

## IFATS Collection: In Vivo Therapeutic Potential of Human Adipose Tissue Mesenchymal Stem Cells After Transplantation into Mice with Liver Injury

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**Key Words.** Adipose • Mesenchymal stem cells • Liver regeneration • Liver function

### ABSTRACT

Mesenchymal stem cells (MSCs), largely present in the adult human body, represent an attractive tool for the establishment of a stem cell-based therapy for liver diseases. Recently, the therapeutic potential and immunomodulatory activity of MSCs have been revealed. Adipose tissue-derived mesenchymal stem cells (AT-MSCs), so-called adipose-derived stem cells or adipose stromal cells, because of their high accessibility with minimal invasiveness, are especially attractive in the context of future clinical applications. The goal of the present study was to evaluate the therapeutic potential of AT-MSCs by their transplantation into nude mice with CCl<sub>4</sub>-caused liver injury. We observed that after transplantation, AT-MSCs can improve liver functions, which we verified by changes in the levels of biochemical parameters. Ammonia, uric acid, glutamic-pyruvic transaminase, and glutamic-oxaloacetic transaminase concentrations returned to a nearly normal level after AT-MSC

transplantation. These results raised the question of how AT-MSCs can achieve this. To discover the possible mechanisms involved in this therapeutic ability of AT-MSCs, in vitro production of cytokines and growth factors was analyzed and compared with MSCs from bone marrow (BM-MSCs) and normal human dermal fibroblasts (NHDFs). As a result we observed that AT-MSCs secrete interleukin 1 receptor  $\alpha$  (IL-1R $\alpha$ ), IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein 1, nerve growth factor, and hepatocyte growth factor in a volume higher than both BM-MSCs and NHDFs. Thus, our findings suggest that AT-MSCs may account for their broad therapeutic efficacy in animal models of liver diseases and in the clinical settings for liver disease treatment. *STEM CELLS* 2008;26:2705–2712

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

The establishment of stem cell therapy for the liver is of great significance. The liver is exposed to many factors such as drugs, xenobiotics, and viruses, which cause chronic hepatitis and liver cirrhosis. In most cases, these illnesses lead to hepatocellular carcinoma and, finally, to organ failure, where there is chronic inflammation, fibrosis, and no longer any ability to regenerate [1]. The only effective treatment to date is orthotopic liver transplantation, but because of the limited number of donors and organ rejection, alternative approaches are needed. One such approach for the treatment of liver failure is stem cells. After

transplantation, they can support a host's liver function and thereby can open the way to further treatment and liver regeneration.

The preeminent candidate stem cells for therapy of an injured liver are mesenchymal stem cells (MSCs), which can be obtained from different sources such as bone marrow (BM) [2], umbilical cord blood [3], amniotic fluid [4], scalp tissue [5], placenta [6], and adipose tissue (AT) [7, 8] of the human body. MSCs possess both a multipotentiality and a semi-infinite proliferation ability [2–8]. Currently, attention is being given to AT as a source of MSCs for regenerative medicine [7–9]. From this tissue, a sufficient number of stem cells for stem cell-based therapy may be obtained without invasiveness or damage to a

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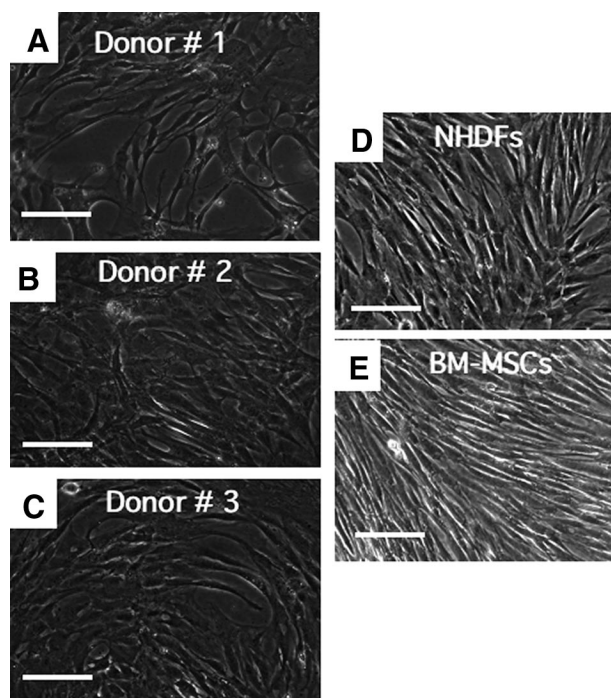
patient's health. The possibility for their future application in the therapy of liver diseases is very promising. MSCs can be easily obtained from a patient's own tissues, isolated *ex vivo*, expanded, and transplanted back into the patient as an autologous transplant. Such a possibility sidesteps the limits governing ethical issues and immunocompatibility problems. Importantly, human MSCs represent an advantageous cell type for allogeneic transplantation as well, since they are immunoprivileged with low human leukocyte antigen (HLA) I and no HLA II expression, thereby reducing the risk of allogeneic transplant rejection and preventing graft-versus-host disease (GVHD) [10–15]. It has been proposed that MSCs act as “trophic mediators” [16–22], which by secretion of bioactive factors act as either immunosuppressors or promoters of regeneration [17]. It is already anticipated that MSCs in animal models can engraft into the liver and ameliorate liver injury caused by  $\text{CCl}_4$  [23–26], allyl-alcohol [27], or retrorsine [28] injection or by combined radiation and  $\text{CCl}_4$  injection [29]. Mechanisms, however, are unknown and opinions remain controversial [24–31]. Among the proposed ideas, very highly convincing is the hypothesis of the paracrine effects of MSCs, by which, through secretion of active factors, amelioration of liver damage occurs [18, 20]. We have already demonstrated that human AT-MSCs have the ability to give rise to hepatocyte-like cells and that CD105 is one of the candidate mesenchymal stem cell markers [32–33].

