

# CCl<sub>4</sub>-induced acute liver injury in mice is inhibited by hepatocyte growth factor overexpression but stimulated by NK2 overexpression

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**Abstract** Hepatocyte growth factor (HGF) inhibits acute liver injury. NK2 acts as an antagonist to HGF *in vitro*, but its *in vivo* function has reached no consensus conclusions. We have investigated *in vivo* effects of HGF and NK2 on CCl<sub>4</sub>-induced acute liver injury. Elevation of the serum alanine aminotransferase level and extension of centrilobular necrosis were inhibited in HGF transgenic mice but were promoted in NK2 transgenic mice. Hepatocyte proliferation after liver injury was not inhibited in NK2 transgenic mice. Thus, this study indicates that HGF inhibits liver injury, and NK2 antagonizes HGF on liver injury, however, NK2 may not antagonize HGF on hepatocyte proliferation.

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**Key words:** Hepatocyte growth factor; NK2; Transgenic mouse; Carbon tetrachloride; Liver injury; Hepatocyte proliferation

## 1. Introduction

Hepatocyte growth factor (HGF) was purified from the plasma of a patient with fulminant hepatic failure [1] and is now known as a pleiotropic cytokine that stimulates mitogenesis, motogenesis, and morphogenesis [2]. The effects of HGF are mediated through the receptor, c-Met [3]. HGF reportedly prevents acute hepatic injury caused by a variety of hepatotoxins including carbon tetrachloride (CCl<sub>4</sub>) [4–10].

Structurally, HGF consists of an  $\alpha$ -subunit of 60 kDa containing an N domain and four kringle domains and a  $\beta$ -subunit of 30 kDa [11]. Naturally, HGF mRNA can undergo alternative splicing to create truncated isoforms, NK1 (consisting of an N domain and the first kringle domain of HGF) and NK2 (consisting of an N domain and the first two kringle domains of HGF) [12,13]. These two isoforms can bind to c-Met with relatively high affinity and both were initially characterized as an HGF antagonist [13–15]. However, the biolog-

ical activity of NK1 was later reported to stimulate mitogenesis, motogenesis, and morphogenesis [16,17]. On the other hand, NK2 has been reported to act as an agonist of mitogenic activity [18,19].

In HGF transgenic mice, using a mouse metallothionein (MT)-1 promoter and associated locus control regions, HGF overexpression led to stimulation of hepatic proliferation and regeneration [20]. Recently, it was reported that NK1 transgenic mice showed phenotypes almost similar to those of HGF transgenic mice [12]. In NK2 transgenic mice, using the same expression construct as HGF and NK1 transgenic mice, there were no overt phenotypic abnormalities. However, in btransgenic mice harboring both HGF and NK2 transgenes, NK2 overexpression inhibited phenotypic abnormalities induced by overexpression of HGF, including liver enlargement and accelerated hepatocyte proliferation [23]. In contrast, when melanoma cells, which exhibited very high c-Met expression and predominantly metastasized to the liver [24], were introduced intravenously into the tail vein of NK2 transgenic mice, NK2 overexpression stimulated the metastatic efficiency of these cells [23]. These *in vivo* data indicate that NK2 acts as an antagonist of HGF in mitogenesis but an agonist of HGF in respect of metastatic activity.

To obtain more direct evidence of the preventive effect of HGF and to elucidate the role of NK2 in CCl<sub>4</sub>-induced acute liver injury, we exploited HGF and NK2 transgenic mice [23].

## 2. Materials and methods

### 2.1. Animals

HGF and NK2 transgenic mice were generated on an albino FVB genetic background as previously described [21,23]. Expressions of the mouse HGF cDNA and the human NK2 cDNA were placed under the control of the mouse MT-1 promoter and associated locus control regions as previously [25]. All studies were performed using only female mice and mice were maintained in compliance with the guidelines for animal care and use established by Gunma University School of Medicine.

