Anti-high mobility group box 1 monoclonal antibody improves ischemia/reperfusion injury and mode of liver regeneration after partial hepatectomy

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Running Title: Anti-HMGB1 mAb in partial hepatectomy

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M.S., H.S., H.K.T., and T.F. conceived the idea for this project, designed all experiments and wrote the manuscript. M.S., Y.S., H.T., K.O., and T.N. performed all laboratory experiments and M.S., S.S., Y.U., R.Y., D.N., and M.U. performed all animal experiments. T.Yo., Y.S., and K.O. performed the histological analysis. H.T. and T.N. contributed to carry out the western blot analysis. T.Yo. and T.Ya. provided crucial ideas and helped with data interpretation. H.T., Y.U., and R.Y., provided technical assistance. M.N. provided the anti-HMGB1 antibody.
Abstract

BACKGROUND: The purpose of this study is to determine the effects of anti-high mobility group box-1 (HMGB1) monoclonal antibody (mAb) on ischemia/reperfusion injury (IRI) and the mode of liver regeneration.

METHODS: Rats underwent 70% hepatectomy with IRI caused by clamping the hepatoduodenal ligament for 20 min, followed by the administration of anti-HMGB1 mAb immediately before declamping the hepatoduodenal ligament. Five animals were used for each time point. We then evaluated IRI and regeneration parameters and the status of HMGB1 in remnant livers.

RESULTS: The anti-HMGB1 mAb significantly ameliorated the degree of IRI in the remnant livers in association with the down-regulation of HMGB1 protein. The ratio of Ki67-positive hepatocytes at 48 h after 70% hepatectomy was significantly improved. Mean hepatocyte size was significantly reduced and p21 expression was significantly attenuated.

CONCLUSIONS: Anti-HMGB1 mAb ameliorated IRI and improved the mode of liver regeneration after IRI followed by 70% hepatectomy in rats.

Key words: ischemia/reperfusion injury; hepatectomy; high mobility group box-1; liver regeneration
Introduction

Partial hepatectomy (PH) is one approach to treating primary and metastatic liver tumors and it is also applied to donors for living donor liver transplantation. Intermittent hepatic inflow occlusion, known as the Pringle maneuver, has been applied to control intra-operative bleeding during PH and increase safety.\(^1\)\(^2\) However, prolonged warm ischemia has been implicated in delayed liver failure in patients who have undergone major hepatectomy.\(^3\) Furthermore, experimental studies have shown that 30 - 90 min of liver ischemia during PH impairs liver regeneration and leads to postoperative liver dysfunction.\(^4\)\(^6\)

The notion that liver regeneration depends mainly on hepatocyte proliferation is generally accepted.\(^7\)\(^-\)\(^9\) Miyaoka et al. recently showed that, in mice, the initial phase of the regenerative process after 70% PH depends solely on hepatocyte hypertrophy, and only about half of hepatocytes undergo cell division after 70% PH.\(^10\) The duration of warm ischemia due to the Pringle maneuver during PH in the present clinical setting has been optimized due to advances in surgical PH.\(^2\)\(^,\)\(^11\) However, little is known about the influence of ischemia/reperfusion injury (IRI) on the mode of remnant liver regeneration in models of major PH where the Pringle maneuver takes < 30 min.

High mobility group box-1 (HMGB1) is a highly conserved non-histone nuclear structural protein that binds chromatin, consequently bends DNA and promotes protein assembly at specific DNA targets.\(^12\) In addition to its intranuclear role, HMGB1 is passively released from necrotic or damaged cells and, when secreted by activated immune cells, functions as an extracellular signaling molecule during infection, injury and inflammation.\(^12\)\(^-\)\(^15\) In the liver, HMGB1 acts as an early mediator of inflammation and organ damage after IRI.\(^16\)\(^-\)\(^18\) However, little is understood about the influence of HMGB1 on IRI and the mode of liver regeneration in remnant liver in models of IRI combined with major
The present study investigates the influence of IRI on the status of HMGB1 and the mode of remnant liver regeneration and whether or not treating IRI with an anti-HMGB1 monoclonal antibody (mAb) would improve the mode of liver regeneration by shifting hepatocyte status from hypertrophy to proliferation.