In this study, we evaluate the therapeutic potential of AT-MSCs with respect to their future clinical usage for the treatment of liver failure. The usage of undifferentiated AT-MSCs, which are minimally manipulated *ex vivo* and easily obtained within a short period of time, is crucial for future clinical emergency usage. We transplanted human AT-MSCs into immunodeficient mice with acute liver failure caused by  $\text{CCl}_4$  injection. Our results show a significant decrease of ammonia, glutamic-oxaloacetic transaminase (GOT), uric acid (UA), and glutamic-pyruvic transaminase (GPT) concentration 24 hours after transplantation of undifferentiated AT-MSCs, and also indicate that undifferentiated AT-MSCs are involved in liver regeneration *in vivo*. Because the mechanisms are still unclear, however, we postulate that involvement may be, in part, due to the pleiotrophic contribution through the direct and/or indirect activity of AT-MSCs. We evaluated *in vitro* production of cytokines/growth factors by undifferentiated AT-MSCs and compared it with BM-MSCs and normal human dermal fibroblasts (NHDFs). We detected higher secretion of interleukin 1 receptor antagonist (IL-1RA), IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP-1), nerve growth factor (NGF), and hepatocyte growth factor (HGF) by AT-MSCs, as opposed to BM-MSCs and NHDFs. The vascular endothelial growth factor (VEGF) was secreted similarly by both types of MSCs, higher, however, than by NHDFs. Summarizing, our *in vitro* study shows a higher production of bioactive factors in AT-MSCs than in BM-MSCs. Our *in vivo* study shows the ability of AT-MSCs to incorporate into the liver and improve its function. The regenerative ability of AT-MSCs may be possibly due to trophic activity of AT-MSCs.

## MATERIALS AND METHODS

### Isolation and Culturing of MSCs

AT-MSCs were derived from abdominal subcutaneous adipose tissue, which was obtained from gastric cancer patients (donor 1: female, 36 years old; donor 2: female, 55 years old; and donor 3: male, 45 years old) undergoing gastrectomy at the International



**Figure 1.** Morphology of adipose tissue-derived mesenchymal stem cells. Donors 1 (A), 2 (B), and 3 (C); NHDFs (D); and BM-MSCs (E). Scale bars = 50  $\mu\text{m}$ . Abbreviations: BM-MSCs, bone marrow mesenchymal stem cells; NHDFs, normal human dermal fibroblasts.

Medical Center of Japan in Tokyo. The hospital's committee of ethics approved this study, and informed consent was obtained from all patients. Adipose tissue was processed as previously described [33], and banked in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Tokyo, <http://www.gibcobl.com>), supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (GIBCO-BRL), and 10% dimethyl sulfoxide (Sigma-Aldrich, Tokyo, <http://www.sigmaaldrich.com>). After thawing, cells were cultured in a DMEM/10% FBS, and at 70%–80% confluence, the cells were harvested with 0.05% trypsin-EDTA (GIBCO-BRL).

Human BM-MSCs (Cambrex Corp., Walkersville, MD, <http://www.cambrex.com>) were cultured in a mesenchymal stem cell growth medium. Normal human dermal fibroblasts (Cambrex Corp.) were cultured in DMEM supplemented with 10% FBS.

