

## Inhibition of Kupffer Cell Activity Improves Transplantation of Human Adipose-Derived Stem Cells and Liver Functions

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Numerous approaches to cell transplantation of the hepatic or the extrahepatic origin into liver tissue have been developed; however, the efficiency of cell transplantation remains low and liver functions are not well corrected. The liver is a highly immunoreactive organ that contains many resident macrophages known as Kupffer cells. Here, we show that the inhibition of Kupffer cell activity improves stem cell transplantation into liver tissue and corrects some of the liver functions under conditions of liver injury. We found that, when Kupffer cells were inhibited by glycine, numerous adipose-derived stem cells (ASCs) were successfully transplanted into livers, and these transplanted cells showed hepatoprotective effects, including decrease of liver injury factors, increase of liver regeneration, and albumin production. On the contrary, injected ASCs without glycine recruited numerous Kupffer cells, not lymphocytes, and showed low transplantation efficiency. Intriguingly, successfully transplanted ASCs in liver tissue modulated Kupffer cell activity to inhibit tumor necrosis factor- $\alpha$  secretion. Thus, our data show that Kupffer cell inactivation is an important step in order to improve ASC transplantation efficiency and therapeutic potential in liver injuries. In addition, the hepatoprotective function of glycine has synergic effects on liver protection and the engraftment of ASCs.

**Key words:** Adipose-derived stem cells (ASCs); Glycine; Kupffer cells; Liver; Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )

### INTRODUCTION

Liver failure represents a major medical problem with significant morbidity and mortality (15,16). However, there are no effective drugs for end-stage liver diseases to date. Liver transplantation is still the only therapeutic option for liver failure. However, liver transplantation is quite limited by the donor availability. Therefore, recent studies are focused on endogenous or exogenous stem cells as a source of possible replacement for diseased hepatocytes through cell transplantation (32). Many literatures have shown in vivo experiments with successful transplantation of allogenic or heterogenic mesenchymal stem cells (MSCs) from various sources into liver tissue (5–8,13,20–22,26,44,47,52,53). Despite this progress, the low efficiency of transplantation and the differentiation of MSCs in liver tissue remain a problem, and the functional role of stem cells in liver injuries is unclear.

We hypothesized that two factors might be considered for successful engraftment of stem cells into liver tissue.

First, the establishment of a proper liver injury models is important for animal experiments to reproduce the pathophysiological condition of humans because the liver of experimental animals has higher enormous regenerative capacity than human tissues. The induction of proper liver injury in animal experiments leads stem cells to participate in liver regeneration. Therefore, we compared the transplantation efficiency of adipose-derived stem cells (ASCs) in two liver injury models induced by 2-acetylaminofluorene (AAF) or by AAF combined with partial hepatectomy (AAF/PHx). In AAF or AAF/PHx liver injury models, proliferation of hepatocytes is inhibited by AAF and then, oval cells, ductular bipolar progenitor cells in the liver, proliferated and differentiated into hepatocytes or bile duct cells to compensate for the inhibition of hepatocyte proliferation. However, if there is continuously severe liver damage by AAF, the differentiation of oval cells into hepatocytes is delayed. Consequently, this may proceed to liver failure or carcinogenesis (2,27,31,34,46).

Received July 26, 2011; final acceptance March 27, 2012. Online prepub date: April 26, 2012.

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The second factor for the successful engraftment of stem cells is the inhibition of immune reactions to stem cells. In previous studies related to stem cell transplantation in the liver, researchers usually used experimental animal models including genetically immune-deficient animals such as nude (T-cell deficiency) (7,8,47,52) and perforin/recombination activating gene 2 knockout (Pfp/Rag2<sup>-/-</sup>) mice [T, B, and natural killer (NK) cell deficiency] (5,6) or animals artificially immunosuppressed by drugs such as cyclosporin A, a T lymphocyte suppressor (44). However, these studies did not solve the problem of the low engraftment of stem cells into the liver. In addition, the role of the transplanted cells on liver functions has not been clear. The liver is a highly immunoreactive organ, and it contains many resident macrophages known as Kupffer cells, rather than T and B lymphocytes. Therefore, we hypothesized that inhibition of Kupffer cell activation might improve stem cell transplantation in liver tissue.

In the present study, we transplanted human (h)-ASCs into rats that have liver injuries by AAF and AAF/PHx. Some rats are treated with glycine, a strong inhibitor of Kupffer cell activity. We demonstrated that h-ASC transplantation recruits Kupffer cells, whereas glycine treatment improves transplantation of h-ASCs into liver tissue through down-regulation of Kupffer cell activity. Intriguingly, once h-ASCs were successfully transplanted into the liver, h-ASCs modulated Kupffer cell activity and had a protective effect against liver injury through differentiation into hepatocyte-like cells that secreted albumin.

## MATERIALS AND METHODS

All chemicals are from Sigma-Aldrich, USA, unless otherwise stated.

### *Isolation, Culture, In Vitro Differentiation, and Characterization of h-ASCs*

Human subcutaneous fat was obtained from female healthy donors (aged 28–44 years) undergoing elective liposuction procedures after informed consent. All sample collection procedures from human beings and experimental protocols were approved by the Institutional Review Board at Yeungnam University Medical Center (Yeungnam Univ. Hosp. IRB PCR-10-95). Fat tissue was digested with 0.2% collagenase type I (Worthington Biochemical, USA) at 37°C and was filtered through a 70-µm nylon cell strainer. The harvested cells were cultured in low-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Gibco, USA) at 37°C with 5% CO<sub>2</sub>. Cells from the third passage were used in this study. We reproduced the same experiment at least three times.

The differentiation medium was made as described previously (3,55). To analyze multipotent differentiation of h-ASCs, several special staining methods were used,

including Oil-Red O, alkaline phosphatase, von Kossa, Alcian blue, and immunofluorescence for  $\alpha$ -actinin, troponin, nestin, and glial fibrillary acidic protein. Finally, h-ASCs were examined for their ability to differentiate into adipocytes, osteogenic, and chondrogenic lineages, and were found to express cardiogenic and neural cell markers. Thus, h-ASCs have a potential for multipotent differentiation (data not shown).

The third-passage (P3) expansion of h-ASCs was incubated with the following fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (Abs): CD13-PE, CD29-PE, CD31-FITC, CD34-FITC, CD44-PE, CD45-FITC, CD117-PE, CD166-PE (BD Pharmingen, USA), human leukocyte antigen (HLA)-DR-PE, HLA-Class I-PE (Abcam, UK), and CD105-FITC (Serotec, UK). Cells were analyzed by flow cytometry (FACS Aria, BD, USA). FITC- or PE-conjugated mouse IgGs were used as negative controls.