Methods

Animals

Eight-week-old male Wistar rats weighing 250 - 300 g (SLC Japan, Kyoto, Japan) were maintained in a temperature-controlled animal facility under a 12-hour light-dark cycle with food and water ad libitum. All protocols and procedures conformed to the guidelines of the Okayama University Committee for the Care and Use of Laboratory Animals and the Animal Experiments Ethics Committee of Okayama University approved the protocols.

Surgical procedures

All manipulations proceeded under anesthesia with inhaled 1.5% – 3.0% isoflurane (Abbott, Tokyo, Japan) with 0.5 L/min oxygen flow. The hepatoduodenal ligament was clamped for 20 min using a vascular clamp (Natsume Seisakusho, Tokyo, Japan) via an upper abdominal midline laparotomy. The abdominal wall was sutured closed during ischemia. The vascular clip was removed after 70% PH and the remnant liver was reperfused. Blood samples were collected, and remnant liver samples were removed for histological, real-time polymerase chain reaction (PCR) and Western blot analyses.

Production and administration of anti-HMGB1 mAb or control mAb
An anti-HMGB1 mAb was produced as described previously. The mAb against HMGB1 (clone #10-22, subclass IgG2a) recognizes the C-terminal sequence of HMGB1 (EEEDDDDE) and is specific for HMGB1, but not for HMGB2. As the control antibody, a monoclonal antibody (IgG2a subclass) against keyhole limpet hemocyanin produced in the same manner was used. Anti-HMGB1 mAb (1.8 mg/kg) or the class-matched control mAb (IgG2a) was administered intravenously immediately before removing the clamp from the hepatoduodenal ligament.

**Experimental protocol**

The protocol and experimental design of the present study was shown in Fig. 1A. IRI was induced by clamping the hepatoduodenal ligament for 20 min. The rats were randomly assigned to undergo 70% PH without 20 min of the hepatoduodenal ligament clamping (Hx) or 20 min of clamping the hepatoduodenal ligament followed by 70% PH and the administration of control mAb (IRHxC) or anti-HMGB1 mAb (IRHxA) immediately before removing the clamp. Five animals were sacrificed at 2, 6, 24, 48, 72 and 96 h after 70% PH in each group. We assessed IRI histologically, as well as by measuring liver enzyme release and the hepatic expression of inflammatory cytokines. The status of HMGB1 was analyzed by Western blotting and immunohistochemically. Liver regeneration was determined by weighing the remnant liver, immunohistochemically staining for Ki67, evaluating hepatocyte size and by Western blotting to analyze signal transducer and activator of transcription 3 (STAT3) and cyclin-dependent kinase inhibitor 1 (p21).

The effect of anti-HMGB1 mAb on mortality was examined using severer experimental model. Although the mortality at 20 min ischemia was observed difference, since it was not significant, we performed survival experiments in a stress model with longer ischemic time (30 minutes). Seven-day survival rates were evaluated after clamping the
hepatoduodenal ligament for 30 min, performing 70% PH and reperfusing the remnant liver (n = 15 per group).

**Immunohistochemical detection of HMGB1**

Immunohistochemical analysis of HMGB1 proceeded as described. Biopsy specimens were fixed in 10% formaldehyde, embedded in paraffin and then 4-µm-thick serial sections cut from each paraffin-embedded tissue block were immunohistochemically assessed using an alkaline phosphatase-labeled rat anti-HMGB1 mAb with the automated Bond-Max stainer (Leica Biosystems, Melbourne, Australia).

**Western blotting of liver tissue**

Whole liver was lysed in 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1% Triton X-100 and Complete Mini protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Proteins resolved by electrophoresis on 10% – 15% sodium dodecyl sulfate polyacrylamide gels were then transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Non-specific binding on blots was blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20, pH 7.4) at room temperature for 30 min. The primary antibodies were: rat anti-HMGB1 mAb, mouse anti-STAT3 mAb (Cell Signaling Technology, Boston, MA, USA), rabbit anti-phospho-STAT3 mAb (Cell Signaling Technology), rabbit anti-caspase 3 polyclonal antibody (pAb) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-p21 pAb (Santa Cruz Biotechnology). The secondary antibodies were: horseradish peroxidase-conjugated antibodies against goat IgG (Chemicon International Inc., Temecula, CA, USA), and rabbit or mouse IgG (GE Healthcare). Immunoreactive bands on blots were visualized using ECL Plus enhanced chemiluminescence substrates (GE Healthcare). Bands
were quantified using Image J (National Institutes of Health, Bethesda, MD, USA).