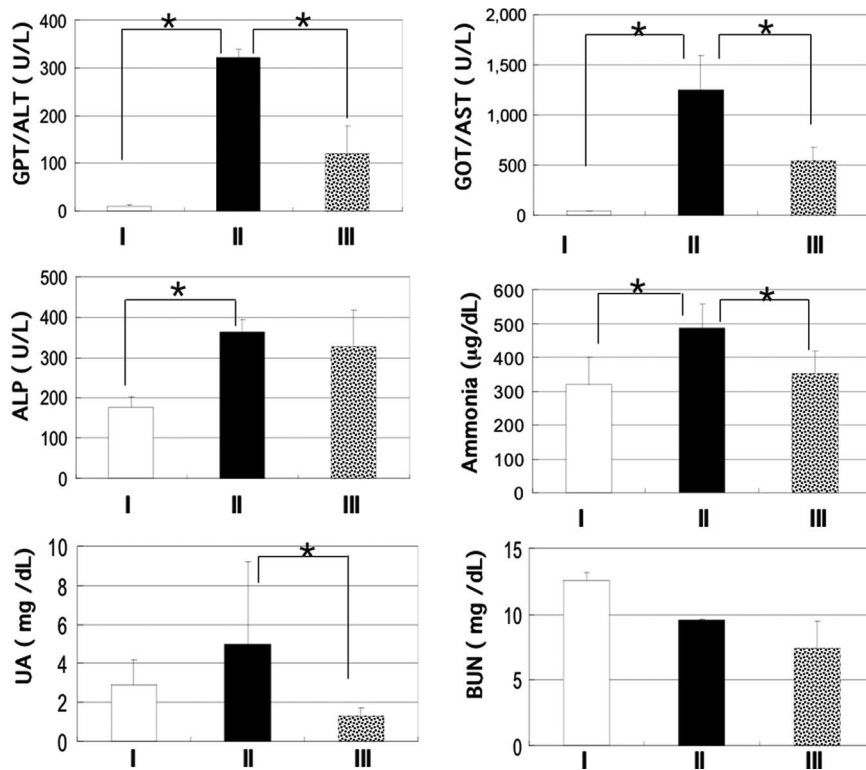
### Flow Cytometry

The phenotype profile of AT-MSCs (passages 5–6) (donors 1 and 3) was evaluated by flow cytometry analysis (fluorescence-activated cell sorting, Epic XL, Software Expo 32; Beckman Coulter, Fullerton, CA, <http://www.beckmancoulter.com>), using CD29 (BD Bioscience Pharmingen, Tokyo, <http://www.bdbiosciences.com>), CD31, CD45 (eBioscience, Tokyo, <http://www.ebioscience.com>), CD34 (DakoCytomation, Carpinteria, CA, <http://www.dakocytomation.com>), and CD105 (Ansell, Bayport, MN, <http://www.ansell.com>) antibodies, coupled to either phycoerythrin or fluorescein isothiocyanate.

### Cytokine/Growth Factor Protein Level Measurement

Cytokine/growth factor protein concentration was evaluated using Multiplex Suspension Array (Genetic Lab Corp. Ltd., Sapporo, Japan, <http://www.gene-lab.com>).

For the analyses cells, AT-MSCs (passage 5–8) (donors 1, 2, 3) ( $n = 3$  of each donor), BM-MSCs (passage five) ( $n = 3$ ), and NHDFs (passage five) ( $n = 3$ ) were plated in the same concentration on 6-well plates ( $3 \times 10^5$  cells/well). Upon confluence, they were washed with phosphate-buffered saline (PBS)(-) and incubated with serum-free DMEM/F-12 medium. After 3 days, supernatant and cells were collected for analyses of interleukins ( $1\beta$ ,  $1\alpha$ , 1RA,



**Figure 2.** Biochemical analysis. Concentration of GPT/ALT, GOT/AST, ALP, ammonia, UA, and BUN in blood serum of sacrificed mice. Group I: noninjured mice that did not undergo transplantation; group II: injured mice that underwent transplantation; group III: injured mice that received a transplant of adipose tissue-derived mesenchymal stem cells (combine data of donors 1 and 2). Data are presented as the mean  $\pm$  SD and were analyzed by the Bonferroni correction;  $n = 3$ . (\*,  $p < .05$ ). Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; UA, uric acid.

2, 4, 5, 6), hepatocyte-stimulating factors (HSFs; 7, 8, 10, 12(p40), 12(p70), 13, 15, 17), interferon (IFN)  $\beta$ , tumor necrosis factor (TNF)  $\alpha$ , GM-CSF, G-CSF, eotaxin, MCP-1, macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , interferon-gamma-inducible protein (IP)-10, TNF-related activation protein (TRAP)/CD40L/CD154, transforming growth factor (TGF)  $\beta$ , fractaline, NGF, HGF, epidermal growth factor (EGF), and VEGF.

### AT-MSC Transplantation into Mice with CCl<sub>4</sub>-Induced Liver Injury

We used 6-week-old female nude BALB/c nude mice (SLC, Tokyo, <http://www.jslc.co.jp>). Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. We applied acute liver failure model, by administration of one dose of CCl<sub>4</sub> (supplemental online Fig. 1). At day 0, mice underwent intraperitoneal injection of 100  $\mu$ L/20g body weight of olive oil containing 10  $\mu$ L of CCl<sub>4</sub>. At day 1, mice underwent transplantation of AT-MSCs (passage 10) (donor 1 [ $n = 3$ ; group IIIA]; donor 2 [ $n = 3$ ; group IIIB]) at a concentration of  $1.5 \times 10^6$  cells per mouse (0.2 ml of the cell suspension was injected through the tail vein). As a control, CCl<sub>4</sub>-treated mice ( $n = 3$ ) only (group II) and nontreated (olive oil) ( $n = 3$ ) and mice that did not undergo transplantation (group I) were used. The details of experimental groups are listed in supplemental online Figure 1. Twenty-four hours after transplantation, blood serum and liver samples were collected. Blood serum was evaluated for biochemical parameters, such as GPT, GOT, alkaline phosphatase (ALP), UA, ammonia, and blood urea nitrogen (BUN) concentration levels. The liver sections were analyzed by immunohistochemistry.

### Immunohistochemical Analyses of Mice Liver Sections After Cell Transplantation

Mice livers were harvested 24 hours after cell transplantation, fixed with 10% formalin, and embedded with paraffin. Histological analyses of liver tissues were conducted by serial tissue section and stained with hematoxylin and eosin (H&E) for conventional morphological evaluation and with anti-HLA-1 antibody (Sigma-Aldrich) for detection of human AT-MSCs in immunodeficient mouse liver.

### Assessment of Liver Functions

Blood samples were obtained from each mouse and centrifuged for 20 minutes at 5,000 rpm and serum was collected. Serum samples were tested for ammonia concentration level using Ammonia Test-Wako (Wako Pure Chemicals Co., Ltd., Japan, <http://www.wakochem.co.jp/english>). Concentration of markers of liver injury such as GPT, GOT, ALP, UA, and BUN was analyzed using FUJIFILM DRI-CHEM 3500 machine (FujiFilm, Tokyo, <http://www.fujifilm.co.jp>) and FUJI DRY CHEM SLIDES (FujiFilm), respectively, for GPT/alanine transaminase (ALT)-PIII, GOT/aspartate aminotransferase (AST)-PIII, ALP-PIII, UA-PIII, and BUN-PIII.

