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Autologous Bone Marrow Infusion Activates the Progenitor Cell Compartment in Patients With Advanced Liver Cirrhosis

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Several clinical trials of bone marrow cell infusion in patients with liver cirrhosis (LC) have shown clinical improvement, despite conflicting results from animal models. We investigated serial pathological features and the clinical impact after autologous bone marrow infusion (ABMI) in patients with advanced LC. Ten patients with advanced LC due to chronic hepatitis B virus infection underwent ABMI. Serological tests, MRI, and liver biopsies were performed, and quality of life was assessed by a questionnaire. Median serum albumin and hemoglobin levels increased significantly after ABMI. All patients showed an improvement in quality of life, with no serious adverse events. Liver volume, measured by MRI, increased in 80% of the patients, and ascites decreased after ABMI. Child-Pugh scores were also significantly improved at 6 months after ABMI. In the serially biopsied livers, a gradually increasing activation of the hepatic progenitor cell (HPC) compartment, including HPC activation (ductular reaction) and HPC differentiation (intermediate hepatocyte), reached a peak after 3 months, with continued proliferation of hepatocytes, and returned to baseline levels after 6 months. There was no significant change in grade or stage of liver fibrosis or stellate cell activation after ABMI. ABMI is suggested to improve liver function and to activate the progenitor cell compartment. Although clinical improvement was sustained for more than 6 months, histological changes in the liver returned to baseline 6 months after ABMI. Further comparative studies are warranted.

Key words: Adult bone marrow stem cell; Autologous bone marrow transplantation; Liver cirrhosis; Liver regeneration; Progenitor cell

INTRODUCTION

Liver cirrhosis (LC) is the end stage of various chronic liver diseases and is extremely difficult to treat. In addition to substitution of parenchyma by fibrous tissue, the gradual loss of cellular function results in the impairment of homeostasis. Until now, liver transplantation has been the only effective method of cure, but due to the several limitations of transplantation, such as a lack of donors, surgical complications, rejection, and high cost, regenerative therapy has been suggested to provide alternative choices using less invasive procedures.

In general, organ regeneration requires the presence of a progenitor cell population in the absence of a resident cell proliferation (15). However, in the liver physiological turnover and regeneration occur predominantly through replication of native hepatocytes. Progenitor-dependent regeneration takes place only if parenchymal hepatocytes are severely damaged and unable to regen-

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erate efficiently, as in cirrhosis (17). Thus, as an external source of hepatocytes, mature hepatocytes, fetal hepatocytes, oval cells (hepatoblasts in humans), hematopoietic stem cells, mesenchymal stem cells, multipotent adult progenitor cells, or embryonic stem cells have been used for regeneration in liver disease models (4).

After Peterson and colleagues (19) first reported bone marrow cells (BMCs) as a potential source of oval cells, supportive data about bone marrow-derived hepatocytes in animals (12,28) and humans (1) have been published. Although recent advances have documented that bone marrow cells can also differentiate into muscle (5), heart (16), pancreas (9), and lung (8) cells, controversy still exists regarding the role of BMCs in organ regeneration, including in the liver. Researchers who have a negative opinion on the plasticity of BMCs insist that little evidence exists for the use of bone marrow-derived hepatocytes in the replacement of injured liver (10), and that no data have demonstrated transdifferentiation to hepatocytes or a major role in regeneration (14,30,31). On the other hand, BMCs have been reported to be a possible source of functional hepatic stellate cells and myofibroblasts contributing to fibrosis (23).

However, in a murine model, transplanted BMCs populated and differentiated into albumin-producing hepatocytes, via hepatoblast intermediates (27). Furthermore, in humans, BMC infusion elevates serum albumin levels (26), and, in mice or rats, it reduces liver fibrosis (7), corrects liver dysfunction (2), and improves survival rate (25). Based on these results, a clinical trial of autologous BMC infusion (ABMI) has been performed (18,24), and it was shown that ABMI administered through a peripheral vein or the hepatic artery improves liver function in patients with LC (13,26).