**Real-time PCR**

Total RNA was extracted from the liver samples using miRNeasy Mini Kits (Qiagen, Hilden, Germany). Levels of interleukin (IL)-6, tumor necrosis factor alpha (TNF-α) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) expression were determined in complementary DNA (cDNA) synthesized from total RNA (100 ng) using quantitative real-time PCR, the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) and TaqMan® Gene Expression Assays (Applied Biosystems). Relative levels of IL-6 and TNF-α mRNA expression were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with reference to GAPDH mRNA expression.

**Parameters of liver regeneration**

Liver regeneration was evaluated by weighing the remnant liver, assessing the ratio of Ki67-positive to total hepatocytes, and measuring the size of immunohistochemically stained hepatocytes in paraffin-embedded liver sections. The ratio of Ki67-positive hepatocytes and hepatocyte size were evaluated in liver sections obtained from identical locations in the right lateral lobe from each animal. Stained sections were further fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and incubated with a rabbit polyclonal antibody against Ki67 antigen (Leica Biosystems, Newcastle, UK). The ratio (%) of Ki67-positive hepatocytes was determined in five high-power fields per animal. Hepatocyte size was determined by immunohistochemical staining for E-cadherin, which is a superfamily of transmembrane glycoproteins located at the membranes of normal hepatocytes. Stained sections were further fixed in PBS containing 4% PFA and incubated with mouse anti-E-cadherin (Invitrogen Corporation, Carlsbad, CA, USA). Hepatocyte size was
calculated in five high-power fields per rat using NIS-Elements Documentation software (Nikon Corporation, Tokyo, Japan).

Statistical analysis

Data are presented as means ± standard error of the mean. Groups were compared using Student’s t-test or analysis of variance (ANOVA). Survival data were generated by Kaplan-Meier survival analysis with the log-rank test. Differences were considered significant at the level of \( p \leq 0.05 \). All data were statistically analyzed using SPSS II statistical software (IBM, Armonk, NY, USA).

Results

Assessment of IRI

Hepatocellular damage following IRI and PH in the rat liver was assessed by measuring serum transaminase levels. As shown in Fig. 1B, the level of serum aspartate aminotransferase (AST) at 6 h after PH was significantly higher in the IRHxC, than in the Hx group, reflecting the effect of 20 min of IRI. The level of serum AST that was elevated in the IRHxC group was significantly reduced in the IRHxA group (2749 ± 397 vs. 1459 ± 201 IU/L; \( p = 0.019 \)). The mean level of gene expression of the inflammatory cytokines IL-6 and TNF-\( \alpha \) in the liver at 2 h after PH were higher in the IRHxC than in the Hx group. Anti-HMGB1 mAb therefore decreased the expression of inflammatory cytokine genes.

Histological assessment of remnant liver samples at 24 h after PH revealed normal hepatic histology in the Hx group (Fig. 1C). Some necrotic foci with hemorrhage, inflammatory cell infiltration and mild sinusoidal and central venous dilation were evident in the IRHxC group (Fig. 1D), whereas some vacuolar degeneration of hepatocytes, small necrotic foci and slight
sinusoidal and central venous dilation were features of the IRHxA group (Fig. 1E).

**Immunohistochemistry for HMGB1**

The normal liver and the remnant livers of the three groups were immunohistochemically stained to determine the cellular localization of HMGB1 (Fig. 2A). The nucleus was the main location of HMGB1 in normal hepatocytes, and it was undetectable in the cytoplasm. In the Hx group, the intranuclear expression of HMGB1 was not increased at 6 h after PH and the cytoplasmic translocation of HMGB1 was minimal in the remnant livers at 24 and 48 h after PH. In the IRHxC group, the intranuclear expression of HMGB1 was increased at 6 h after PH and the cytoplasmic translocation of HMGB1 was increased and decreased, respectively, at 24 and 48 h after PH. On the other hand, a small amount of cytoplasmic HMGB1 that was translocated at 6 h after PH decreased at 24 and 48 h after PH in the IRHxA group.