### 2.2. Assessment of liver injury

Wild-type (WT) and transgenic mice were maintained on 25 mM zinc sulfate (ZnSO<sub>4</sub>) in their drinking water, starting 4 weeks after birth, to enhance transgene expressions. WT mice were littermates of transgenic mice. Mice were injected intraperitoneally with 0.25 ml/kg CCl<sub>4</sub> (Kanto Chemistry, Tokyo, Japan) dissolved in olive oil (Kanto Chemistry, Tokyo, Japan) at 6 weeks of age and were sacrificed, and their sera and livers were collected at specified time points. Serum alanine aminotransferase (ALT) levels were measured at each point, and liver tissues were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin–eosin. At a magnification of  $\times 40$ , 30 areas of centrilobular necrosis and the cross section of central vein in the

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**Abbreviations:** HGF, hepatocyte growth factor; CCl<sub>4</sub>, carbon tetrachloride; MT, metallothionein; WT, wild-type; ZnSO<sub>4</sub>, zinc sulfate; ALT, alanine aminotransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; ANOVA, analysis of variance

necrosis were measured using a software, NIH Image 1.62, provided by the National Institute of Health (MD, USA) for each group. Each area is indicated by a Pixels number. The index of centrilobular necrosis was scored as follows: the area of centrilobular necrosis divided by the area of cross section of the central vein in the necrosis.

### 2.3. Northern blot analysis

Total RNAs from liver tissues were prepared using TRIzol (Gibco BRL, Tokyo, Japan) according to the manufacturer's instructions, and 20 µg of total RNA was loaded per lane onto a 1% agarose-formaldehyde gel and transferred to a nylon membrane (Amersham Pharmacia Biotech, Tokyo, Japan). Transcripts of transgenes and endogenous HGF were detected with a 2.2-kbp mouse HGF cDNA probe as previously described [22]. Transcripts of c-met were detected with a 1.5-kbp mouse c-met cDNA probe as previously described [22]. MT transcripts were detected with a 355-bp mouse MT-1 cDNA probe (generously provided by Richard Palmiter, University of Washington, Seattle, WA, USA). The membrane was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, and RNA levels of transgenes and endogenous c-met and MT were quantified by densitometric analysis related to GAPDH levels.

### 2.4. Immunohistochemistry

Liver tissues were removed on days 0 and 2 for proliferating cell nuclear antigen (PCNA) staining using monoclonal mouse anti-PCNA antibody (PC-10; Dako Japan, Tokyo, Japan). The labeled hepatocyte nuclei were scored by counting 30 high-power light microscope fields ( $\times 1000$ ) for each group.

### 2.5. Statistical analysis

All experimental data are shown as means  $\pm$  S.D. Differences in serum ALT levels were determined by two-way factorial analysis of variance (ANOVA) for each group. Differences in the index of centrilobular necrosis and PCNA-labeled hepatocytes were determined by one-way ANOVA and the Fisher test as a post-hoc test for each group. The level of significance for all statistical analyses was set at  $P < 0.01$ .

## 3. Results

### 3.1. Liver injury after injection of CCl<sub>4</sub>

As shown in Fig. 1, serum ALT levels in NK2 transgenic mice were approximately twice and three times as high as those in WT mice on days 1 and 2, respectively. In contrast, the serum ALT levels in HGF transgenic mice were much lower than those in WT mice on days 1 and 2. Histological examination revealed that CCl<sub>4</sub> administration induced centrilobular necrosis with ballooning degeneration of hepatocytes on day 2 in the WT mice (Fig. 2A,B). The extent of liver injury in the NK2 transgenic liver was larger than that in WT mice (Fig. 2C,D). The indices of centrilobular necrosis in NK2 transgenic mice and WT mice were  $30.1 \pm 16.2$  and  $25.2 \pm 9.60$ , respectively ( $P = 0.10$ ). In contrast, the hepatic damage was much less severe in HGF transgenic mice than that in WT mice on day 2 (Fig. 2E,F). The index in HGF transgenic mice was significantly decreased (HGF,  $9.59 \pm 5.3$ ; WT,  $30.1 \pm 16.2$ ,  $P < 0.01$ ). In all mice, the damage had resolved by day 7 (data not shown).

