver was fixed in 4% phosphate-buffered formaldehyde. Paraffin-embedded liver sections were immunostained with monoclonal antibody of BrdU conjugated with peroxidase. The proliferation index was determined by counting stained nucleus in 3 different sections of the same rats.

Evaluation of apoptosis. Apoptotic cells on liver sections were stained with the terminal-deoxynucleotidyl-transferase-mediated deoxyuridine nick-end labeling (TUNEL) methods and detected. A commercially available kit of reagents for the TUNEL methods (ApopTag; Oncor; Gautherborg, MD, USA) was used according to the manufacturer's instruction. The apoptotic index was determined by counting stained nucleus in three different liver sections of the same rats.

Statistical analysis. Survival of rats was determined by the Kaplan-Meier method and compared by the Log-rank test. The difference in experi-

mental parameters and proliferation index among the treatment groups was analyzed using the one-way ANOVA test. For all tests, $p < 0.05$ was considered significant.

Results

Establishment of animal models. A 70% partial hepatectomy model of rats has been established with the removal of median lobe and left lateral lobe [13], and additional resection of right lateral lobe results in $85 \pm 3\%$ partial hepatectomy, based on our preliminary study ($n = 6$). With this model, 66% rats of the Control group died within 1 week of hepatectomy (Fig. 1). Dead rats underwent laparotomy and were found to have small and firm livers and ascites in most cases; they were therefore considered to have died of liver failure. Additional resection of the upper half of the caudal lobe created a 90%-resection model, but all rats died within 3 days (data not shown). We used an 85%-partial hepatectomy procedure as a massive hepatectomy model for further examination.

Transfectional rate of hepatocytes. A 1×10^9 pfu of AdLacZ was administered intravenously, and liver tissue was stained with X-gal at

48 h after viral administration. Nearly 80% of hepatocytes showed expression (data not shown).

Survival of rats. In the Control group ($n = 18$), which was administered only PBS, 66% of rats died within a week (Fig. 1). But all rats that were administered AdLacZ ($n = 6$) died within 4 days. In the HGF group ($n = 18$), 72% of rats survived for a week and the survival rate was significantly higher than the Control and LacZ groups. The deterioration of survival in the LacZ group was considered to be due to the toxicity of adenovirus toward liver. We used the HGF and Control groups for further examination, because the aim of our study was to prove that the benefits of HGF could overwhelm the toxic effect of adenovirus.

Regeneration of liver. All survival rats were sacrificed on the 7th day following hepatectomy, and the remnant liver weight was examined. The liver weights of each group were 6.6 ± 0.6 grams in the Control group and 7.9 ± 0.5 grams in the HGF group, which were 63.4% and 75.5% of estimated whole liver weight at the time of surgery, respectively (Fig. 2). There was no data for the LacZ group because no rats in the LacZ group survived for 7 days. To clarify the liver regeneration in the early stage, rats in each group were sacrificed at 24, 48, and 72 h

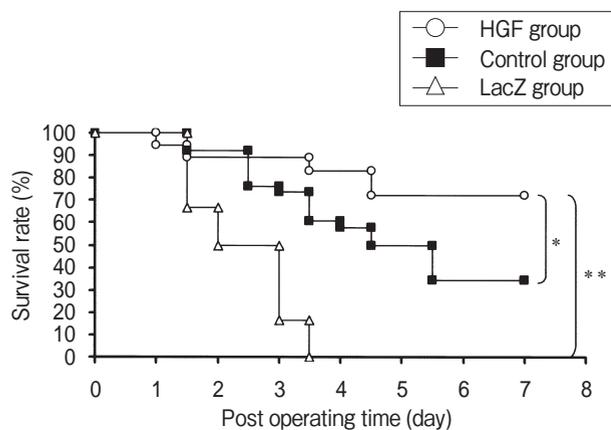


Fig. 1 Survival curve of rats after hepatectomy in Control, LacZ, and HGF groups. The HGF group ($n = 18$) had a significantly better survival rate than the Control group ($n = 18$). On the other hand, all rats of the LacZ group were dead within 4 days ($n = 6$). *: $p < 0.05$, **: $p < 0.01$.