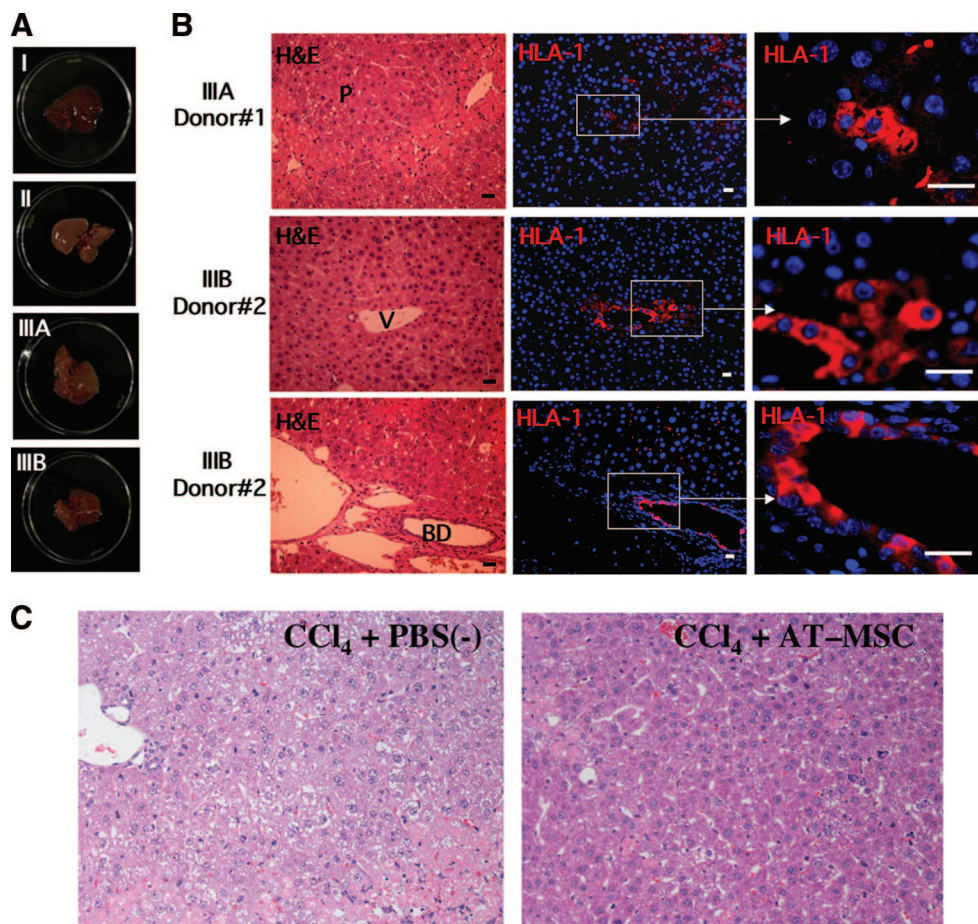
### Statistical Analysis

Results are given as the mean  $\pm$  SD. Statistical analysis were conducted or by using the use of variance with the Bonferroni correction for multiple comparisons. A  $p$  value  $< .05$  was considered significant.

## RESULTS

### Preparation and Characterization of AT-MSCs

AT-MSCs were cultivated in DMEM/10% FBS, and were replated when they reached subconfluence. While cultivated in expansion medium containing 10% FBS, the AT-MSCs of all three donors revealed similar morphology (Fig. 1A–C). AT-MSCs of donors 1 and 3 after expansion were trypsinized and analyzed by flow cytometry, which revealed that the cells of donor 1 expressed CD31 (30%), CD34 (5.3%), CD45 (0.06%), CD29 (99%), and CD105 (98.5%) and those of donor 3, CD31 (12.9%), CD34 (9.9%), CD45 (8.4%), CD29 (96%), and CD105 (63.9%). AT-MSCs of donors 1 and 2 were trypsinized and transplanted into nude mice with liver injury. AT-MSCs of all donors (1, 2, 3), as well as BM-MSCs, and NHDFs were plated in the same concentration (Fig. 1A–E). Upon reaching confluence, they were cultured for 3 days in serum-free medium and processed in vitro in a cytokine/growth factor protein array.