### 3.2. Expression of transgenes and endogenous HGF, c-met and MT in livers following CCl<sub>4</sub> administration

High expression of each transgene was detected at each time point in each transgenic liver (Fig. 3). In particular, higher expression of each transgene was detected on day 1. Because CCl<sub>4</sub> up-regulates the endogenous MT level in the liver suggesting that CCl<sub>4</sub> activates the MT promoter in their constructs [26], we investigated MT expression. However, MT expression was not increased after injection of CCl<sub>4</sub> (Fig. 3).

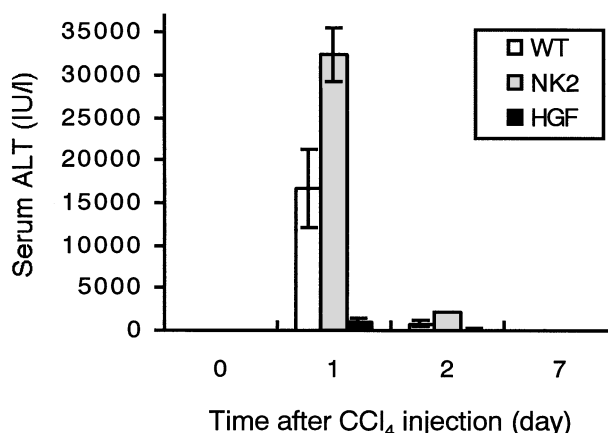


Fig. 1. Serum ALT levels in WT, NK2 transgenic, and HGF transgenic mice on days 0, 1, 2, and 7. CCl<sub>4</sub>-induced acute liver injury was inhibited in HGF transgenic mice but was stimulated in NK2 transgenic mice, as compared to WT mice ( $n = 3-9$  per group). The  $P$  value is  $< 0.01$ .

Endogenous HGF expression increased in WT and NK2 transgenic mice but did not increase in HGF transgenic mice on day 1. On the other hand, endogenous c-met expression decreased in HGF transgenic mice but did not decrease in WT and NK2 transgenic mice on days 1 and 2 (Fig. 3).

### 3.3. Hepatocyte proliferation after CCl<sub>4</sub> administration

PCNA staining was performed to analyze hepatocyte proliferation of each mouse on days 0 and 2 (Fig. 4). The labeling index of NK2 transgenic hepatocytes was similar to that of WT hepatocytes on day 0 (WT,  $2.70 \pm 2.18$ ; NK2,  $2.13 \pm 1.80$ ) and day 2 (WT,  $45.5 \pm 7.60$ ; NK2,  $45.5 \pm 7.13$ ) (Fig. 5). In contrast, the labeling index of HGF transgenic hepatocytes was significantly increased relative to that of WT on day 0 (WT,  $2.70 \pm 2.18$ ; HGF,  $9.70 \pm 4.96$ ,  $P < 0.01$ ). Moreover, the labeling index of HGF transgenic hepatocytes was significantly decreased relative to that of WT on day 2 (WT,  $45.5 \pm 7.60$ ; HGF,  $21.0 \pm 5.46$ ,  $P < 0.01$ ) (Fig. 5).