Although recent clinical trials have shown transient improvements in liver function, there has been no report about the duration of effect and what happens in the liver after cell transplantation. The aims of this study were to investigate the clinical impact and serial histological changes that contribute to liver regeneration and to determine how long such changes were sustained after ABMI in patients with advanced LC.

MATERIALS AND METHODS

Patients

Patients were enrolled between November 2006 and February 2008. All patients had biopsy-proven LC. Child-Pugh class B or C patients aged between 18 and 75 years with total bilirubin ≤3.0 mg/dl, platelet count ≥50,000/μl, and no viable hepatocellular carcinoma (HCC) on magnetic resonance imaging (MRI) were eligible. Patients were excluded from the study if they had problems with organs other than the liver, required general anesthesia, or had any uncontrolled malignancies. We advised all patients to maintain their current medications, except diuretics, during the study period. After ABMI, doses of diuretics were adjusted according to the patient's condition. Antiviral therapy was individualized, according to the guidelines. No patient was given albumin during the study.

BMC Harvest and Infusion

BMCs (500–750 ml) were harvested from both ilia according to standard procedures under general anesthesia and were collected in a plastic bag containing heparin. After fat and bony particles were removed by filtration, collected cells were moved to a cell-processing device. The automatic cell washer 2991 (Gambro, Lakewood, CO, USA) was used for collecting mononucleated cells (MNCs), as well as depleting red blood cells and plasma in a closed system. The final concentrated cell product was made up to a final volume of about 100 ml. Five milliliters of the final cell product was subjected to trypan blue dye exclusion testing, bacterial culture, cell counting, and fluorescence-activated cell sorting analysis by flow cytometry (Cytomics FC 500; Beckman Coulter, Inc., Fullerton, CA, USA). Because no specific cell marker has been proven useful in estimating contributing cells for hepatic regeneration, hematopoietic stem cell markers (CD34, CD45, CD133; Miltenyi Biotech, Inc., Auburn, CA, USA) and an epithelial cell marker (CD 117; Immunotech, Beckman Coulter, Marseille, France) were used to assess cell composition. At 4–8 h after harvesting the BMCs, the final MNC preparation was administered into a peripheral vein over a 1-h infusion.

Clinical Follow-up Protocol

Before ABMI, laboratory tests with MRI and liver biopsy were conducted. After ABMI, patients were discharged within 5 days and followed up every week for 2 weeks, and monthly for 6 months. Laboratory tests, body weight check, and a questionnaire for performance status, subjective well-being, and quality of life were checked at every visit. Performance status was scored using a 0–100% grading scale. Subjective well-being and quality of life were scored using a 1–7 grading scale. Indocyanine green clearance was also measured before and 1, 3, and 6 months after the procedure.

MRI Evaluation

All MRI to measure liver volume and ascites, and to screen for HCC, was performed using 1.5-T MR scanners (Gyroscan Intera; Philips Medical Systems Best, The Netherlands). Routine T1-weighted gradient echo, T2-weighted turbo spin echo, T2*-weighted gradient echo, and three-phase, dynamic three-dimensional (3D) T1-weighted images after injection of gadobenate di-

meglumine (dose: 0.1 mmol/kg of body weight; Multi-Hance; Bracco SpA, Milan, Italy) were obtained.

The total liver volume was measured with commercially available image postprocessing software (Voxel plus 2; Mevisys, Daeduk, Korea) using the summation-of-areas technique with a 10-mm reconstruction thickness by one radiologist. Right perihepatic ascites was estimated by a 3D measurement [the largest length (x) of ascites × the greatest perpendicular length (y) × sum of the vertical length (z)] on a picture archiving and communication system (PACS) monitor by another radiologist. The radiologist was unaware of the patient history, laboratory results, or biopsy findings.

Liver Biopsy, Tissue Preparation, and Immunohistochemistry

Liver biopsies were performed before and 1, 3, and 6 months after ABMI using an ultrasound-guided 16-gauge gun, if the patient consented. Transjugular liver biopsy was attempted as an alternative biopsy in cases with massive ascites or a high risk of bleeding.