### *Experimental Design and h-ASC Transplantation*

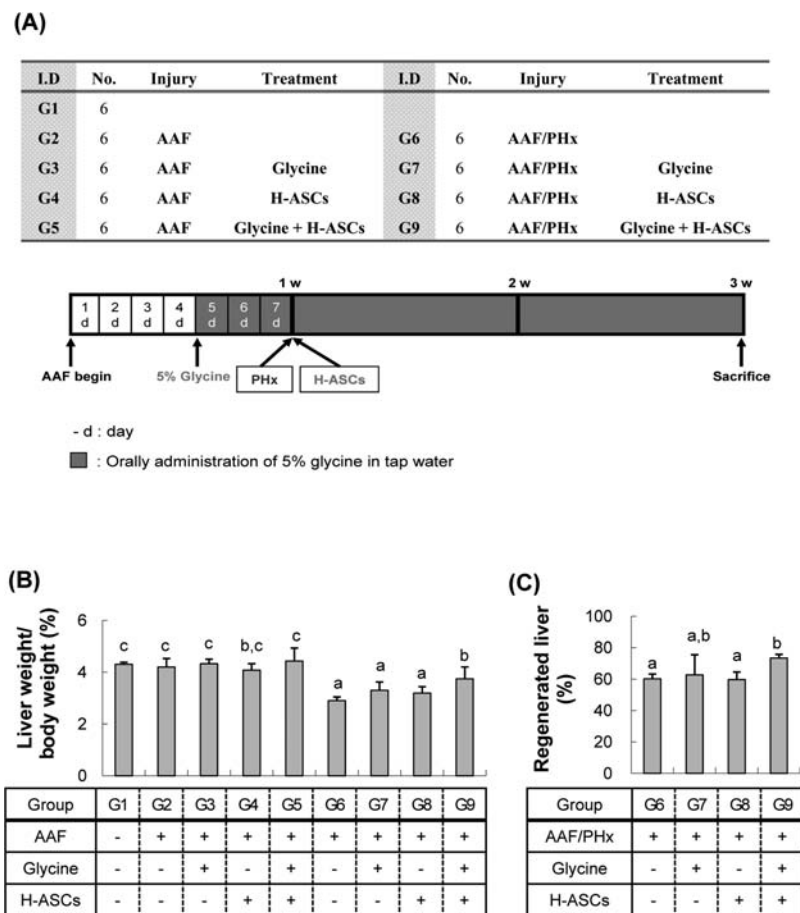
Male Sprague-Dawley rats (Orient Bio Inc., Korea), weighing 240–260 g, were maintained in a room at 22±2°C with a 12-h light–dark cycle and were given standard chow and had access to tap water ad libitum. The animal experiments were performed in accordance with the Kyungpook National University guidelines for the care and use of laboratory animals. Rats were divided into nine groups (Fig. 1A).

The AAF model of rats was developed as follows: AAF was dissolved in a small volume of dimethylsulfoxide (DMSO) and suspended in a 1% aqueous solution of carboxymethylcellulose to obtain a final concentration of 1.5 mg/ml. The animals received a daily oral gavage of 10 mg/kg of AAF for a period of up to 3 weeks until being euthanized. Seven days after the AAF treatment began, 1×10<sup>6</sup> h-ASCs were slowly injected into the spleen under anesthesia with ketamin and xylazine.

The AAF/PHx model of rats was developed as follows: Preparation of AAF was the same as with the AAF model. The rats received a daily oral gavage of 10 mg/kg AAF for a period of up to 7 days. Seven days after AAF treatment began, rats underwent 70% PHx, consisting of resection of median and left lateral lobes after placing a thread knot surrounding the hilum and the hepatic vein. On the same day, 1×10<sup>6</sup> h-ASCs were slowly injected into the spleen, followed by administration of 3.3 mg/kg AAF for 2 weeks until euthanization.

The h-ASCs (P3) were labeled with a 4',6-diamidino-2-phenylindole (DAPI) solution. DAPI stock solution was added to the culture medium at a final concentration of 50 µg/ml and incubated for 30 min.

Glycine was dissolved in tap water with 5% (w/v) and was orally administered into rats ad libitum for 3 days before the h-ASC injection and until euthanization.



**Figure 1.** Experimental design of rats. (A) Group 1 (G1) is normal control. Group 2 (G2) received a daily oral gavage of 10 mg/kg 2-acetylaminofluorene (AAF) for a period of up to 3 weeks. Group 3 (G3) received AAF and 5% glycine. Group 4 (G4) received AAF and human adipose-derived stem cells (h-ASCs). Group 5 (G5) received AAF, 5% glycine, and h-ASCs. Group 6 (G6) received a daily oral gavage of 10 mg/kg AAF for a period of up to 7 days and received 3.3 mg/kg AAF for 2 weeks after partial hepatectomy (PHx; 70%). Group 7 (G7) received AAF/PHx and 5% glycine. Group 8 (G8) received AAF/PHx and h-ASCs. Group 9 (G9) received AAF/PHx, 5% glycine, and h-ASCs. (B) Liver regeneration ratio (R1).  $R1 (\%) = [\text{liver weight/body weight (g)}] \times 100$ . (C) Liver regeneration ratio (R2) in the AAF/PHx groups (G6–G9).  $R2 (\%) = 100 \times 0.7 \times (W2/W1)$ . W1 is the weight of the liver resected at PHx, W2 is the weight of the regenerated liver. Each value is presented as the mean  $\pm$  SD. <sup>a-c</sup>Values not sharing a common superscript letter differ significantly at  $p < 0.05$  by Duncan's multiple comparison test.

### Liver Regeneration Ratio

The regeneration ratio (R1) was calculated using the wet weights of whole livers and their body weights at euthanasia.  $R1 (\%) = [\text{liver weight/body weight (g)}] \times 100$ . In the AAF/PHx groups (G6–G9), the regeneration ratio (R2) (%) =  $100 \times 0.7 \times (W2/W1)$ . W1 is the weight of the liver resected at PHx. W2 is the weight of remnant livers at euthanasia.

### Histology and Immunohistochemistry (IHC)

Liver tissues from each rat were rapidly removed, fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax for sectioning. The sectioned livers were subjected to H&E staining and IHC. The other liver tissues were cryopreserved in tissue

freezing medium for cryosectioning. The sectioned livers were fixed in 4% paraformaldehyde. DAPI-labeled h-ASCs were examined in the cryosectioned livers using an Axioplan 2 upright/Fluorescence microscope (Carl Zeiss, Germany). Three liver pieces of each rat were collected and put on a slide. DAPI-labeled h-ASCs were counted in the liver tissue by analyzing five random fields (200 $\times$ ) per slide of each animal. The number of h-ASCs is presented mean  $\pm$  SD of each group.