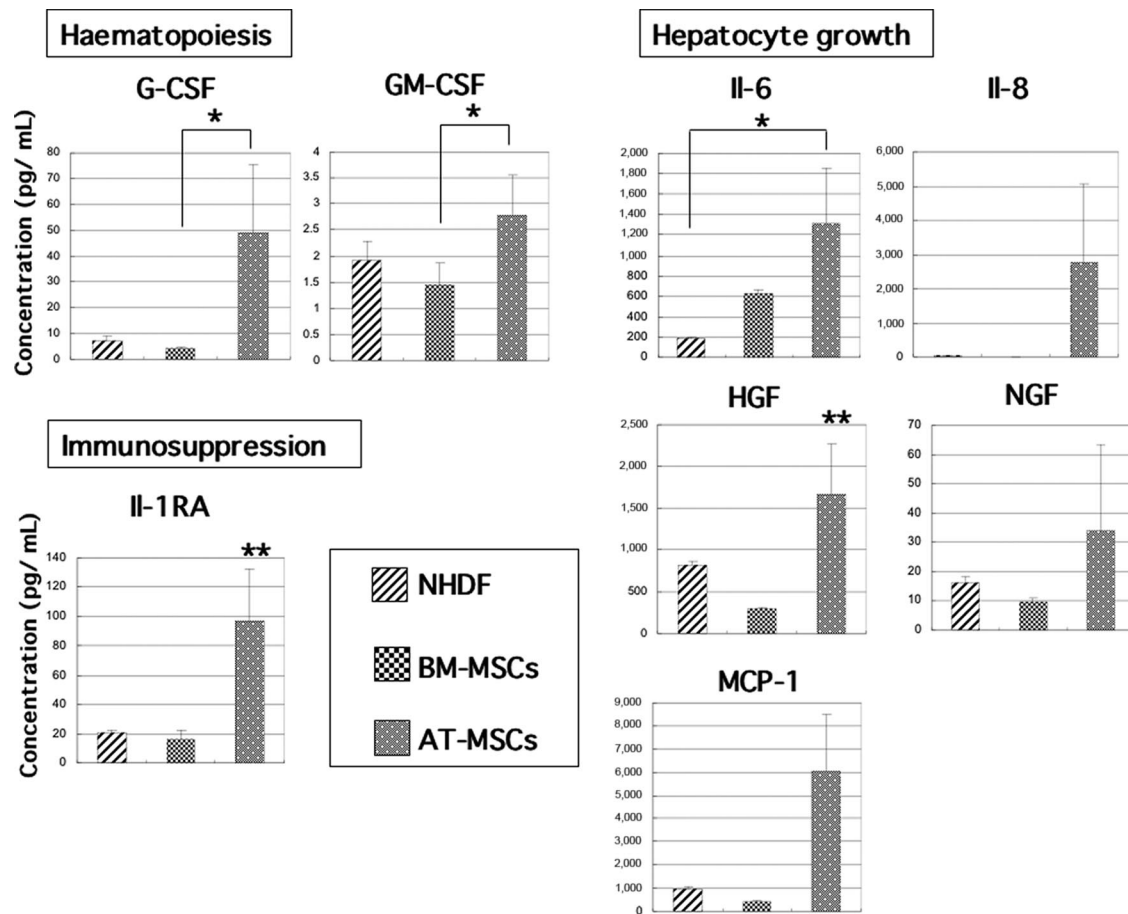
**Figure 3.** Morphological analysis. (A): Symptomatic macromorphology changes in the livers of each experimental group (I–III). (B): Representative immunohistological analyses for human leukocyte antigen 1 (HLA-1)-positive cells in liver sections after adipose tissue-derived mesenchymal stem cell (AT-MSC) transplantation (group IIIA and IIIB). HLA-1-positive cells were detected within P, V, and BD. On the left, H&E staining of liver sections of the same selected areas. Scale bars = 500  $\mu$ m. (C): Hematoxylin eosin staining of  $\text{CCl}_4$ -injured liver sections from control PBS(-) (left panel) and AT-MSC transplant (right panel). Abbreviations: BD, bile duct; P, parenchyma; PBS, phosphate-buffered saline; V, vessel.

### Transplantation of AT-MSCs into Mice with Liver Injury

To address our goal, whether undifferentiated AT-MSCs reveal therapeutic abilities to regenerate an injured liver, we transplanted AT-MSCs into mice with acute liver failure.  $\text{CCl}_4$  injury generated oxidative stress and hepatocyte necrosis. Twenty-four hours after  $\text{CCl}_4$  injection, mice revealed serious liver injury. Biochemical parameters such as GPT, GOT, ALP, UA, and ammonia were increased in mice with  $\text{CCl}_4$  injury (group II) compared with noninjured mice (group I) (Fig. 2). We transplanted  $1.5 \times 10^6$  cells of AT-MSCs into  $\text{CCl}_4$ -injured mice. After AT-MSC transplantation, GOT and GPT were significantly decreased to a value more than 50% lower than in group II (Fig. 2). Likewise, ammonia concentration was significantly decreased after AT-MSC transplantation (group III). ALP was also decreased (but not significantly). UA, a marker of oxidative stress, was significantly decreased up to a normal level. Markers of liver injury GOT and GPT were decreased after AT-MSC transplantation (group III), yet the liver sections were not morphologically distinguishable (H&E) from sections of the injured group II (data not shown). We also checked the concentration of BUN and detected no improvement in concentration after AT-MSC transplantation (Fig. 2). Supplemental online Figure 2 demonstrates, separately, the donor 1- and donor 2-derived AT-MSC *in vivo* effect on the above parameters. We could notice variations between donor 1 and donor 2 regarding their effect on ALP and ammonia concentrations. Twenty-four

hours after transplantation of AT-MSCs, livers were collected and sections were examined by H&E and against HLA-1 staining. In Figure 3A, we can notice macromorphological changes in the livers of each experimental group, as claret (healthy) or beige (injured) colors. Photographs of H&E staining of the liver sections of injured mice that received a transplant of AT-MSCs (group IIIA, IIIB) show visible liver injury (Fig. 3B). Immunostaining revealed that some human AT-MSCs were detected within the host livers (Fig. 3B). Low-magnification photographs reveal HLA-1-positive cells in different areas of host livers, such as parenchyma, vessel, and bile duct. The above presented data indicate that transplanted AT-MSCs migrate into the injured liver, as well as improve markers of liver injury. Furthermore, H&E staining revealed significant morphological changes in the hepatocytes of the non-necrotic area. As shown Figure 3C, the injured livers of mice that received a transplant of AT-MSCs revealed less vacuolar degeneration caused by dilatation of mitochondria and rough endoplasmic reticulum. These observations reflect the data of the decrease of ALT and AST levels in injured mice that underwent transplantation.