The biopsied liver tissues were routinely processed and hematoxylin and eosin (H&E) stained. Tissue sections (4 μm) of formalin-fixed, paraffin-embedded liver specimens were deparaffinized with xylene and rehydrated through graded alcohols. After washing in distilled water, the sections were immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed by boiling the sections in 100 mM sodium citrate (pH 6.0) for 15 min in a microwave oven. Primary monoclonal antibodies [cytokeratin 7, clone OV-TL 12/30, Dako, Glostrup, Denmark, 1:100 dilution; proliferating cell nuclear antigen (PCNA), clone PC10, Dako, 1:60 dilution; p21WAF1/Cip1, Clone SX118, Dako, 1:50 dilution] and α-smooth muscle actin (Clone 1A4, Dako, 1:100 dilution) were applied for 30 min at room temperature, followed by washing in PBS. Incubation with the secondary antibody was carried out using the Dako EnVision Rabbit/Mouse kit for 30 min at room temperature. Sections were then developed with diaminobenzidine (Dako) and counterstained with hematoxylin.

The hepatic progenitor cell (HPC) compartments, including HPC activation (ductular reaction) and HPC differentiation (intermediate hepatocyte), were assessed on cytokeratin 7-stained sections (22). Positively stained ductular cells and intermediate hepatoyctes were counted in at least five non-overlapping high-power fields (HPFs; ×400), and the average of these scores/HPF was taken. The proliferating hepatocytes were evaluated in PCNA-stained sections; the hepatocytes with nuclear PCNA expression were counted in at least five non-overlapping HPFs, and the average of these scores/HPF was taken. The hepatocytes in replicative arrest were evaluated on p21-stained sections and the p21 labeling index was determined as the percentage of positive nuclei per total number of nuclei counted in at least five non-overlapping HPFs. Stellate cell activation was evaluated in smooth muscle actin-stained sections. Activated hepatic stellate cells were evaluated semiquantitatively, as follows: grade 0, no positive cells; 1+, rare positive cells that required a careful search at high power; 2+, scattered positive cells readily identified at medium power; 3+, scattered or clustered positive cells apparent at low power; 4+, widespread positive cells apparent at low power.

Statistical Analyses

Changes in clinical indices from baseline to months after ABMI were analyzed. Wilcoxon's signed-ranks test with Bonferroni correction was used to compare the values of paired samples. Descriptive statistics were also used. Changes in proportion from baseline to last follow-up were plotted and compared. The analyses were performed using the SPSS 13.0 statistical package (SPSS Inc., Chicago, IL, USA). All statistical analyses were based on two-tailed hypothesis tests with a significance level of $p < 0.05$.

Ethical Considerations

The protocol for the clinical trial conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board (No. 4-2006-0087; approved June 20, 2006) of Severance Hospital in the Yonsei University Health System. Written informed consent was obtained from all patients. This investigational study was performed with the cooperation of Yamaguchi University Graduate School of Medicine in Japan, under a Memorandum of Understanding. The costs related to this study were supported by a grant for the Liver Cirrhosis Clinical Research Center, Seoul, Korea.

RESULTS

Patient Characteristics

Between November 2006 and February 2008, 12 patients were screened. Ten patients were finally enrolled; one patient was excluded due to continuous hyperbilirubinemia, and another patient decided to withdraw due to family opposition. The baseline demographic features and clinical characteristics are listed in Table 1. The median age was 56 (range 43–64) years old. Three patients were male and seven were female. All patients had a history of ascites, and four patients had ascites at enrollment. All patients had Child-Pugh class B cirrhosis. Eight of the 10 patients had been on antiviral treatments,