For IHC, the following antibodies were used: ED1 (CD68; Serotec, USA), proliferating cell nuclear antigen (PCNA, Santa Cruz Biotechnology, USA),  $\alpha$ -fetoprotein (AFP, Novus Biologicals, USA), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , Cell Signaling, USA), CD79a and CD3 (Santa Cruz Biotechnology, USA). Anti-senescence marker

protein-30 (SMP30) was obtained from Akihito Ishigami (Molecular Regulation of Aging, Metropolitan Institute of Gerontology, Tokyo, Japan). The antigen–antibody complex was visualized by an avidin–biotin peroxidase complex solution using an ABC kit (Vector Laboratories, USA), and color was developed using a commercial 3,3'-diaminobenzidine (Invitrogen, USA). Sections were then counter stained with hematoxylin.

Anti-human albumin (clone HAS-11, Sigma-Aldrich) was used to detect human albumin in rat livers. This antibody has been shown to have no cross-reaction with rat livers in IHC according to the manufacturer's information and a previous study (44). FITC-conjugated anti-mouse (National Veterinary Research and Quarantine Service, Korea) was used as a secondary antibody. Photomicrographs were taken using a confocal microscopy of LSM 700 CLSM system (Carl Zeiss, Germany).

#### *Immunoblot Analysis*

Snap-frozen liver tissues were homogenized in RIPA buffer to obtain soluble protein. Protein lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The antibodies used for immunoblot were as follows: ED1, PCNA, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1; Santa Cruz Biotechnology, USA), TNF- $\alpha$ , GAPDH (Cell Signaling Technology, USA), and SMP30. The primary antibodies were detected using horseradish peroxidase-conjugated second antibodies.

#### *Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis*

Total RNA was extracted from snap-frozen liver tissues using Trizol reagent (Gibco, USA). cDNA synthesis and mRNA isolation were performed using AccuPower™ RT/PCR premix (Bioneer, Korea) following the manufacturer's protocol. Human albumin primers were designed following Seo et al. (47).

#### *Serum Biochemical Analysis*

The blood of rats was collected from caudal vena cava under euthanasia. The blood was centrifuged at 3,000 rpm for 15 min to separate the serum, which was immediately frozen and stored at  $-70^{\circ}\text{C}$  until analysis. Albumin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were analyzed by a blood chemistry analyzer (Konelab 20, Thermo Clinical Labsystems, Vantaa, Finland).

#### *Statistical Analysis*

All values are presented as means  $\pm$  SD. For multiple comparisons, ANOVA with Duncan's or Waller-Duncan's multiple range test was used. All calculation were carried out using SPSS 12.0K software. Statistical significance was assumed when  $p < 0.05$ .

## RESULTS

### *Characterization of h-ASCs*

Flow cytometry analysis showed that h-ASCs expressed high levels of stromal cell-related markers such as CD13, CD29, CD44, CD105, and CD166. No expression of CD31 (endothelial cell-related marker), CD34, CD117 (hematopoietic stem cell-related marker), and CD45 (pan-hematopoietic cell-related marker) was detected in these cells. We also performed analysis of HLA antigens and found that h-ASCs express HLA-class I, but not HLA-DR (data not shown).

### *Liver Regeneration Ratio of Rats After Transplantation of h-ASCs*

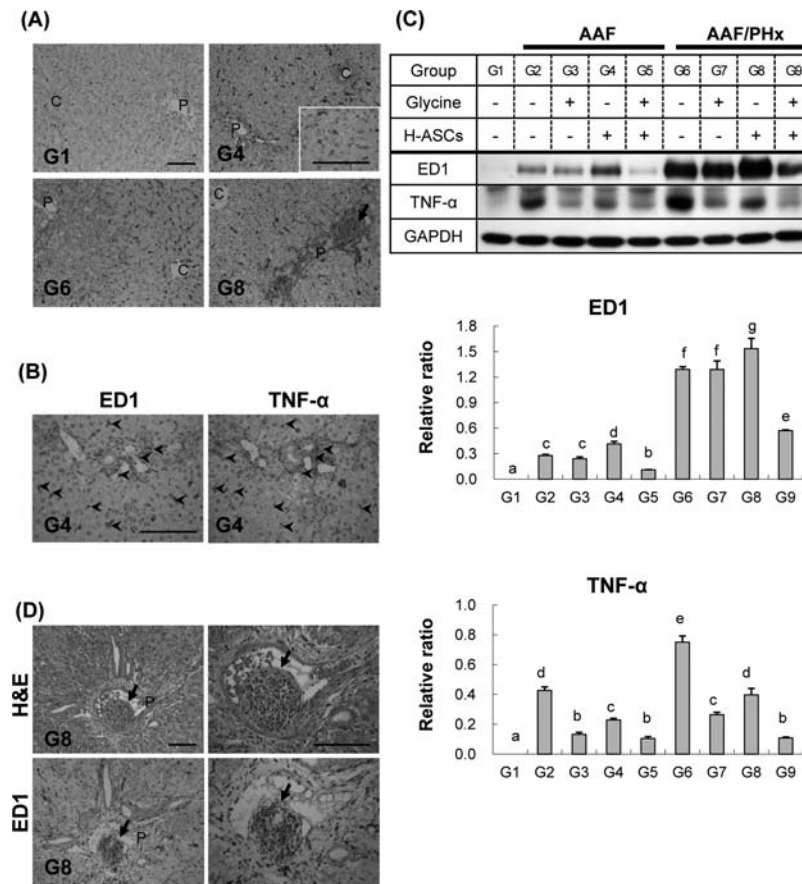
To understand the role of inactivation of Kupffer cells in transplantation, we used several animal models of liver injury. The general strategy and animal groups are shown in Figure 1A.

To examine if h-ASC transplantation might change liver regeneration ratio, we determined this ratio in all experimental groups (Fig. 1B, C). In the AAF groups (G2–G5), there was no significant change of liver/body weight ratio among groups. The regeneration ratio was  $4.08\text{--}4.43 \pm 0.18\text{--}0.50$ . These values were similar to the control group (G1) ( $4.31 \pm 0.08$ ) (Fig. 1B). In the AAF/PHx groups (G6–G9), the liver regeneration ratio had no significant change by the sole treatment of glycine (G7) (R1:  $3.30 \pm 0.32$ , R2:  $62.70 \pm 12.84$ ) or h-ASCs (G8) (R1:  $3.19 \pm 0.25$ , R2:  $59.74 \pm 4.78$ ) compared to the AAF/PHx group (G6) (R1:  $2.90 \pm 0.15$ , R2:  $60.26 \pm 2.98$ ). However, cotreatment of glycine and h-ASCs significantly increased regeneration ratio as R1:  $3.72 \pm 0.46$ , R2:  $73.42 \pm 2.41$  in the AAF/PHx+Glycine+ASCs group (G9) compared to the AAF/PHx group (G6) (Fig. 1B, C). Thus, these studies revealed that transplantation of h-ASCs with glycine increased liver regeneration ratio in animals.