## 4. Discussion

We investigated effects of HGF and NK2 on CCl<sub>4</sub>-induced acute liver injury using HGF and NK2 transgenic mice. Reportedly, intermittent injection of recombinant HGF and the implantation of HGF-producing cells into the spleen protect against CCl<sub>4</sub>-induced acute liver injury [4,8]. However, there has been no report which describes continuous HGF overexpression in vivo. We exploited HGF transgenic mice to provide more direct evidence that HGF protects from CCl<sub>4</sub>-induced acute liver injury in vivo. After CCl<sub>4</sub> administration, serum ALT levels in HGF transgenic mice were dramatically lower than those in WT mice and the degree of the centrilobular necrosis in HGF transgenic mice was remarkably reduced relative to that in WT mice. These results indicate that HGF overexpression protects hepatocytes from damage induced by CCl<sub>4</sub> administration in vivo. In contrast, serum ALT levels in NK2 transgenic mice were significantly higher than those in WT mice and the degree of the centrilobular necrosis in NK2 transgenic mice was more severe than that in WT mice after CCl<sub>4</sub> administration. These data indicate that NK2 overexpression antagonizes the protective effect of endogenous



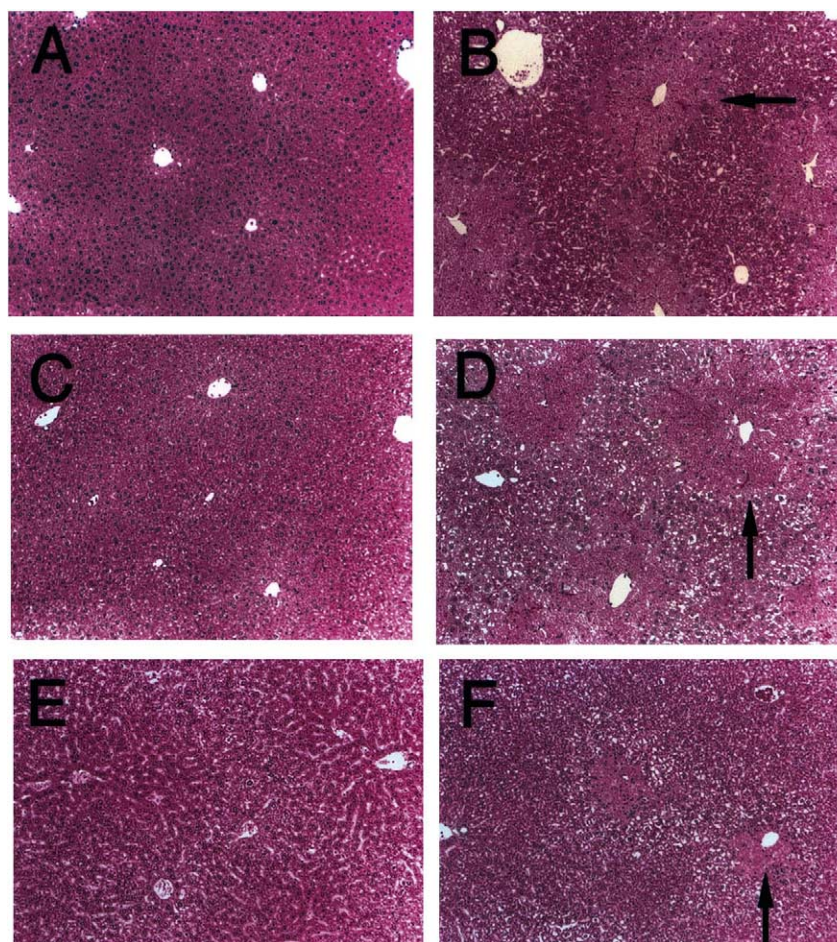


Fig. 2. Histological findings in livers of WT mice (A and B), NK2 transgenic mice (C and D), and HGF transgenic mice (E and F) before  $\text{CCl}_4$  administration (A, C, and E) and 2 days after  $\text{CCl}_4$  administration (B, D, and F). There are no necrotic areas in the livers, in any of the groups, before  $\text{CCl}_4$  administration. On day 2, the degrees of centrilobular necrosis in NK2 transgenic mice are larger than that in WT mice, but the necrosis in HGF transgenic mice is much less severe than that in WT mice. The arrows indicate centrilobular necrosis. Magnification,  $\times 100$ .