Table 1. Patient Characteristics at Screening

Patient	Age	Gender	Etiology	Ascites	ALT (IU/L)	Albumin (g/dl)	TB (mg/dl)	PT (INR)	ICG R15 (%)	MELD
YC01	59	M	HBV	large	40	3.1	1.0	1.30	45.8	12
YC02	55	M	HBV	none	41	2.7	0.9	1.18	33.9	9
YC03	47	M	HBV	large	39	3.4	0.7	1.26	25.5	9
YC04	64	F	HBV	small	39	3.6	1.6	1.18	46.3	10
YC05	56	F	HBV	none	58	2.6	1.0	1.14	30.3	7
YC06	60	F	HBV	large	130	2.3	1.6	1.12	71.4	9
YC07	52	F	HBV	minimal	28	2.5	2.4	1.31	41.7	13
YC08	43	F	HBV	minimal	21	3.1	1.6	1.27	37.3	11
YC09	49	F	HBV	none	39	2.3	1.8	1.20	40.5	11
YC10	52	F	HBV	minimal	47	2.7	1.7	1.13	35.5	8

ALT, alanine aminotransferase; TB, total bilirubin; PT, prothrombin time; INR, international normalized ratio; ICG R15, indocyanine green clearance; MELD, the Model for End-Stage Liver Disease score; HBV, hepatitis B virus.

with oral nucleoside/nucleotide analogs. All patients were positive for HBsAg and negative for antibodies against hepatitis C.

Characteristics of Processed Total Mononucleated Cells

Infused MNC characteristics and composition, according to cellular markers, are summarized in Table 2. The median infused MNC count per body weight was 0.995×10^8 cells/kg (range, $0.48–1.48 \times 10^8$). The median infused volume of concentrated MNCs was 112.5 ml (range, 88–150). CD45-positive hematopoietic stem cells were the major cell component (median, 79.85%). CD133-positive hematopoietic stem cells composed 1.05% of total infused cells. CD34-positive stem cells and CD117-positive epithelial cells represented 0.53% and 1.0% of infused cells, respectively.

Changes in Clinical Data

Serial clinical data from each patient were recorded. The initial median albumin level at screening was 2.7 g/dl. The median albumin level during the 6 months before ABMI was 2.9 g/dl, and this slightly decreased with conservative management, including use of antiviral agents. Beginning at the first month after ABMI, the serum albumin levels gradually increased and reached at peak level to 3.5 g/dl at 9 months, which was maintained at 12 months after ABMI. Significant increase was noted at 6 months after ABMI (Fig. 1A). The median hemoglobin level showed a gradual but significant increase at each time point, from 11.7 g/dl at baseline to 13.8 g/dl at 12 months after ABMI (Fig. 1B). The median cholesterol level improved significantly at 6 and 12 months after ABMI (Fig. 1C). The median prothrombin time

Table 2. Infused Mononuclear Cell Characteristics

Patient	Infused Nucleated Cells/Weight ($\times 10^8$ Cells/kg)	Final Volume (ml)	$CD34^+$ Cells (%)	$CD45^+$ Cells (%)	$CD117^+$ Cells (%)	$CD133^+$ Cells (%)
YC01	1.31	110	0.44	98.3	1.8	2.2
YC02	0.76	88	0.45	95.8	0.7	0.7
YC03	0.81	105	0.60	73.3	1.1	1.0
YC04	0.99	120	0.68	56.2	1.3	1.1
YC05	1.35	111	0.63	77.8	0.9	0.7
YC06	1.00	100	1.17	53.4	0.9	2.0
YC07	1.34	114	0.44	74.9	1.9	2.4
YC08	0.48	136	1.23	82.2	1.7	1.5
YC09	0.50	130	0.24	82.2	0.1	0
YC10	1.48	150	0.40	81.9	0.3	0.1