### *Kupffer Cell/Macrophage Activation Is Inhibited by Glycine and h-ASC Treatments*

Given the established delivery of h-ASCs to the liver, we asked if the treatment with glycine inhibits activity of Kupffer cells in rats. It has been shown that Kupffer cells are resident macrophages in the liver and that Kupffer cells release TNF- $\alpha$ , which in turn induces oval cell proliferation in AAF/PHx-induced liver injuries (27). Therefore, we examined the secretion of TNF- $\alpha$  as a function of Kupffer cells. IHC with antibodies to ED1 (Kupffer cell marker) showed that ED1 was expressed in the cytoplasm of Kupffer cells (Fig. 2A), and TNF- $\alpha$  expression was colocalized with ED1-positive cells (Fig. 2B). ED1 and TNF- $\alpha$  were increased by AAF-induced liver injuries (G2) and were increased even more by AAF/PHx-induced liver injuries (G6) compared to the





**Figure 2.** Kupffer cell/macrophage activation is inhibited by glycine and h-ASC treatments. (A) Kupffer cells locate in sinusoids and are detected by ED1 (CD68). The arrow in G8 group shows the infiltrating macrophages from the portal vein. (B) Tumor necrosis factor (TNF)- $\alpha$  expression is colocalized with ED1-positive cells (arrowhead). (C) The panels show relative ratio of ED1 and TNF- $\alpha$  to GAPDH (glyceraldehyde 3-phosphate dehydrogenase). (D) Numerous macrophages (arrow) from the portal vein infiltrate into liver tissue in G8. G1, control group; G2, AAF group; G3, AAF+Glycine group; G4, AAF+ASCs group; G5, AAF+Glycine+ASCs group; G6, AAF/PHx group; G7, AAF/PHx+Glycine group; G8, AAF/PHx+ASCs group; G9, AAF/PHx+Glycine+ASCs group. C, central vein; P, portal vein. IHC for ED1 (A, B, D) and TNF- $\alpha$  (B). Scale bar: 100  $\mu$ m. Each value represents mean  $\pm$  SD. <sup>a-g</sup>Values not sharing a common superscript letter differ significantly at  $p < 0.05$  by Duncan's multiple comparison test.

control group (G1). In agreement with our hypothesis, the number of Kupffer cells was significantly increased by h-ASC transplantation in both the AAF+ASCs (G4) and AAF/PHx+ASCs (G8) groups, compared to the other groups (Fig. 2C). It is important to note that numerous macrophages from portal veins infiltrated into the liver in the AAF/PHx+ASCs group (G8) (Fig. 2A, D). The number of infiltrated Kupffer cells was unchanged by glycine treatment; however, TNF- $\alpha$  secretion was markedly decreased by glycine treatment in both the AAF+Glycine (G3) and AAF/PHx+Glycine (G7) groups, compared to the AAF (G2) and AAF/PHx (G6) groups, respectively. Interestingly, TNF- $\alpha$  secretion was decreased by h-ASC transplantation in the AAF+ASCs (G4) and AAF/PHx+ASCs (G8) groups, although these groups had the largest increase in the number of Kupffer cells at the same time. Furthermore, cotreatment of glycine and h-ASCs

significantly decreased the number of Kupffer cells and TNF- $\alpha$  secretion in the AAF+Glycine+ASCs (G5) and AAF/PHx+Glycine+ASCs (G9) groups; much more than in comparison to the AAF+Glycine (G3) and AAF/PHx+Glycine (G7) groups, respectively (Fig. 2C). Taken together, these studies revealed that h-ASC transplantation recruits numerous Kupffer cells into the liver, and glycine treatment inhibits the activity of Kupffer cells. Moreover, successfully transplanted ASCs in liver tissue by glycine treatment also inhibit the activity of Kupffer cells.

#### *h-ASC Transplantation Does Not Recruit B and T Lymphocytes in the Liver*

To examine whether h-ASC transplantation recruits other inflammatory cells into the liver, IHC with CD79a (B lymphocyte marker) and CD3 (T lymphocyte marker)

was performed (data not shown). The spleen was used as a positive control for IHC of B and T lymphocytes. In IHC studies with CD79a, the spleen of the control group showed numerous CD79a-positive cells, which were normally detected in coronal areas that surround the germinal center of white pulp, whereas CD79a-positive cells were not detected in their livers. CD79a-positive cells were also not detected in liver tissue of the other groups. In IHC studies with CD3, the spleen of the control group showed some CD3-positive cells, which were normally detected in the germinal center of white pulp, whereas these cells were not detected in their livers. IHC results were also the same in the other groups. Thus, there were no significant changes in the B and T lymphocyte populations by glycine and h-ASC treatments in the liver.

#### *Livers of Recipient Rats Contain High Numbers of Transplanted h-ASCs and Express Human Albumin*

To examine if the transplantation of h-ASCs was successful, we have determined the number of h-ASCs and the expression of human albumin as a marker of these cells. The h-ASCs were prestained with DAPI to distinguish the transplanted cells from endogenous ones. Numerous scattered DAPI-labeled h-ASCs were found in rat livers (Fig. 3A). They were usually found to flock together around large vessels. The number of transplanted h-ASCs was more abundant in the AAF/PHx groups (G6–G9) than in the AAF groups (G2–G5). Glycine treatment significantly increased the transplantation numbers of h-ASCs into rat recipient livers in both the AAF+Glycine+ASCs (G5) and AAF/PHx+Glycine+ASCs (G9) groups (Fig. 3B). The human albumin mRNA was detected only in h-ASC-recipient livers of rats through RT-PCR analysis (Fig. 3C). To confirm this observation at the protein level, we performed IHC with antibodies specific to human albumin. This approach showed that DAPI-labeled h-ASCs express human albumin protein in their cytoplasm (Fig. 3D). Thus, these experiments revealed that h-ASCs are successfully transplanted in liver tissues through Kupffer cell inactivation, and then transplanted h-ASCs secrete albumin protein.