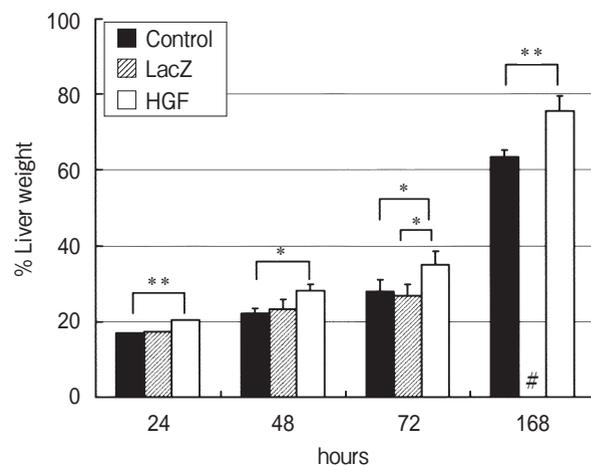


Fig. 2 Liver weight after massive hepatectomy. The estimated liver weight at the time of surgery is indicated as 100%, and the % liver weights at each time point are shown ($n = 6$; except $n = 13$ for HGF group at 168 h). Values are expressed as the mean \pm SD. *: $p < 0.05$, **: $p < 0.01$. #: no data at 168 h of the LacZ group.

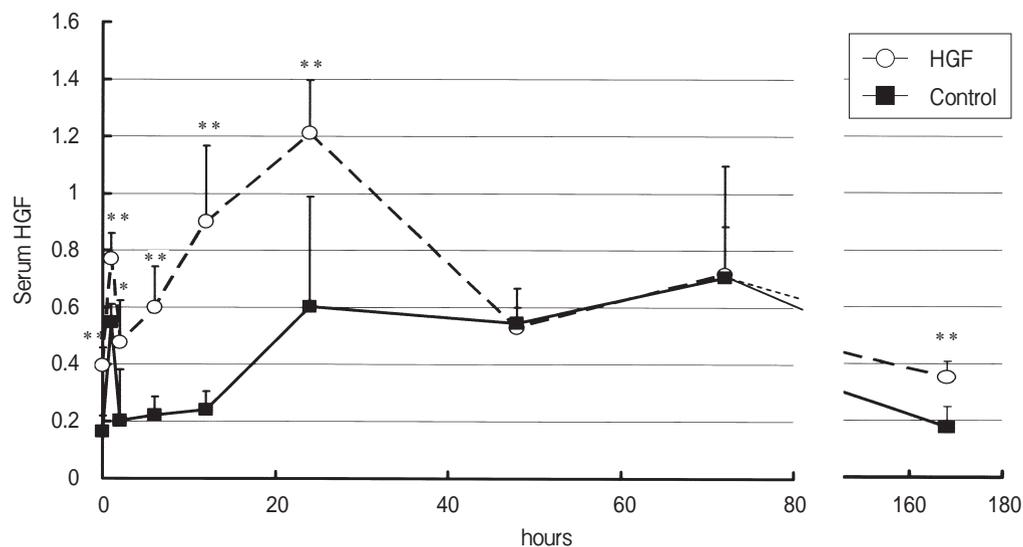


Fig. 3 Serum HGF levels at 0, 1, 2, 6, 12, 24, 48, 72, and 168 h following hepatectomy in Control and HGF groups ($n = 5$). Serum HGF levels were significantly higher in the HGF group from 0 to 24 h following the surgery. Each point represents the mean \pm SD. *: $p < 0.05$, **: $p < 0.01$.