HGF in terms of promoting liver injury induced by  $\text{CCl}_4$  administration in vivo. The hepatic expressions of transgene transcripts were very high at each time point after  $\text{CCl}_4$  administration. We previously checked the relative levels of NK2 and HGF proteins expressed in the transgenic mouse livers and NK2 was present in a seven-fold molar excess relative to HGF [23]. The expression of endogenous HGF was increased on day 1 but had returned to the initial level by day 2 in WT mice. This result is consistent with the observation that the plasma HGF level peaked at 24 h after  $\text{CCl}_4$  administration, then decreased to the initial level at 72 h, in the rat [27]. On day 1, although the expression of endogenous HGF was induced in NK2 transgenic mice, it was not induced in HGF transgenic mice. This suggested that HGF overexpression inhibited  $\text{CCl}_4$ -induced acute liver injury resulting in reduced expression of endogenous HGF. The expression of endogenous c-met RNA in the livers of WT and NK2 transgenic mice was unchanged at each time point after  $\text{CCl}_4$  administration. The expression in HGF transgenic mice was reduced on days 1 and 2, implying that marked overexpression of HGF decreased c-met expression. In fact, the HGF increase induced by  $\text{CCl}_4$  administration down-regulated c-Met [28].

HGF is required for liver regeneration following necrosis

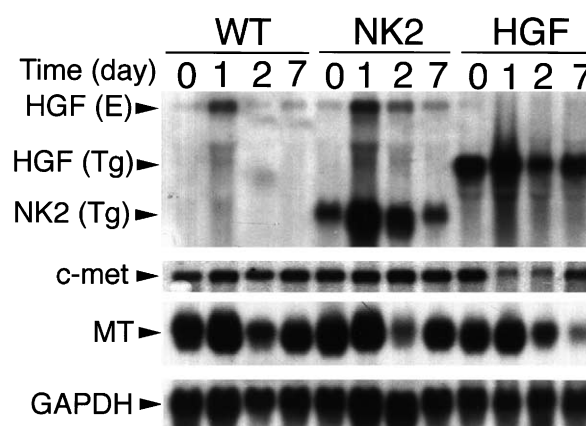


Fig. 3. RNA expressions of transgenic (Tg) HGF and NK2, and endogenous (E) HGF, c-met and MT in livers from WT, transgenic mice on days 0, 1, 2, and 7. All transgene expressions were high at each time point. After  $\text{CCl}_4$  administration, endogenous HGF expression increased in WT and NK2 transgenic mice, and endogenous c-met expression decreased in HGF transgenic mice, and endogenous MT expression was unaltered in WT and transgenic mice.