**Liver expression of HMGB1 protein**

The amount of HMGB1 protein in the liver was quantified using Western blotting. The level of hepatic HMGB1 protein in the IRHxC group increased about 4-fold at 6 and 24 h after PH compared with the Hx group (Figs. 2B and D). The level of liver HMGB1 expression was significantly reduced in the IRHxA compared with the IRHxC group at 24 h after PH (Figs. 2C and D), indicating that the anti-HMGB1 mAb prevented the HMGB1 upregulation that was evident in the IRHxC group.

**Hepatocyte proliferation and hypertrophy during liver regeneration**

The weight of the remnant liver similarly increased in all groups from 24 h after PH and reached about a 3.8-fold increase in weight at 96 h after PH (Fig. 3A). There was no
difference in weight of the remnant livers in the three groups.

Hepatocyte proliferation was immunohistochemically quantified in remnant livers using Ki67 (Fig. 3B). The ratio of Ki67-positive hepatocytes increased to 62.5% ± 3.2% at 48 h after PH and fell to 43.6% ± 3.8% at 96 h thereafter in the Hx group. The ratio at 48 h after PH was significantly lower in the IRHxC than in the Hx group (30.7% ± 2.4% vs. 62.5% ± 3.2%; p < 0.01), and decreased to 19.1% ± 0.7% at 96 h after PH. These ratios at 48, 72 and 96 h after PH were significantly increased in the IRHxA compared with the IRHxC group, indicating that the anti-HMGB1 mAb stimulated hepatocyte proliferation in the remnant liver.

Immunohistochemical staining to detect E-cadherin in the remnant liver at 48 h after PH was shown in Fig. 3C. The mean size of hepatocytes equally increased from 24 h after PH in all groups. At 48, 72 and 96 h after PH, the mean hepatocyte size that was significantly increased in the IRHxC, compared with the Hx group was ameliorated in the IRHxA group at all three time points after PH, indicating that that anti-HMGB1 mAb prevented the increase in hepatocyte size caused by IRI (Fig. 3D).

**Activation of STAT3, caspase 3 and p21**

The crucial mitotic transcription factor in liver regeneration, unphosphorylated STAT3, was basally expressed in the rat liver before PH. At 2 h after PH, STAT3 was rapidly phosphorylated and then recovered to the basal level within 24 h in the Hx group. In contrast, STAT3 in the IRHxC group was persistently and markedly phosphorylated for up to 24 h, with a marked peak at 2 h. On the other hand, in the IRHxA group, STAT3 was more strongly phosphorylated at 2 h to 6 h than the IRHxC group, and then remained elevated for up to 24 h thereafter (Fig. 4A). Western blotting and immunohistochemical staining did not detect cleaved caspase 3 at 2, 6, 24, 48, 72 or 96 h after PH in any group.

The cyclin-dependent kinase inhibitor p21 halts the cell cycle at the G1 phase and
contributes to cellular hypertrophy in several pathological states.20-22 The level of hepatic p21 was slightly increased in Hx livers. In contrast, p21 was up-regulated about 3.5- and 2.5-fold in the livers of the IRHxC group at 24 and 48 h after PH, respectively, compared with the Hx group. The level was significantly lower at 24 h after PH in the livers of the IRHxA, than the IRHxC group (Fig. 4B), indicating that the anti-HMGB1 mAb ameliorated cellular hypertrophy and prevented pathological progression.

Effects of anti-HMGB1 mAb on mortality

We investigated the effects of anti-HMGB1 mAb on mortality in a rat 70% PH model after prolonged IRI (30 min). The one-week survival rate of this model treated with the control mAb was only 33.3%, although all rats survived simple 70% PH. Mean survival was significantly longer in the IRHxA than the IRHxC group (5.08 ± 0.73 vs. 2.85 ± 0.79 days; \( p < 0.05 \); Fig. 5). The anti-HMGB1 mAb significantly improved the mortality rates of rats after IRI for 30 min followed by 70% PH (n = 15 per group).