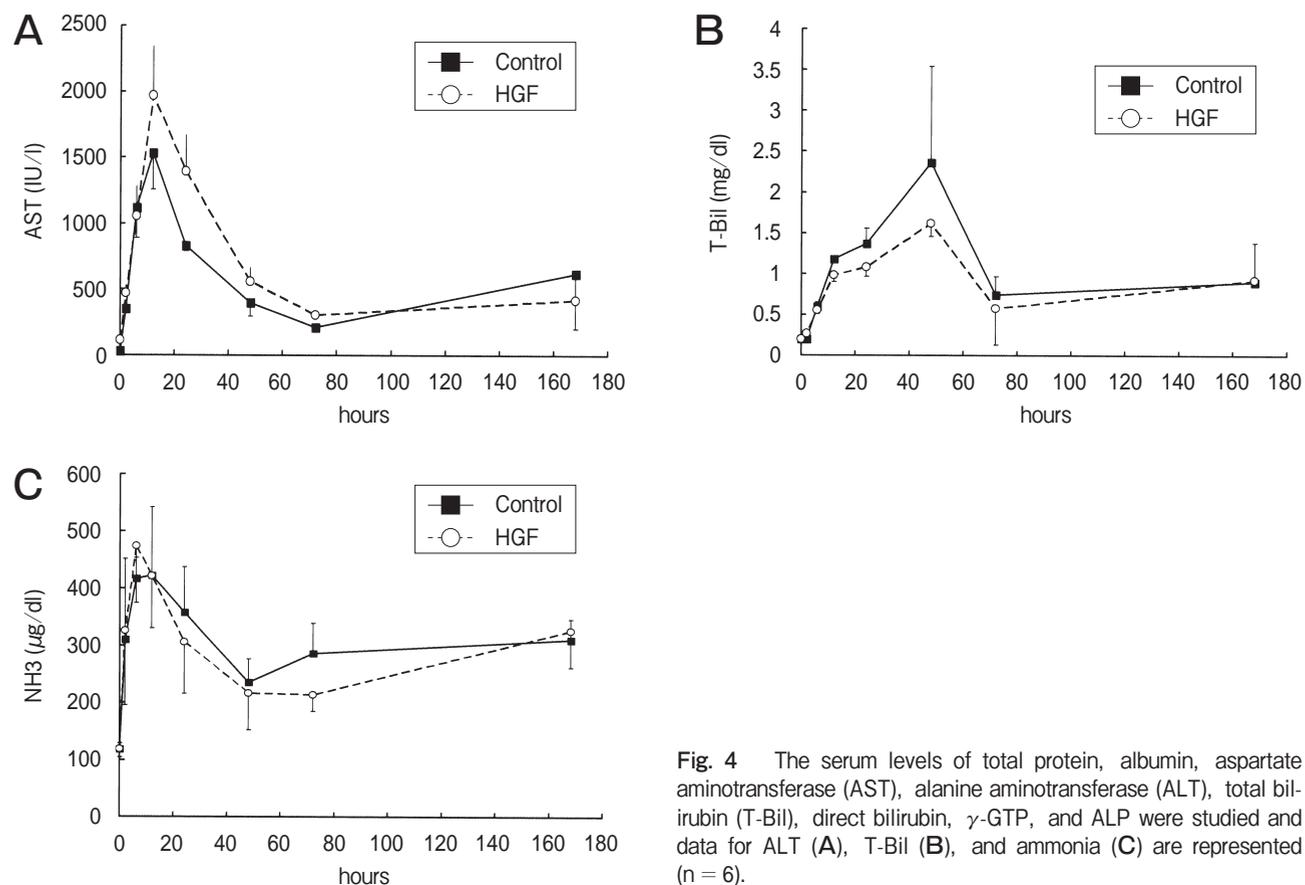


Fig. 4 The serum levels of total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-Bil), direct bilirubin, γ -GTP, and ALP were studied and data for ALT (A), T-Bil (B), and ammonia (C) are represented ($n = 6$).

following hepatectomy, and their livers were weighed ($n = 6$). The HGF group showed significantly higher liver regeneration than the Control group from 24 to 168 h after surgery. The liver weight of the LacZ group at 72 h was 26.8% and was almost the same as that of the Control group, which was 27.9%.