In addition, to evaluate how long, and whether, the transplantation effect of AT-MSCs is sustained in a longer experimental period, kinetic analysis was performed. As shown in supplemental online Figure 4, ALT and ammonia levels were monitored for 120 hours after the cell transplantation. As a result, ALT and ammonia levels in animals that underwent AT-MSC transplantation were significantly decreased compared with levels in PBS(-) control



**Figure 4.** Proteome profile of cytokine/growth factors interleukin 1 (IL-1) receptor antagonist (RA), IL-6, IL-8, MCP-1, G-CSF, GM-CSF, HGF, and NGF concentration (pg/mL) in conditioned medium of normal human dermal fibroblasts (NHDFs), bone marrow mesenchymal stem cells (BM-MSCs), and AT-MSCs (combined data of 3 donors: 1, 2, and 3). Cells were incubated ( $n = 3$ ) in serum-free medium for 3 days, and supernatant was collected and analyzed for the presence of selected cytokines and growth factors. Data are presented as the mean  $\pm$  SD and were analyzed by the Bonferroni correction;  $n = 3$ . (\*,  $p < .05$ ; \*\*,  $p < .01$ , vs. both NHDFs and BM-MSCs). Abbreviations: AT-MSCs, adipose tissue-derived mesenchymal stem cells; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; MCP-1, monocyte chemoattractant protein 1; NGF, nerve growth factor.

animals at 24 hours, and then levels returned to normal by 48 hours. There is no change at 72, 96, and 120 hours after the transplantation. These results suggest that the recovery effect of AT-MSC transplantation is not transient.

### Proteome Profile of Cytokines/Growth Factors Secreted by AT-MSCs

To determine the cytokine and growth factor profile of AT-MSCs, conditioned media of AT-MSCs (donors 1, 2, and 3) were evaluated for cytokine/growth factor protein concentration (Fig. 4, supplemental online Fig. 3, Table 1). As a control, BM-MSCs and NHDFs were analyzed. Differences in secretion between AT-MSCs and BM-MSCs were observed in the concentrations of IL-1RA, IL-6, a so-called hepatocyte stimulating factor, IL-8, G-CSF, GM-CSF, MCP-1, NGF, and HGF. These factors responsible for immunosuppression, hepatocyte growth, and hematopoiesis were secreted in a higher concentration by AT-MSCs versus BM-MSCs and NHDFs. These results suggest strong differences between AT-MSCs and BM-MSCs with respect to their stem cell properties in vitro and in vivo. High secretion of proangiogenic VEGF by AT-MSCs and BM-MSCs was similar, in contrast to NHDFs, which do not secrete VEGF. In addition, other factors such as EGF, IL-10, IP-10, MIP-1 $\alpha$ , MIP-1 $\beta$ , IL-7, IL-15, eotaxin, fractaline, IL-12p40, IL-12p70,

and IL-17 were also secreted, although not strongly (supplemental online Fig. 3). Importantly, the fact that undifferentiated AT-MSCs revealed regeneration ability after transplantation into mice with liver injury may be explained by their ability to secrete a spectrum of bioactive factors. In addition, we observed quite a difference between analyzed AT-MSCs from different donors (supplemental online Fig. 3). For example, NGF was highly produced by donor 1, but anti-inflammatory IL-10 was secreted only by donor 2. The concentration of secreted IL-1RA, IL-6, IL-8, G-CSF, GM-CSF, HGF, NGF, VEGF, IP-10, IL-15, IL-7, and eotaxin by the three donors was significantly different (supplemental online Fig. 3). We did not detect secretion of IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-13, IFN $\gamma$ , TNF $\alpha$ , and TRAP/CD40L/CD154 by any of the analyzed cells (Table 1). In conclusion, our results revealed a subset of cytokines/growth factors specifically excreted by AT-MSCs. Expression and secretion of those trophic proteins may likely contribute to the AT-MSC-induced therapeutic effects on animal liver injury models.

### CONCLUSION

We have shown here that undifferentiated AT-MSCs have the ability to improve liver functions. We observed that in mice with

**Table 1.** Cytokine and growth factor concentration (pg/ml) in conditioned medium of NHDF BM-MSCs and AT-MSCs of three donors