Discussion

Several factors, including IRI and liver regeneration, combine to generate stress and morbidity in most patients with major liver resection. Prolonged warm ischemia during PH in both experimental and clinical studies3-6 apparently results in impaired liver regeneration and leads to liver failure. However, little is known about the influence of warm ischemia for < 30 min on the mode of liver regeneration. In addition, liver IRI causes the release of endogenous danger signals from damaged cells and activated immune cells,16-18, 23-25 among which HMGB1 is the most widely investigated.16,18,25-29 Therefore, we designed the experimental protocol shown in Figure 1 and investigated how warm ischemia for 20 min affects the status
of HMGB1, the mode of remnant liver regeneration and whether or not an anti-HMGB1 mAb could modify these factors in a rat model of 70% PH.

Warm IRI of the liver leads to the up-regulation of HMGB1 expression and its cytoplasmic translocation in the liver. However, the status of HMGB1 in models of IRI combined with major PH remains obscure. The present study pointed out that HMGB1 protein is upregulated in the remnant liver and that cytoplasmic HMGB1 translocation is followed by the intranuclear expression of HMGB1 in hepatocytes of remnant livers mainly at 24 h after IRI and 70% PH. Furthermore, anti-HMGB1 mAb can reduce the up-regulation of HMGB1 protein expression in remnant livers at 24 h after IRI and 70% PH.

We analyzed the effects of an anti-HMGB1 mAb on the dynamic status of HMGB1 by immunohistochemical staining and Western blotting. The therapeutic effects of mAb are reportedly generated via the inhibition of cell signaling by binding to the extracellular domain of the targeted receptor, stimulation of antibody-dependent cell-mediated cytotoxicity and the inhibition of protein function by binding to a specific region of the targeted protein. Antibodies generally cannot bind to or inhibit proteins in the nucleus and cytoplasm of cells. We found that anti-HMGB1 mAb could bind to extracellular HMGB1, but not to HMGB1 in the nucleus and cytoplasm. Thus, the administration of anti-HMGB1 mAb might affect the detection of extracellular HMGB1, especially at 6 and 24 h after IRI and 70% PH. However, we assumed that the administered anti-HMGB1 mAb did not interfere with the detection of nuclear and cytoplasmic HMGB1 in hepatocytes by immunohistochemical staining and Western blotting.

Hepatocytes enter the cell cycle and regenerate the liver in response to the loss of liver mass. Liver regeneration depends mainly on the proliferation of hepatocytes. Miyaoka et al. recently demonstrated that the initial phase of the regenerative process depends mainly on hepatocyte hypertrophy after 70% PH, and that only about half of hepatocytes undergo
cell division after 70% PH in mice. We showed here that 20 min of warm ischemia affects the mode of liver regeneration in rat models of 70% PH. Hepatocyte hypertrophy rather than proliferation occurred in the remnant liver after 20 min of warm ischemia and 70% PH. Miyaoka et al.\textsuperscript{10} showed that hepatocyte hypertrophy occurs first, and then hepatocytes divide to increase numbers in models of 70% PH alone. On the other hand, only about 30% of hepatocytes underwent cell division and hepatocytes continued to enlarge until 96 h after 20 min of warm ischemia and 70% PH in the present study. We also showed that treating IRI with an anti-HMGB1 mAb improved the mode of liver regeneration by shifting from a regeneration mode that mainly depends on hepatocyte hypertrophy to another that depends mainly on proliferation.

The cyclin-dependent kinase inhibitor p21 contributes to cell cycle arrest during the G0/G1 phase and cellular hypertrophy under various conditions and organs.\textsuperscript{20-22} We found that p21 was remarkably upregulated at 24 h after 20 min of warm ischemia and 70% PH in the IRHxC group compared with Hx group (70% PH alone). Hepatocyte hypertrophy after 70% PH can somewhat compensate for the loss of function and volume of the remnant liver, but the effects of hepatocyte hypertrophy on the massive loss of liver tissue might be limited compared with hepatocyte proliferation. Our findings show that an anti-HMGB1 mAb can ameliorate the upregulation of p21 expression in the remnant liver at 24 h after IRI and 70% PH. This suggests that the infusion of anti-HMGB1 mAb can prevent the signaling progression of hepatocyte hypertrophy and generate a shift to a state in which liver regeneration mainly depends on hepatocyte proliferation.