Expression of HGF. HGF was found to be rapidly up-regulated by the stimulation of hepatectomy, but temporally decreased between 2 and 6 h in both groups (Fig. 3), and this phenomenon is considered to be due to the early consumption of HGF [6]. In the Control group, HGF gradually increased again from 12 h and peaked at 72 h after hepatectomy. In contrast, rats of the HGF group had HGF levels more than twice as high at the time of surgery as those of the Control group and showed an earlier re-increase in serum HGF, which peaked at 24 h after hepatectomy. HGF levels became the same in both groups after 48 h following surgery.

Liver function after hepatectomy. The serum levels of total protein, albumin, AST, ALT, total bilirubin, direct bilirubin, γ -GTP, and ALP were studied. The liver functions of both groups were significantly worse than those presurgery, even at 168 h after surgery, but there was no significant difference between the Control and HGF groups (Fig. 4). The absence of difference between these groups may be due to the bias of data collection, because only survival rats in both groups were used for analysis.

Proliferation and apoptosis in remnant liver. To clarify the mechanisms of earlier liver regeneration in the HGF group, we evaluated proliferation and apoptosis of the liver. The proliferation rates of hepatocytes in the Control and HGF groups were determined by BrdU incorporation and were evaluated histologically (Fig. 5A, B). The proliferating indexes were gradually increased in both

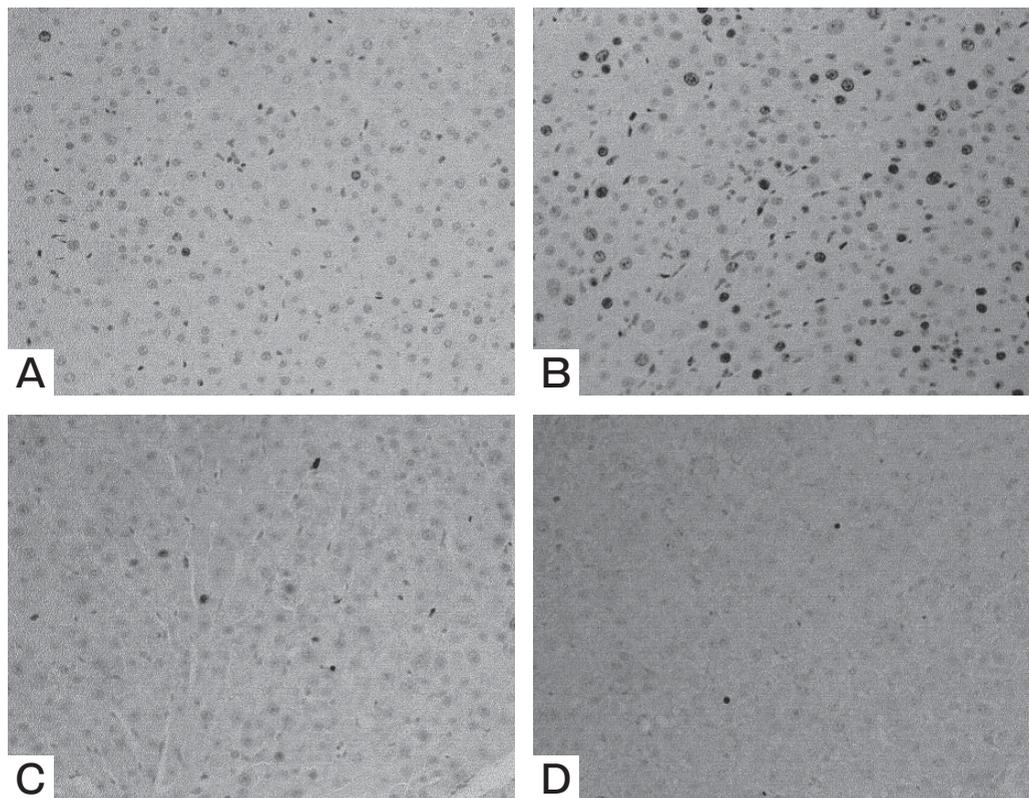


Fig. 5 Paraffin-embedded liver sections with BrdU immunostaining and TUNEL staining. The Control (A) and HGF groups (B) at 72 h for BrdU staining, and the Control (C) and HGF groups (D) for TUNEL staining at 72 h ($\times 200$).