#### *Glycine and h-ASC Treatments Decrease Injury Factors Induced by AAF/PHx*

Given the successful transplantation of h-ASCs in the liver, we asked if this transplantation changes liver morphology and the proliferation of other cells in the liver. The control group had a normal liver tissue structure, and there were no significant lesions (Fig. 4A). Administration of AAF slightly induced oval cell proliferation in periportal regions of the liver (G2), whereas oval cell proliferation in periportal regions was highly increased by AAF/PHx-induced injury (G6) (Fig. 4A). It is known that administration of AAF induces TGF- $\beta$

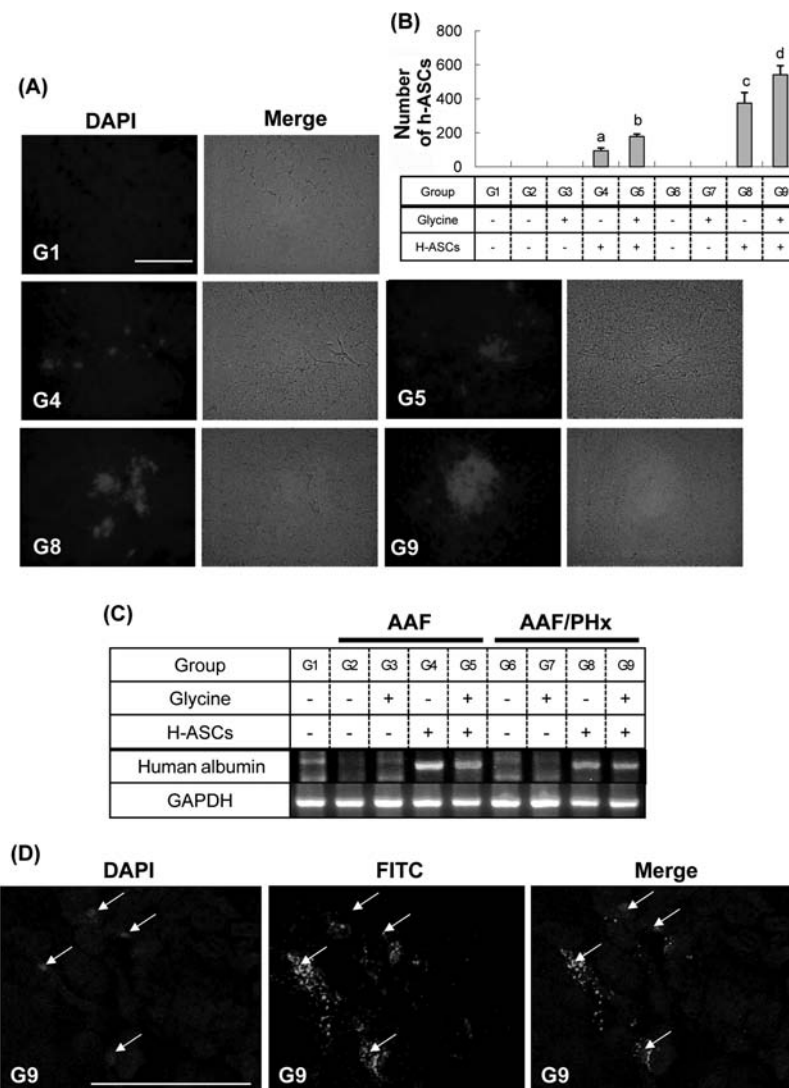
expression in the liver, and TGF- $\beta$  causes marked growth inhibition to hepatocytes (37). Immunoblot analysis of TGF- $\beta$ 1 in the liver (Fig. 4B) showed only rare expression of TGF- $\beta$ 1 in the AAF groups (G2–G5) compared with the control group (G1), whereas TGF- $\beta$ 1 expression was high in the AAF/PHx group (G6) and was significantly decreased by glycine (G7) and h-ASC treatments (G8). The expression of TGF- $\beta$ 1 was hardly seen in the AAF/PHx+Glycine+ASCs group (G9).

Under growth inhibition of hepatocytes by AAF, oval cells proliferate to compensate for the damaged liver (34). AFP is an oval cell marker, and its expression decreases following oval cell differentiation into hepatocytes during liver regeneration in AAF/PHx-induced liver injuries (35). We found that AFP was expressed in the cytoplasm of oval cells, which was occasionally observed in periportal regions of control livers (Fig. 4C). In the AAF groups (G2–G5), a few AFP-expressing oval cells were observed in periportal regions with no significant differences among the AAF groups. In the AAF/PHx groups (G6–G9), the expression of AFP highly increased in the AAF/PHx group (G6) and was then significantly decreased by glycine (G7) and h-ASC (G8) treatments. Cotreatment of glycine and h-ASCs (G9) significantly decreased AFP expression compared to the other groups (Fig. 4B, C).

We next examined the proliferation of liver cells by measuring PCNA because it is a marker expressed in the nucleus of proliferating cells. PCNA has been shown to be expressed in proliferative oval cells in the AAF/PHx-induced liver injury model (43). In the control liver, PCNA expression was occasionally observed in binuclear hepatocytes undergoing proliferation. In the AAF and AAF/PHx groups (G2–G9), while there was no expression of PCNA in hepatocytes, PCNA was expressed in the nucleus of oval cells in the periportal areas (Fig. 4D). AFP expression is relatively colocalized with PCNA-positive cells (Fig. 4E). In the AAF groups (G2–G5), there were no significant changes in AFP and PCNA expression. Among the AAF/PHx groups (G6–G9), expression of both AFP and PCNA was highest in the AAF/PHx group (G6) and was decreased by glycine (G7) and h-ASC (G8) treatment. Cotreatment of glycine and h-ASCs significantly decreased PCNA expression along with decreased oval cell proliferation in the AAF/PHx+Glycine+ASCs group (G9) compared to the other groups (Fig. 4B). Taken together, our studies showed that transplantation of h-ASCs and glycine treatment decrease injury factors in the liver induced by AAF/PHx.