STAT3 was rapidly phosphorylated and then recovered to the basal level within 24 h in the Hx group, but remained phosphorylated in the IRHxC group. The anti-HMGB1 mAb augmented STAT3 phosphorylation at 2 h and sustained it for up to 24 h after IRI and 70% PH. The IL-6/STAT3 pathway plays pivotal roles in regulating hepatocyte proliferation, at
least in the acute liver response after hepatectomy in rodents. In addition, the target genes of STAT3 include the anti-apoptotic FLIP, Bcl-2, and Bcl-xL genes, and therefore STAT3 potentially has anti-apoptotic capacity. Hepatocyte apoptosis was undetectable by cleaved caspase 3 in all groups. Therefore, the persistent STAT3 phosphorylation induced by the anti-HMGB1 mAb might have contributed not only to hepatocyte proliferation but also to the prevention of apoptosis.

In the IRHxC group, although STAT3 which is crucial mitotic transcription factor of liver regeneration was more phosphorylated than the Hx group, p21 (which suppresses cell division) was up-regulated. As a result, hypertrophy occurred in compensation. In the IRHxA group, STAT3 was more strongly phosphorylated than in the IRHxC group, but p21 was down-regulated, the cells of the remnant liver were able to divide (Fig. 6). In this study, treatment for IR injury with anti-HMGB1 mAb could improve the mode of liver regeneration through shifting to the proliferation of hepatocytes from a state mainly depending on the hypertrophy of hepatocytes.

Conclusions

Anti-HMGB1 mAb appears to ameliorate IRI and improve the mode of liver regeneration by generating a shift from a state that mainly depends on hepatocyte hypertrophy to another that depends on proliferation. These effects of anti-HMGB1 mAb are associated with the attenuation of p21 upregulation induced by 20 min of warm IRI and the persistent augmentation of STAT3 phosphorylation after 70% PH. Anti-HMGB1 mAb might have therapeutic potential for IRI and liver regeneration when major hepatectomy requires frequent Pringle maneuvers to control blood loss.
References


FIGURE LEGENDS

Figure 1. (A) Study protocol. Rats were randomly assigned to the following groups: Hx, 70% hepatectomy without ischemia/reperfusion injury (IRI); IRHxC, IRI (hepatoduodenal ligament clamped for 20 min) and 70% hepatectomy and administration of control mAb; IRHxA, IRI and 70% hepatectomy and administration of anti-HMGB1 mAb. Antibodies were intravenously administered immediately before removing clamp. (B) Serum aspartate aminotransferase (AST) level. Serum AST level at 6 h after PH was significantly higher in the IRHxC, than in the Hx group. Serum AST level that was elevated in the IRHxC group was significantly reduced in the IRHxA group. Data are shown as mean ± SD. Five rats were used for each time point. *p < 0.05 vs. Hx; †p < 0.05 vs. IRHxA. (C-E) Histological findings in each group. Hematoxylin-eosin-stained liver section 24 h after PH in Hx (C), IRHxC (D) and IRHxA (E) groups (Original magnification ×200). The Hx group showed normal hepatic histology. Some necrotic foci with hemorrhage, inflammatory cell infiltration and mild sinusoidal and central venous dilation are evident in the IRHxC group, whereas some vacuolar degeneration of hepatocytes, small necrotic foci and slight sinusoidal and central venous dilation are features of the IRHxA group. Representative images from 5 rats per group were selected.