Factor	NHDFs (n = 3)		BM-MSCs (n = 3)		AT-MSCs					
	Mean	SD	Mean	SD	Donor 1 (n = 3)		Donor 2 (n = 3)		Donor 3 (n = 3)	
					Mean	SD	Mean	SD	Mean	SD
IL-1RA	20.14	2.46	15.61	6.57	59.37	8.00	105.28	22.93	127.74	16.01
IL-6	179.67	17.72	624.22	42.15	909.76	196.98	1110.53	316.67	1931.50	180.90
IL-7	16.67	1.19	12.61	1.66	20.27	1.17	18.19	2.11	13.29	1.64
IL-8	33.53	7.54	7.91	0.35	5222.06	2910.35	2414.26	839.00	676.27	60.64
IL-15	6.98	1.09	1.46	0.29	4.11	0.83	3.48	0.73	2.34	0.74
G-CSF	7.24	1.64	3.76	0.99	67.23	14.76	61.22	25.21	18.50	1.85
GM-CSF	1.91	0.37	1.45	0.42	3.38	0.54	3.06	0.70	1.93	0.22
Fractaline	21.85	3.34	20.42	2.60	18.64	3.65	17.54	1.28	17.60	4.18
Eotaxin	34.67	1.83	6.04	8.38	47.64	8.58	19.93	1.14	92.55	10.73
MCP-1	977.07	108.99	380.88	39.30	3414.30	3357.48	6373.97	11439.28	8289.72	10966.12
VEGF	N.D.	0	837.96	39.94	741.98	124.40	685.79	130.13	415.92	39.31
HGF	821.50	43.43	291.21	15.45	2198.45	187.50	1795.05	133.51	1026.17	51.49
NGF	16.12	2.12	9.49	1.25	68.09	7.46	14.03	1.97	19.98	1.69
IP-10	7.76	1.22	N.D.	0	1.24	1.46	5.37	0.85	6.81	2.67
IL-12p40	2.04	3.42	6.65	5.27	4.24	5.68	2.32	2.70	6.40	8.20
MIP-1 $\alpha$	1.15	1.24	3.76	0.28	5.04	0.85	2.61	1.53	2.69	1.62
MIP-1 $\beta$	2.09	3.55	N.D.	0	2.09	3.55	6.83	4.80	5.12	4.91
EGF	N.D.	0	1.87	1.41	1.84	1.34	2.10	1.16	N.D.	0
IL-10	N.D.	0	N.D.	0	N.D.	0	43.95	1247.08	N.D.	0
IL-12p70	3.19	0.09	3.07	0.05	3.11	0.07	3.14	0.04	3.05	0.07
IL-17	0.73	0.22	N.D.	0	0.86	0.24	1.03	0.20	N.D.	0
IL-1 $\alpha$	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-1 $\beta$	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-2	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-4	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-5	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-13	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IFN $\gamma$	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
TGF $\alpha$	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
TRAP/CD154	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0

Abbreviations: AT-MSCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow mesenchymal stem cells; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IFN $\gamma$ , interferon  $\gamma$ ; IL-1RA, interleukin 1 receptor antagonist; IP-10, interferon-gamma-inducible protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1 $\alpha$ , macrophage inflammatory protein 1 $\alpha$ ; N.D., not detected; NGF, nerve growth factor; NHDFs, normal human dermal fibroblasts; TGF $\alpha$ , transforming growth factor  $\alpha$ ; TRAP, tumor necrosis factor-related activation protein; VEGF, vascular endothelial growth factor.

liver injury, AT-MSC transplantation promotes liver recovery. Parameters such as GOT, GPT, ALP, UA, and ammonia were decreased in injured mice after AT-MSC transplantation, suggesting that AT-MSCs have the ability to regenerate the liver. We did not, however, observe morphological improvement of the liver after AT-MSC transplantation. Although a long time course experiment with histological analyses as well as safety issues should be carefully evaluated, effect of transplantation of AT-MSCs was not transient. A lack of knowledge regarding post-transplantation modifications and risk of carcinogenesis still exists. At present, we know that AT-MSCs may undergo spontaneous transformation in vitro; however, it was observed in cells at senescent phase after a prolonged period of culturing [34]. Nevertheless, MSCs from different sources were shown to have great therapeutic potentiality [35–37], especially in stroke, myocardial infarct, and meniscus repair. However, questions such as what kind of MSCs represent the best therapeutic ability and what MSCs actually do to effect regeneration remain unclear.

By HLA-1 immunostaining, we have detected human AT-MSCs within the liver of immunodeficient mice 24 hours after transplantation. We found few positive cells in different areas of the liver such as parenchyma, vessel, and bile duct. We cannot, however, exclude the cell fusion event. Monitoring AT-MSC migration, mobilization, and engraftment for a longer time is essential and very informative. It is interesting to evaluate the effect of AT-MSCs on endogenous progenitor activation [38]. It

has been proposed that there is a different response of oval cells in vivo between mice and rats as well as the injury model [39]. The possible actions of AT-MSCs in vivo are that they act directly, by causing intracellular signaling and/or indirectly by secreting or making other cells in the vicinity secrete functionally active agents. Recently, MSCs were thought to be “multi-drug delivery vehicles that are injury-site sensitive and/or responsive” [17]. Liver disease is a complex, heterogeneous condition accompanied by inflammation and fibrosis, and, at present, there is no proper treatment strategy. Kupffer cells and liver macrophages are major sources of IL-1 $\beta$  and TNF $\alpha$  production. These cytokines are thought to enhance the expression of adhesion molecules in endothelial cells, which mediate the neutrophil adhesion to endothelial cells causing cell dysfunction and destruction, leading to hepatocyte necrosis [40]. It has already been demonstrated that MSCs secrete a broad spectrum of active cytokines, chemokines, and growth factors [16–22]. We observed that AT-MSCs produce significantly more bioactive factors than do BM-MSCs, and, therefore, they may have an equal or even stronger regenerative effect than BM-MSCs. This issue is very interesting and needs precise evaluation, using MSCs isolated from adipose tissue and bone marrow from the same donor. We demonstrated that in vitro AT-MSCs secrete many active factors, among them, (a) immunosuppressive IL-1RA, (b) hepatocyte-growth promoting IL-6, IL-8, HGF, NGF, and MCP-1, and (c) hematopoiesis-promoting G-CSF and GM-