#### *Hepatocyte Regeneration Is Improved by h-ASC and Glycine Treatments*

We have examined SMP30 expression in the liver to evaluate the regeneration rate of hepatocytes. SMP30

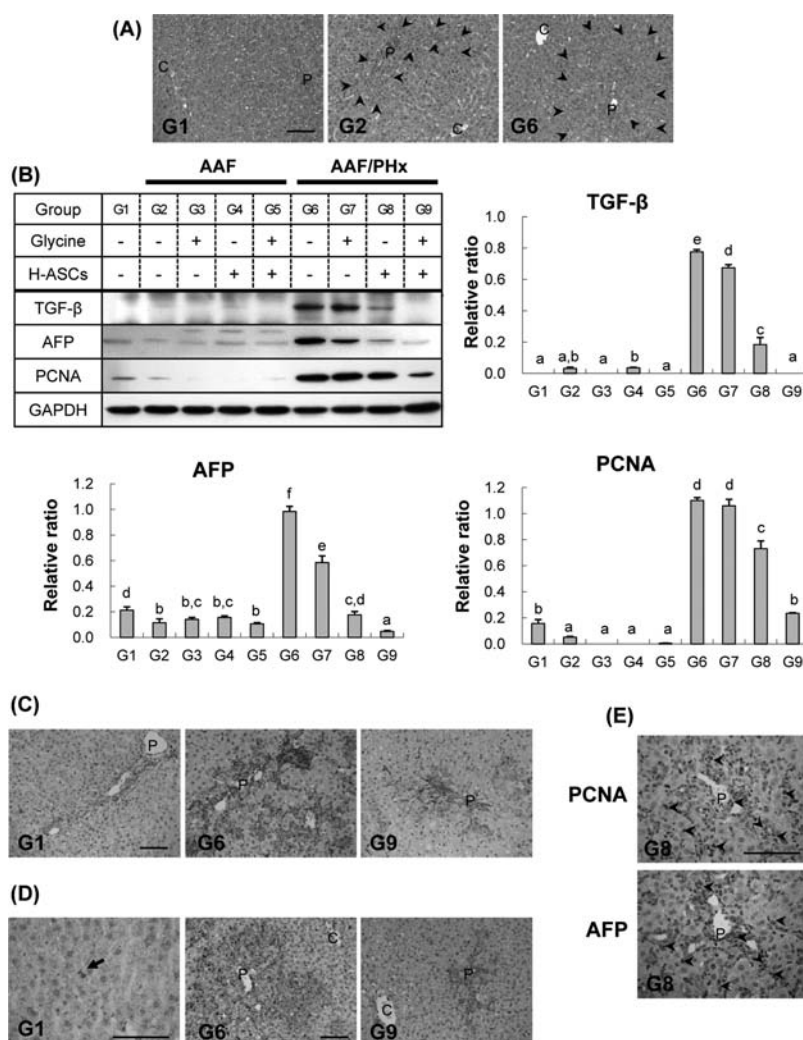


**Figure 3.** The number of transplanted h-ASCs and human albumin expression in rat recipient livers. The h-ASCs were prestained with DAPI prior to transplantation. (A) Left panels show fluorescence, and right panels show bright-field merged with fluorescence. There is numerous scattered nuclear blue fluorescence (DAPI) in rat recipient livers except in the control group (G1). (B) The number of transplanted h-ASCs is counted by analyzing five random fields (200 $\times$ ) per slide of each animal. (C) Human albumin mRNA was analyzed by RT-PCR. GAPDH was used as a loading control. (D) By IHC, human albumin (FITC) is expressed in the cytoplasm of DAPI-labeled h-ASCs (arrow). G1, control group; G2, AAF group; G3, AAF+Glycine group; G4, AAF+ASCs group; G5, AAF+Glycine+ASCs group; G6, AAF/PHx group; G7, AAF/PHx+Glycine group; G8, AAF/PHx+ASCs group; G9, AAF/PHx+Glycine+ASCs group. Scale bar: 100  $\mu$ m. Each value is presented as mean  $\pm$  SD. <sup>a-d</sup>Values not sharing a common superscript letter differ significantly at  $p < 0.05$  by Duncan's multiple comparison test.

has been regarded as a multifunctional protein, providing protection to cellular functions from oxidative stress in various organs (33,36,48). By IHC, SMP30 is normally expressed in the cytoplasm and nuclei of uninjured hepatocytes, whereas it is decreased in injured liver with a recovery of expression in regenerated hepatocytes (33).

In the control group (G1), SMP30 was highly expressed in the cytoplasm and nuclei of hepatocytes

around the central veins. Its expression decreased in the AAF (G2) and AAF/PHx (G6) groups; however, SMP30 expression was recovered by glycine (G3, G7) and h-ASC treatments (G4, G8). Cotreatment of glycine and h-ASCs significantly increased SMP30 expression in both the AAF+Glycine+ASCs (G5) and AAF/PHx+Glycine+ASCs (G9) groups compared to the other groups (Fig. 5). Thus, transplantation of h-ASCs and glycine treatment induce hepatocyte regeneration effectively.



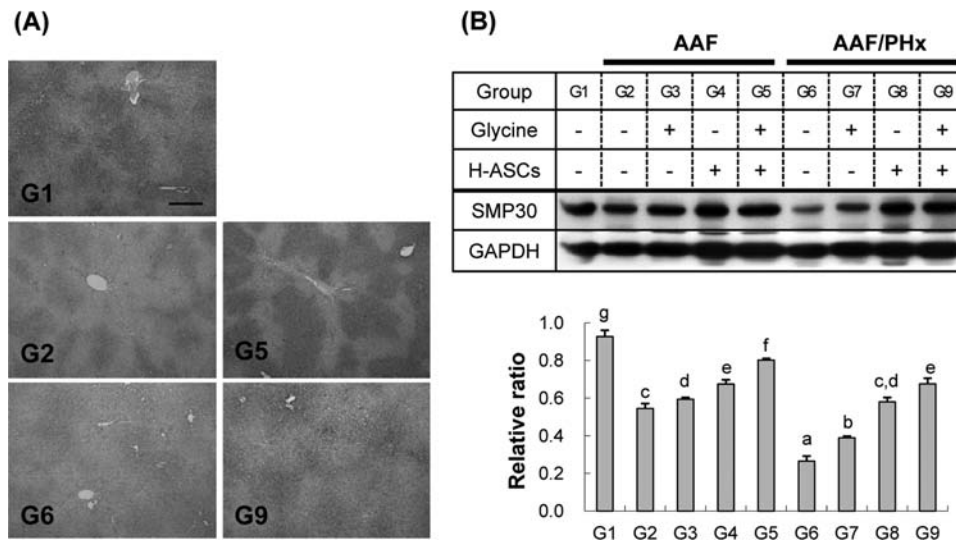
**Figure 4.** Transplantation of h-ASCs and glycine treatment decrease the levels of TGF- $\beta$ 1, AFP, and PCNA induced by AAF/PHx. (A) Proliferation of oval cells (arrowhead) in the periportal regions is evident. The population of oval cells appears as dark areas due to their large nuclei and small amount of cytoplasm. (B) The panels show the relative ratio of transforming growth factor (TGF)- $\beta$ 1,  $\alpha$ -fetoprotein (AFP), and proliferating cell nuclear antigen (PCNA). (C) AFP is expressed in the cytoplasm of oval cells around portal veins. (D, E) PCNA is occasionally expressed in binuclear hepatocytes (arrow) of the control liver. However, in AAF and AAF/PHx-induced liver injuries, PCNA is expressed in proliferating oval cells (arrowhead) that are also positive for AFP (arrowhead). C, central vein; P, portal vein. G1, control group; G2, AAF group; G3, AAF+Glycine group; G4, AAF+ASCs group; G5, AAF+Glycine+ASCs group; G6, AAF/PHx group; G7, AAF/PHx+Glycine group; G8, AAF/PHx+ASCs group; G9, AAF/PHx+Glycine+ASCs group. H&E (A), IHC for AFP (C, E) and PCNA (D, E). Scale bar: 100  $\mu$ m. Each value represents mean  $\pm$  SD. <sup>a–f</sup>Values not sharing a common superscript letter differ significantly at  $p < 0.05$  by Duncan's multiple comparison test.