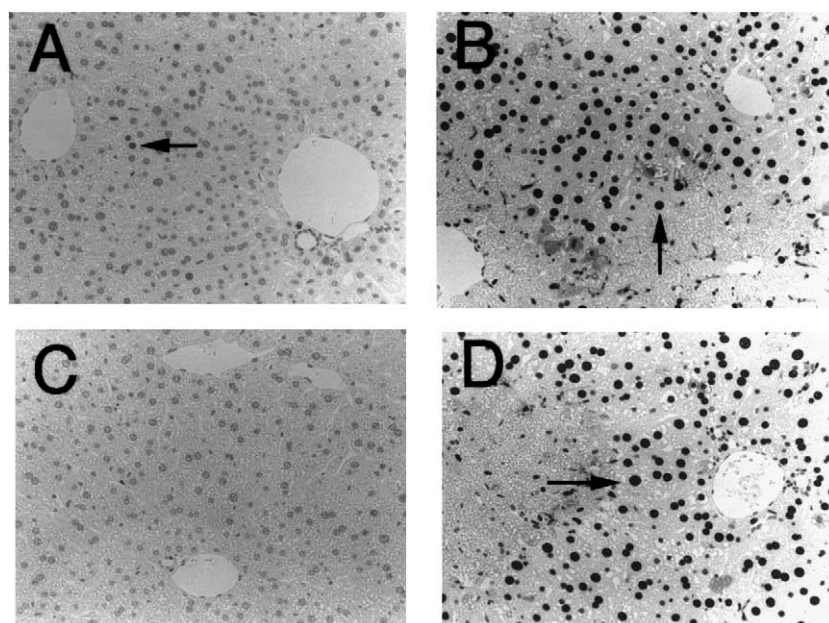


Fig. 4. Hepatocyte DNA synthesis in WT mice (A and B) and NK2 transgenic mice (C and D) on day 0 (A and C) and on day 2 (B and D). Data shown are representative for PCNA staining in each group. The arrows show hepatocytes undergoing DNA synthesis. Magnification,  $\times 200$ .

induced by  $\text{CCl}_4$  administration [29] but the role of NK2 in liver regeneration is not known. The PCNA labeling index of HGF transgenic mouse livers is 3.6-fold higher than that of WT mouse livers before  $\text{CCl}_4$  administration. On the other hand, the index of NK2 transgenic mouse livers is the same level as that of WT mouse livers before  $\text{CCl}_4$  administration. These results are consistent with previous data using BrdU incorporation to estimate hepatocyte proliferation in these mice [23]. The index of HGF transgenic mouse livers is significantly smaller than that of WT mouse livers on day 2. This result suggests that HGF protects hepatocytes from  $\text{CCl}_4$ -induced damage in vivo, because the proliferative response takes place in severely damaged liver tissue [30]. When NK2 transgenic mice were treated with  $\text{ZnSO}_4$  in water, a small reduction in liver size was observed [23]. This result indicated that

NK2 was able to inhibit endogenous HGF-mediated hepatocyte proliferation in vivo. We examined whether NK2 overexpression inhibits the proliferative effect of endogenous HGF induced by  $\text{CCl}_4$  administration. There was no difference in cell proliferation between NK2 transgenic mice and WT mice on day 2. This result suggests that the hepatocyte proliferative response to  $\text{CCl}_4$  may not require HGF alone and another factor may play a role in liver regeneration following necrosis induced by  $\text{CCl}_4$  administration.

In conclusion, this study shows that overexpression of HGF prevents  $\text{CCl}_4$ -induced acute liver injury, while that of NK2 stimulates in vivo. This provides direct evidence that HGF exerts a protective effect and NK2 behaves as an antagonist of HGF in respect of the protective effect on liver injury. However, overexpression of NK2 did not inhibit hepatocyte proliferation after liver damage. Further studies are required to determine the function of NK2 in vivo.

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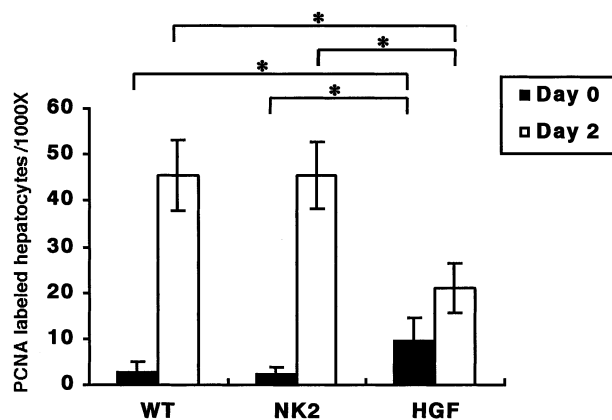


Fig. 5. The PCNA labeling index of WT, NK2 transgenic, and HGF transgenic mice on day 0 (black bars) and on day 2 (white bars) ( $n=30$  per group). There were no significant differences between WT and NK2 transgenic mice on days 0 and 2, but the index of HGF transgenic livers was significantly lower than that of WT or NK2 livers on day 2, respectively.  $*P<0.01$ .

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