groups but were significantly higher in the HGF than the Control group from 24 to 72 h following massive hepatectomy (Table 1). Apoptotic cells were found in both the Control and HGF groups (Fig. 5C, D), but the number of apoptotic cells in the HGF group decreased significantly in comparison with the Control group (Table 2).

Discussion

In an effort to prevent liver failure after hepatectomy, various therapies have been proposed. Portal vein embolization and liver transplantation have been adapted to nonresectable tumors, but their indication is still limited. Attempts to stimulate the proliferation of residual viable hepatocytes after hepatectomy have been studied [6-8, 14-17]. Hepatectomy and liver injury trigger hepatocyte proliferation. The onset of mitosis follows 6-8 h later, reaching a maximum 48 h after surgery [4, 17]. The priming phase after hepatectomy is characterized by the expression of immediate early genes. Among these genes, TNF and IL-6 cytokines work for priming and growth factors; HGF and TGF- α , control cell cycle progression [4]. Thus the previous administration of HGF would effectively promote proliferation of hepatocytes. It has been reported that the injection of HGF is able to induce liver proliferation even in normal rats without liver injury or hepatectomy [17].

This study showed that gene transfection of *HGF*

stimulates liver regeneration *in vivo*, which improves rat survival from massive hepatectomy. The HGF group showed significantly higher liver regeneration than the Control group at all times. Our data may still underestimate the effects of HGF upon liver regeneration because only surviving rats were analyzed and dead rats might have lower liver regeneration. This problem may also account for the similarity of liver function tests between the Control and HGF groups. With previous *in vitro* examinations, the expression of HGF delivered by adenoviral vectors has been started 12 h after viral administration and has reached a peak at 48 h; we therefore administered adenovirus 48 h preceding hepatectomy. The HGF group had HGF levels more than twice as high the time of surgery than the Control group, and overexpression of HGF at the point of hepatectomy may promote early proliferation of hepatocytes and improve rat survival. We found no significant difference in serum HGF levels between surviving rats and dead ones in the HGF group at the preoperative time point. Because the overexpression of HGF was shown in all rats in the HGF group, the gene transfection rate may not explain the nonsurvival rats in the HGF group.

In our model, the single injection of adenoviral *HGF* maintained higher serum levels of HGF for at least 3 days; virus was administered 48 h preceding surgery, and HGF was up-regulated until 24 h after hepatectomy. The previous paper suggested that

Table 1 Proliferation index

Post operative time (hour)	1	24	48	72
Control group (%)	1.5 \pm 0.2	3.3 \pm 0.2	7.4 \pm 0.2	28.4 \pm 2.7
HGF group (%)	1.7 \pm 0.3 n.s.	6.0 \pm 0.8**	8.5 \pm 1.6*	36.8 \pm 3.8**

Proliferation index were determined by BrdU labeling of nucleus after massive hepatectomy in the Control and HGF groups (n = 6). n.s.: not significant, *: $p < 0.05$, **: $p < 0.01$.