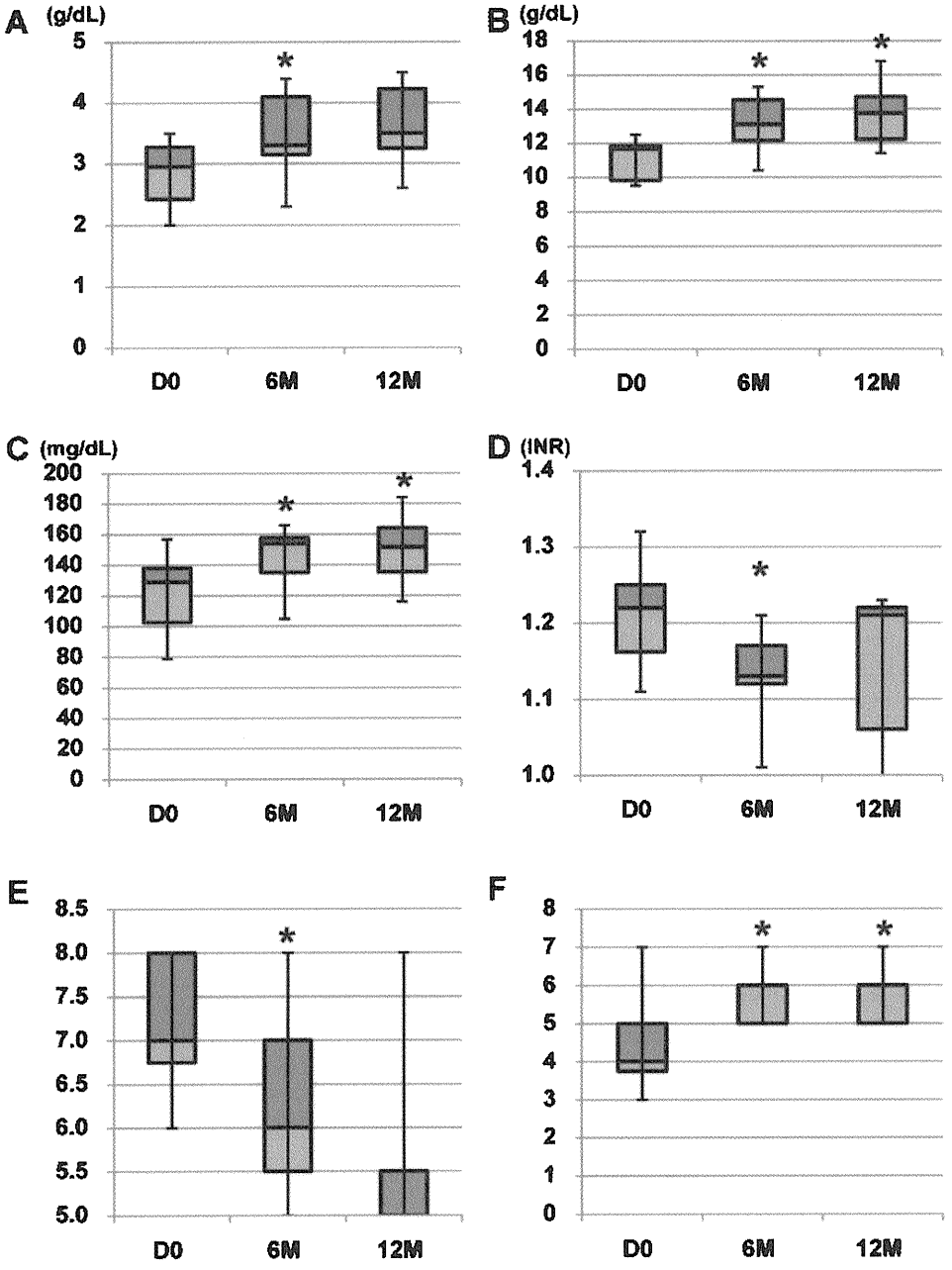


Figure 1. Serial changes in clinical parameters. Serum albumin (A), hemoglobin (B), serum cholesterol (C), prothrombin time (D), Child-Pugh score (E), and feeling of well-being (F) were serially checked according to the follow-up schedule. Gradual but significant improvements in serum albumin (A), hemoglobin (B), and serum cholesterol (C) were observed. The Child-Pugh score improved significantly at 6 months after ABMI (E). Feeling of well-being improved (F) significantly up to 12 months after ABMI. *$p < 0.05$, Wilcoxon signed ranks test with Bonferroni correction.

tended to improve; a significant difference was seen only at 6 months after ABMI (Fig. 1D). Serum alanine aminotransferase, total bilirubin, and α-fetoprotein levels showed no statistically significant change. The Child-Pugh score improved significantly after 6 months (Fig. 1E); however, there was no change in MELD scores. Performance status reflecting objective daily activity, subjective scoring about feeling of well-being

(Fig. 1F), and quality of life increased significantly at 6 months, at 6 and 12 months, and at 6 months after ABMI, respectively.