CSF are secreted in a larger volume than both BM-MSCs and NHDFs (Fig. 4). IL-1RA is a strong anti-inflammatory cytokine, which binds to IL-1 receptors and inhibits the binding of IL-1 $\alpha$  and IL-1 $\beta$ . Therefore, the immune activity of IL-1 $\alpha$  and IL-1 $\beta$  is neutralized. Inflammatory cytokines such as IL-1 and TNF $\alpha$  are not produced by AT-MSCs or by BM-MSCs or NHDFs (Table 1). On the other hand, IL-6, identical to HSF, elicits an acute phase response in liver cells; IL-8 and MCP-1 are inflammatory-related factors, however, it is difficult to predict their actual *in vivo* activity. In fact, IL-6 together with TNF $\alpha$  is responsible for a hepatocyte entering to the state of replication competence [41]. It has been demonstrated that IL-6, IL-8, and MCP-1 are produced by human intrahepatic biliary epithelial cells through toll-like receptor 4, nuclear factor-kappa B, and mitogen activated protein kinase signaling pathways, and therefore are possibly mediating an innate immune system function and modulating hepatic regeneration *in vivo* [42, 43]. Then in the proliferation phase, HGF and TGF $\alpha$  stimulate proliferation of hepatocytes. HGF, considered as proapoptotic for liver stellate cells [18] as well as for myofibroblasts [44], acts as an anticirrhotic agent. Stellate cells are a major source of collagens and other extracellular matrix proteins in liver fibrosis. In addition, they regulate matrix metalloproteinases. Other highly secreted factors by AT-MSCs were C-CSF, GM-CSF, and IL-6, considered as a supporting process of hematopoiesis. The pleiotrophic G-CSF is a mobilizing factor, which promoted migration of BM-MSCs into the heart after myocardial infarction [45]. Again, in the light of much evidence that tissues contain their own multipotential stem cells [46–47], it should be emphasized that endogenous stem cells (oval cells, liver epithelial progenitors, etc.) can be the target of MSC-derived direct or indirect actions. Likewise, it is possible that bioactive factors act on oval cells and therefore provoke hepatocyte generation *in vivo*. Recently, G-CSF has been shown to promote rat liver repair and induce oval cell migration and proliferation [48, 49]; however, there are controversial opinions regarding the G-CSF effect [50]. Recent evidence suggests that neurotrophins, such as NGF, may have a role in hepatic regeneration [51–55], and their mRNA and protein levels become elevated in association with hepatocyte proliferation induced by, for example, administration of CCl<sub>4</sub> [52]. NGF, similarly to HGF, induces the apoptosis of stellate cells [52–54]. NGF is secreted by cholangiocytes and also induces their proliferation [55]. In addition, it is reported that MSCs can inhibit the proliferative and fibrogenic function of activated stellate cells in a paracrine manner [18]. This inhibition was caused by MSC-derived IL-10 and TNF $\alpha$ , which acted synergistically. The secretion of IL-10 by MSCs was found to be a response to IL-6 secretion by activated stellate cells. These events were not detected while stellate cells were cultured with fibroblasts [18]. The production of trophic agents by AT-MSCs exhibits donor to donor variations (supplemental online Fig. 3). In fact, we observed that the secretion of IL-10 was detected in AT-MSCs of one donor only. Recently, Akt-modified MSCs were proposed as candidate cells for paracrine-mediated actions [56]. Akt-modified MSCs are capable of up-

regulating the expression of several candidate mediators such as VEGF, FGF2, HGF, and insulin-like growth factor 1 [56]. Secreted frizzled related protein 2 is the key Akt-modified MSC-released paracrine factor [56]. Of note, our data show only *in vitro* production of bioactive factors. *In vivo* MSCs may act through multiple yet unknown mechanisms to coordinate a dynamic integrated response to fibrosis. It is also likely that similar immunomodulatory mechanisms may influence the phenotype of resident hepatocytes, stellate cells, Kupffer cells, sinusoidal endothelial cells, and immune cells that infiltrate the liver during inflammation. An interesting topic for further evaluation is the effects of AT-MSCs on GVHD. It has been proposed that HGF may suppress GVHD after allogenic BM-MSC transplantation into patients with hepatocellular carcinoma [57]. In our studies we showed that AT-MSCs produce significantly more HGF than BM-MSCs and NHDFs. We propose a careful consideration of the stem cell type/source for the therapy of certain diseases, because of the composition and concentration of secreted bioactive factors by MSCs from different sources, which may promote or inhibit the state of injury.

In conclusion, our study revealed that transplantation of human AT-MSCs has functional benefits, in part because of the cells' ability to produce a large number and volume of bioactive factors. This trophic activity may result in future therapeutic usage of AT-MSCs for liver disease. We propose that trophic activity may be one of the possible actions *in vivo* of AT-MSCs or MSCs in general; however, this issue needs to be precisely confirmed and analyzed with a long-term course of experiments. Other mechanisms have not been excluded. In addition, further characterization, whether AT-MSCs can produce factors that inhibit fibrosis and apoptosis, promote angiogenesis, and stimulate host progenitors to divide and differentiate into functional regenerative units, is required to establish a novel therapeutic approach for liver disease treatment.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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