#### *h-ASC Transplantation and Inhibition of Kupffer Cells Correct Liver Dysfunctions Mediated by Liver Injury*

One of the main functions of the liver is the secretion of proteins into the blood to support body homeostasis. Therefore, we examined if the glycine-mediated improvement of h-ASC transplantation might correct the protein secretion function of the liver that is decreased by injury. For this goal, we measured levels of albumin in the serum of experimental rats. The serum albumin was significantly decreased in the AAF groups (G2–G5)

compared to the control group (G1). Although albumin was slightly increased by glycine and h-ASC treatments, there was no significant change among the AAF groups (Fig. 6A). The AAF/PHx group (G6) showed a severe decrease in serum albumin compared to the control and AAF groups (G2–G5), whereas the amount of albumin was slightly increased by the h-ASC transplantation in the AAF/PHx+ASCs group (G8) compared to the AAF/PHx group (G6). Furthermore, cotreatment of glycine and h-ASCs showed a significant increase of serum albumin





**Figure 5.** SMP30 expression is improved by h-ASCs and glycine treatments. (A) The Senescence marker protein 30 (SMP30) expression is observed in the cytoplasm and nucleus of hepatocytes around the central veins. Scale bar: 100  $\mu$ m. (B) The panel shows the relative ratio of SMP30 compared to GAPDH. G1, control group; G2, AAF group; G3, AAF+Glycine group; G4, AAF+ASCs group; G5, AAF+Glycine+ASCs group; G6, AAF/PHx group; G7, AAF/PHx+Glycine group; G8, AAF/PHx+ASCs group; G9, AAF/PHx+Glycine+ASCs group. Each value represents mean  $\pm$  SD. <sup>a-g</sup>Values not sharing a common superscript letter differ significantly at  $p < 0.05$  by Duncan's multiple comparison test.

in the AAF/PHx+Glycine+ASCs group (G9) compared to the AAF/PHx (G6) and AAF/PHx+Glycine groups (G7). Thus, these studies showed that the glycine-mediated successful transplantation of h-ASCs partially corrects the protein secretion function of the liver.

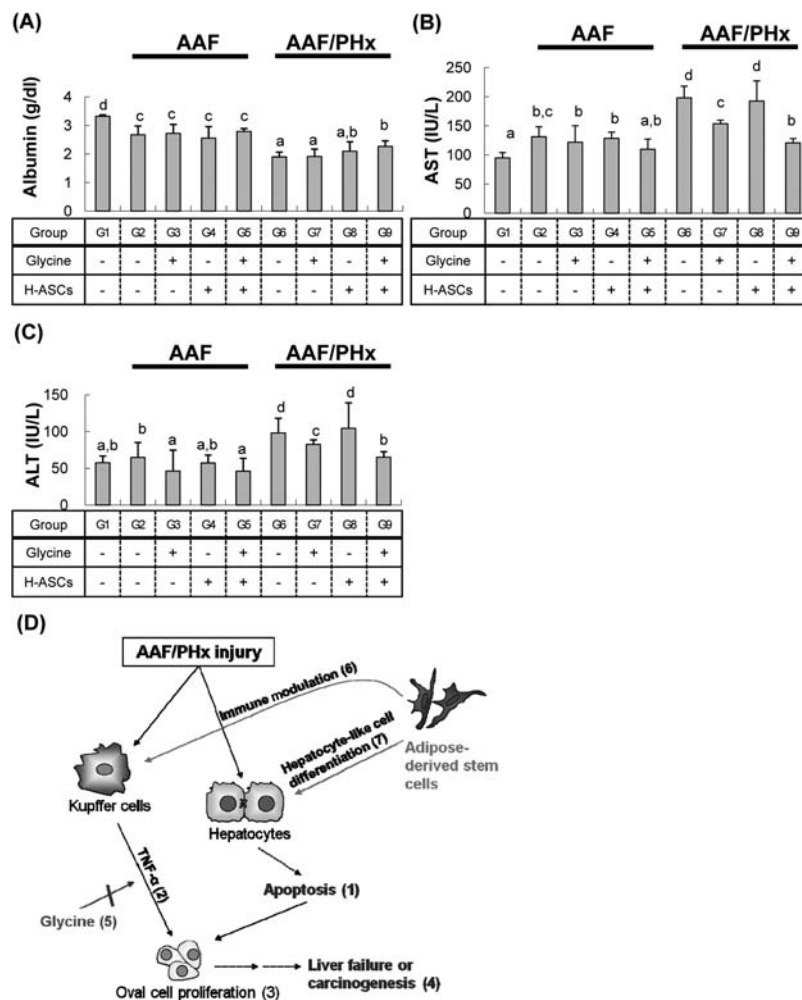
It has been demonstrated that liver injury causes elevation of the activities of AST and ALT in serum (30). We next asked if the transplantation of h-ASCs under the condition of Kupffer cell inhibition might correct this liver dysfunction. The AAF group (G2) showed an increase of AST and ALT compared to the control group (G1). AST was decreased in the AAF+Glycine+ASCs group (G5), and ALT was significantly decreased by glycine treatment in the AAF+Glycine (G3) and AAF+Glycine+ASCs (G5) groups, compared to the AAF group (G2). AST and ALT were significantly increased in all AAF/PHx groups (G6–G9) compared to the control group (G1). Both were significantly decreased by glycine treatment in the AAF/PHx+Glycine group (G7); furthermore, cotreatment of glycine and h-ASCs significantly decreased AST and ALT in the AAF/PHx+Glycine+ASCs group (G9) compared to the AAF/PHx+Glycine (G7) and AAF/PHx+ASCs groups (G8) (Fig. 6B, C). Taken together, our studies showed that the inhibition of Kupffer cell activity leads to an improvement of transplantation of h-ASCs, and that this improvement corrects several liver functions.