Changes in Liver Volume and the Amount of Ascites, by MRI

Hepatic volumes before and at 6 months after ABMI were compared. The median liver volume increased by 5% after 6 months of ABMI (Fig. 2). Patient 1's liver, in particular, grew by 42% in the 6 months after ABMI.

The relative amount of ascites between baseline and the last visit was also compared. Among four patients with ascites at baseline, three showed a decreased amount of ascites in spite of reduction or discontinuation of diuretics. The amount of diuretics required was reduced in all patients without weight gain or peripheral edema. None of the patients developed new ascites.

Liver Pathological Features in Serially Biopsied Liver

The HPC compartment, including HPC activation (ductular reaction) and HPC differentiation (intermediate hepatocytes), which showed a positive reaction for cytokeratin-7, was evaluated at baseline and in serial liver biopsies after ABMI (Fig. 3). The baseline liver biopsy in all patients showed a low level of HPC activation and differentiation. The HPC compartment showed a gradual increase in all patients after ABMI. It peaked at 3 months after ABMI and there were 1.4–6.8-fold increases, when compared with the baseline liver biopsy.

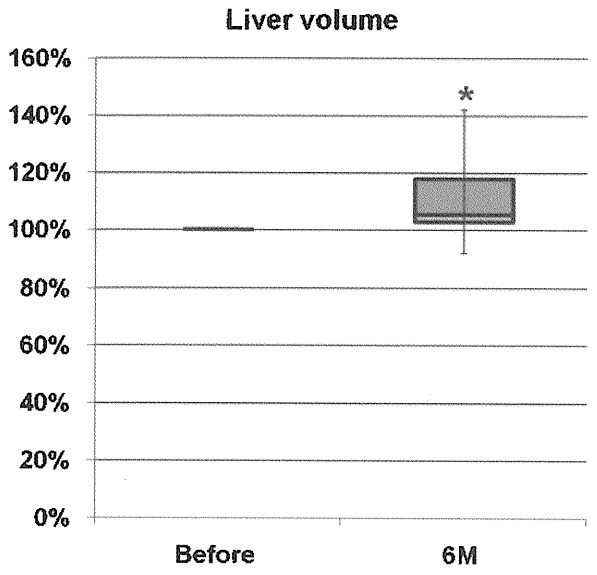


Figure 2. Changes in liver volume before and at 6 months after ABMI. MRIs taken in the sixth month were analyzed to compare liver volume. Significant liver volume enlargement was observed after 6 month of ABMI. $*p < 0.05$, Wilcoxon signed ranks test.

Liver biopsy at 6 months after ABMI showed a decrease in the HPC compartment to baseline levels. This pattern of HPC compartment activation was consistent in all patients (Fig. 4).

The proliferating hepatocytes showed nuclear expression of PCNA. The mean number of proliferating hepatocytes per HPF gradually increased after ABMI and peaked at 3 months after ABMI (Fig. 4B). The number of hepatocytes in replicative arrest was evaluated using the p21 labeling index (Fig. 4C). The p21 labeling indexes were very low, and the maximal value was less than 3.5% in all serially biopsied liver tissues; there was no significant change before and after ABMI.

All liver biopsies at baseline and after ABMI showed cirrhosis with mild necroinflammatory activity. There was no significant change in grade or stage of cirrhosis in the serially biopsied livers after ABMI. Additionally, stellate cell activation was grade 2+ in most biopsies, and there was no significant alteration in the activation of stellate cells among baseline and serially biopsied livers after ABMI in any patient.

Toxicity and Complications

No serious adverse events occurred during or after ABMI. Two patients complained of pain at the puncture site on the first day, which subsided without medication.