Figure 2. HMGB1 expression in the liver. (A) Immunohistochemical staining of HMGB1 in each group (Original magnification ×400). Hx group: the intranuclear expression of HMGB1 was not increased at 6 h and the cytoplasmic translocation of HMGB1 was minimal in the remnant livers at 24 and 48 h. IRHxC: the intranuclear expression of HMGB1 was increased at 6 h and the cytoplasmic translocation of HMGB1 was increased and decreased at 24h and 48h. IRHxA: a small amount of cytoplasmic HMGB1 that was
translocated at 6 h decreased at 24 and 48 h. Representative images from 5 rats per group were shown. **(B, C)** Western blot detection of HMGB1 protein in each group. The level of hepatic HMGB1 protein in the IRHxC group increased at 6 and 24 h after PH compared with the Hx group (B). The level of liver HMGB1 expression was significantly reduced in the IRHxA compared with the IRHxC group at 24 h after PH (C). **(D)** Densitometric quantitation of Western blot signal intensity. Blot shown is representative of three experiments with similar results. Data are shown as mean ± SD. Five rats were used for each time point. *p < 0.01 vs. Hx; †p < 0.05 vs. IRHxA.

**Figure 3.** Analysis of the mode of liver regeneration. **(A)** Remnant liver weight in each group. The weight of the remnant liver similarly increased in all groups from 24 h to 96 h after PH. There was no difference in weight of the remnant livers in the three groups. Data are shown as mean ± SD. Five rats were used for each time point. **(B)** Ratio of Ki67 positive hepatocytes in each group. The ratios of Ki67-positive hepatocytes from 48h to 96h after PH increased in the Hx compared with IRHxC group. These ratios from 48 to 96 h after PH were significantly increased in the IRHxA compared with the IRHxC group. Data are shown as mean ± SD. Five rats were used for each time point. *p < 0.01 vs. Hx; †p < 0.05 vs. IRHxA. **(C)** Immunohistochemical staining of E-cadherin at 48 h after PH in the control liver and each group (Original magnification ×400). The size of hepatocytes increased at 48h after PH in the IRHxC group compared with the Hx group, and the size of hepatocytes was ameliorated in the IRHxA group. Representative images from 5 rats per group were shown. **(D)** Mean size of hepatocytes in each group. At 48, 72 and 96 h after PH, the mean hepatocyte size that was significantly increased in the IRHxC, compared with the Hx group was ameliorated in the IRHxA group at all three time points after PH. Data are shown as mean ± SD. Five rats were used for each time point. *p < 0.01 vs. Hx; †p < 0.05 vs. IRHxA.
**Figure 4.** (A) Western blot analysis of STAT3 phosphorylation after PH. In the Hx group, At 2 h after PH, STAT3 was rapidly phosphorylated and then recovered to the basal level within 24 h. In the IRHxC group, STAT3 was persistently and markedly phosphorylated for up to 24 h, with a marked peak at 2 h. In the IRHxA group, STAT3 was more strongly phosphorylated at 2 h to 6 h than the IRHxC group, and then remained elevated for up to 24 h thereafter. Each lane represents a separate animal. Blot shown is representative of three experiments with similar results. (B) Western blot analysis of p21 protein expression. In the Hx group, the level of hepatic p21 was slightly increased. In the IRHxC group, the level of hepatic p21 was up-regulated at 24 h after PH, compared with the Hx group. In the IRHxA group, the level was significantly lower at 24 h after PH than the IRHxC group, indicating that the anti-HMGB1 mAb ameliorated cellular hypertrophy and prevented pathological progression. Each lane represents a separate animal. Blot shown is representative of three experiments with similar results.

**Figure 5.** Effect of anti-HMGB1 mAb on mortality. Hepatoduodenal ligament was clamped for 30 min before hepatectomy. Remnant liver was reperfused after 70% hepatectomy and rat survival was assessed for seven days (n = 15 per group) thereafter. Statistical differences between the groups were evaluated using Kaplan-Meier survival analysis. †p < 0.05 vs. IRHxC.

**Figure 6.** Pattern diagram of liver regeneration in each group. In the IRHxC group, although STAT3 was more phosphorylated than the Hx group, p21 was up-regulated. As a result, hypertrophy occurred in compensation. In the IRHxA group, STAT3 was more
strongly phosphorylated than in the IRHxC group, but p21 was down-regulated, the cells of the remnant liver were able to divide.