## DISCUSSION

The present study has begun based on two hypotheses to improve transplantation efficiency and verify functional

role of stem cells in liver injury. The major findings in our study were as follows: (i) the establishment of proper liver injury is important for the homing of h-ASCs into injured tissue in animal experiments; (ii) transplanted h-ASCs in liver tissue recruit numerous Kupffer cells; (iii) the number of transplanted h-ASCs in the liver is significantly increased when Kupffer cell activity is inhibited by glycine; (iv) successfully transplanted h-ASCs in liver tissue modulate Kupffer cell activity to inhibit TNF- $\alpha$  secretion; (v) transplanted h-ASCs in liver tissue differentiate into hepatocyte-like cells secreting albumin, and play a role in liver regeneration and reduce AST and ALT.

When it comes to transplantation immunology, matching of donor and recipient for major histocompatibility complex (MHC) antigens has been shown to have a positive effect on graft acceptance. The leukocyte antigens are controlled by the closely linked genes of the MHC. Differences in the MHC antigens or HLAs lead to the tolerance or rejection of grafts and graft-versus-host diseases in humans due to induction of antibodies, antigen-presenting cells, helper and cytotoxic T-cell subsets, immune cell-surface molecules, signaling mechanisms, and cytokines (12). However, human MSCs are poorly immunogenic in spite of constitutive HLA-class I expression, and it has been suggested that they could be transplantable between HLA-incompatible individuals (24,25). Moreover, MSCs from various sources have similar phenotypes and comparable differentiation potentials and immunosuppressive properties in culture (37). MSCs mediate immunoregulatory activities by inhibiting



**Figure 6.** h-ASC transplantation and inhibition of Kupffer cell activity correct liver dysfunctions. Levels of albumin (A), aspartate aminotransferase (AST) (B), and alanine aminotransferase (ALT) (C) in serum of experimental rats. G1, control group; G2, AAF group; G3, AAF+Glycine group; G4, AAF+ASCs group; G5, AAF+Glycine+ASCs group; G6, AAF/PHx group; G7, AAF/PHx+Glycine group; G8, AAF/PHx+ASCs group; G9, AAF/PHx+Glycine+ASCs group. Each value is presented as the mean  $\pm$  SD. <sup>a-d</sup>Values not sharing a common superscript letter differ significantly at  $p < 0.05$  by Waller-Duncan's multiple comparison test. (D) A diagram showing a hypothetical mechanism for the role of h-ASCs in AAF/PHx-induced liver injury of rats. AAF/PHx induces damage of hepatocytes, inhibition of hepatocyte replication (1), and TNF- $\alpha$  secretion from Kupffer cells (2). Oval cells may retain the ability to proliferate in the presence of TNF- $\alpha$  (3). If there is continuously severe liver damage by AAF/PHx, liver failure or carcinogenesis may occur (4). However, glycine administration decreases TNF- $\alpha$  and improves transplantation of h-ASCs into the liver through down-regulation of Kupffer cell activity (5). Transplanted h-ASCs may inhibit TNF- $\alpha$  secretion by modulation of Kupffer cell activity (6) and have protective effects on the liver through differentiation into hepatocyte-like cells that secrete albumin (7). Therefore, h-ASC transplantation with glycine administration gives synergic effects to engraftment of ASCs and liver protection.

the functions of different cell types, including T and B lymphocytes, dendritic cells (DCs), and NK cells (28,38), and they can exert immunosuppressive and immunostimulatory effects depending on the extent of the stimulus (39,40). These biological mechanisms remain largely unclear to date. Suppression of T-cell proliferation and cytokine production by MSCs has been observed in many studies (1,14,17,40,42). MSCs inhibit the in vitro differentiation of DCs from progenitors and reduce proinflammatory cytokine secretion [interleukin (IL)-12, interferon

(IFN)- $\gamma$ , TNF- $\alpha$ ], which may lead to an immunologic tolerance (1,9,19,49). Furthermore, the immunosuppressive functions of MSCs support their therapeutic interest in a variety of diseases related to alloreactive immunity or autoimmunity (4,23). However, only a few studies have investigated the ability of MSCs to suppress immune response following in vivo transplantation (28,29).

Macrophages participate in initial capture and processing of potential antigens (innate immunity) and then in activation of specific T and B lymphocyte effector

mechanisms (adaptive immunity) (46). Xia et al. observed the presence of macrophages with implanted human bone marrow MSCs in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (T- and B-cell deficiency) (51). Chen et al. have reported that MSCs recruit macrophages, but not T lymphocytes, by secretion of monocyte chemoattractive cytokines in the skin-wounded mouse model (11). We consider that the innate immunity of macrophages against MSCs has been disregarded since they have focused on the immunomodulative actions of MSCs. Our study demonstrated that ASC injection recruits Kupffer cells/macrophages into liver tissue without changing T and B lymphocyte expression, whereas when Kupffer cell activity is inhibited, the number of ASCs transplanted into livers is increased. Moreover, the successfully transplanted ASCs inhibit TNF- $\alpha$  secretion from Kupffer cells and improve liver regeneration.

Our study shows that glycine is a good candidate for the inhibition of Kupffer cell activity in ASC transplantation for liver diseases. Glycine has been reported to protect liver functions in a variety of diseases of experimental models such as ischemia-reperfusion injury, shock, arthritis, tumor, drug toxicity, alcoholic hepatitis, hepatic fibrosis, and liver transplantation (10,18,41,45,50,54). The mechanisms of this protection include suppression of Ca<sup>2+</sup> signaling, inhibition of inflammatory cell activation, decreased formation of free radicals, other toxic mediators, and blockage of plasma membrane permeabilization preceding oncotic necrosis (54). Rentsch et al. suggested that glycine down-regulates Kupffer cell activity without alteration of Kupffer cells number (41). Ito et al. showed that glycine administration prevents the elevation of serum AST, ALT, and TNF- $\alpha$  in ischemia-reperfusion injuries with PHx (18). Glycine seems to be nontoxic compared to other Kupffer cell inhibitors including gadolinium chloride and methyl palmitate (18,45) and can be clinically used with ASC transplantation for injured liver.

Taking together the data of our study, we suggest that h-ASC transplantation recruits Kupffer cells and that Kupffer cells may destroy many of the h-ASCs through a phagocytic response, whereas glycine treatment improves transplantation of h-ASCs into liver tissue through down-regulation of Kupffer cell activity. Once h-ASCs have successfully transplanted into the liver, the h-ASCs can modulate Kupffer cell activity to inhibit TNF- $\alpha$  secretion and have a protective effect through differentiation into hepatocyte-like cells that secrete albumin (Fig. 6D). Thus, Kupffer cell inactivation is important to improve h-ASC transplantation efficiency and therapeutic potentials in liver injuries.

**ACKNOWLEDGMENTS:** This research was supported by Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology (2010-0022587) and Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111551). The authors declare no conflict of interest.

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