DISCUSSION

We found that ABMI could induce clinical improvement and increase HPC and hepatocyte proliferation in patients with advanced cirrhosis. To our knowledge, this is the first report to show serial clinicopathological changes, such as activation of the liver progenitor cell compartment, and to reveal how long these alterations were sustained after ABMI. We also demonstrated clinical improvements, consistent with previous data (3, 13,26).

Clinical trials of ABMI in chronic liver disease have begun in a few institutes, although the basic mechanism of hepatic regeneration is unclear in the human liver. Peripheral infusion of concentrated autologous BMCs has been reported to improve liver function in patients with cirrhosis (13,26). In this study, we also found that it could improve liver function and quality of life, with objective liver volume increases in patients with advanced LC. The increase in liver volume was especially marked in patient 1, who showed a final liver volume 1.42-fold larger than the initial volume. This patient had undergone a right lobectomy due to HCC 16 months before the ABMI, which was a distinct clinical history among these patients. His liver volume showed no significant changes during the 12 months prior to the ABMI. When hepatocyte loss, such as that caused by partial hepatectomy in experimental models and clinical

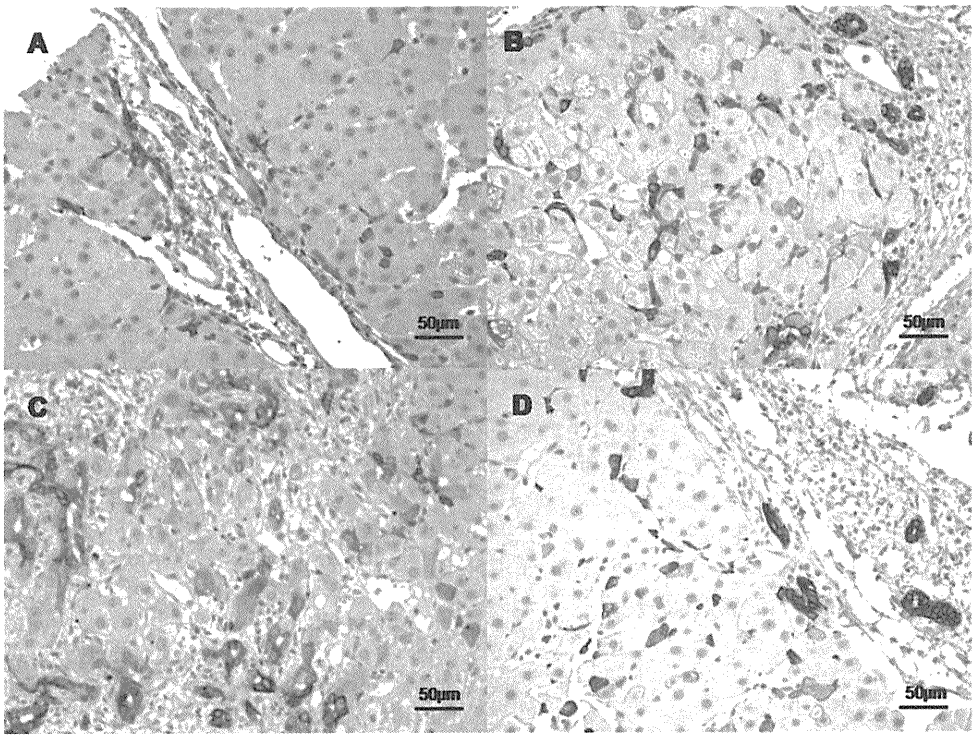
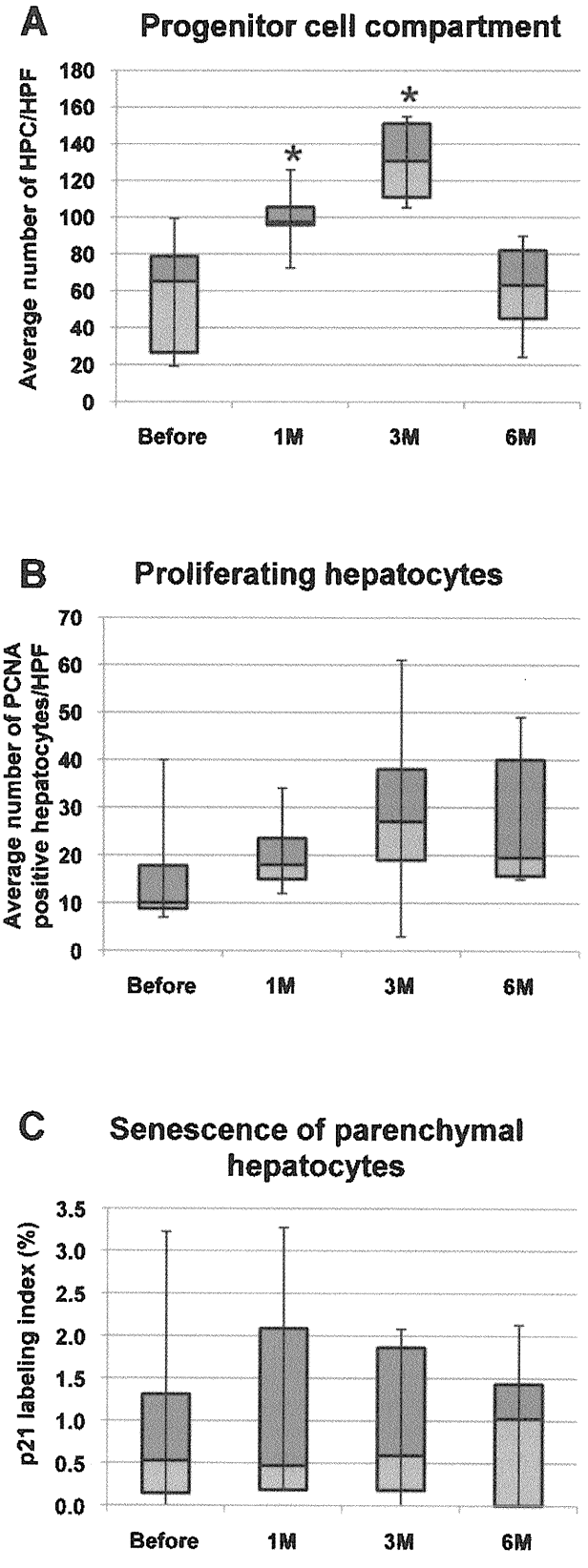