Table 2 Apoptotic index

Post operative time (hour)	1	24	48	72
Control group (%)	2.6 \pm 1.5	1.9 \pm 0.1	1.5 \pm 0.1	2.8 \pm 0.2
HGF group (%)	1.7 \pm 0.2 n.s.	1.1 \pm 0.3**	0.5 \pm 0.2**	1.2 \pm 0.4**

Apoptotic index of hepatocytes after massive hepatectomy in the Control and HGF groups (n = 6). n.s.: not significant, **: $p < 0.01$.

after partial hepatectomy, the first phase from 0 to 3 h is the consumption phase, in which HGF decreases, while the second phase at over 6 h is a productive phase, in which HGF reappears [6]. In our model, a sharp drop of serum HGF was seen 2 h after hepatectomy. These phenomena were also observed in clinical patients after hepatectomy; the maximum value of serum HGF was found the next day after surgery, while serum interleukin-6, the other early parameter of liver dysfunction, reached its maximum on day 0 [18, 19]. Thus the previous administration of *HGF* gene may also be beneficial to clinical patients. But the expression of genes administered by adenovirus may be different *in vitro* and *in vivo*. The expression of HGF peaked at 48 h after viral administration in an *in vitro* study, but serum HGF in the HGF group reached the highest point at 24 h after hepatectomy, 72 h after viral administration. These findings indicate that expression of HGF from transfected HGF may also be stimulated by hepatectomy.

The HGF group showed significantly higher liver regeneration until the 7th day after surgery, while HGF levels became almost the same as those of the Control group at 48 h. Because HGF is the most potent stimulator of cell growth and DNA synthesis of hepatocytes, higher HGF in the early phase of hepatectomy may promote hepatic regeneration and result in higher liver weight at 7 days. The cell protection and anti-apoptotic effects of HGF may also be the reason for higher liver weight. We also showed that HGF has cytoprotective effects. The number of apoptotic cells in the HGF group significantly decreased in comparison with the Control group. The protective activity against hepatic injury has been demonstrated in the fulminant liver failure model induced by endotoxin [9]. HGF has also been proved to suppress caspase-3, which is the key factor to induce apoptosis [11].

One of the obstacles to clinical use of adenoviral vector is its toxicity. The LacZ group in this study showed deterioration of survival compared with the Control group, though the live regeneration rate was the same as that of the Control group. The adenoviral infection, even nonreplicating virus, is known to have toxicity to hepatocytes because of the production of viral proteins and induction of immunological reaction [20]. Because of the high affinity of adeno-

virus to hepatocytes, venous viral administration showed dominant infection into hepatocytes as well as hepatocellular cytotoxicity [21, 22]. There are several previously reported alternative methods to using viral vectors. There is a report that intramuscular induction of naked HGF plasmids by electrophoretion stimulates liver proliferation and functional recovery regeneration in cirrhotic liver following partial hepatectomy [23]. The recombinant HGF protein has preventive effects on liver injury, but a large amount of HGF is required because of the rapid clearance of HGF from blood circulation [12, 24]; the half-life of HGF in plasma is considered to be less than several minutes. The controlled release of HGF by biodegradable gelatin hydrogels has recently been reported to maintain serum HGF at sufficient levels [25], but the cost of recombinant HGF could be a problem. The use of helper-dependent adenoviral vector is another solution to reducing toxicity without losing the effectiveness of gene delivery to hepatocytes and the ease of manipulating viral vectors [26, 27]. Those vectors are completely devoid of viral coding sequences and are able to sustain high-level transgene expression with negligible chronic toxicity. Many experimental studies comparing these techniques would be required before adapting HGF treatments in clinics.

There have been reports that HGF inhibits the proliferation of tumor cell growth and that adenoviral gene transduction of *HGF* has inhibitory effects against hepatoma [28, 29]. But there also have been reports that HGF might be implicated in HCC growth and may stimulate colorectal or lung adenocarcinomas [30-32]. Though the effects of HGF on cancer cell growth are still controversial, the correlation between HGF and tumor growth must be clarified before adapting these techniques to clinics because most hepatectomies are performed for liver malignancies.

In summary, use of a preceding gene transfection of *HGF* stimulates liver regeneration and improves animal survival after massive hepatectomy. Upregulation of HGF stimulates cell proliferation and suppresses apoptosis of remnant liver. The difficulty of clinical use of recombinant HGF, adenoviral *HGF* gene transfection, could provide an alternative technique for clinical application to support liver function after hepatectomy.

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