Figure 3. Progenitor cell compartment in the baseline and serially biopsied liver tissue. Ductular reactions and intermediate hepatocytes, which showed positive expression of cytokeratin 7, were counted as the progenitor cell compartment. Before ABMI, the progenitor cell compartment showed a low level of activation (A). The progenitor cell compartment increased gradually at 1 month after ABMI (B) and was markedly activated at 3 months after ABMI (C). At 6 months after ABMI (D), activation of the progenitor cell compartment had decreased to baseline levels. Positive staining is shown as the brown color (original magnification 200×).

situations, evokes a rapid regenerative response to restore liver volume (6), we suspect that a suboptimal regeneration after a previous partial resection of the liver may alter the effect of ABMI on the patient's liver growth. Although in our study total MNCs were infused via a peripheral administration route at a different time point long after the operation, the portal administration of autologous CD133-positive BMCs before an operation also accelerates liver regeneration and is a novel therapy supporting hepatic resection (3).

Serum albumin as the most important marker of hepatic function and hemoglobin showed a gradual increase and reached a significant level from 1 month after ABMI. These findings were sustained up to 12 months, although liver biopsies showed that significant activation of progenitor cell compartment had lasted only for 3 months. Serum cholesterol and prothrombin time as other markers representing hepatic function also showed improvement. In four patients who had ascites initially, the amount decreased, even after reducing the dose of diuretics. This is also an indirect marker of hepatic functional improvement. Quality of life and daily activities improved in all patients, and all experienced a better sense of well-being and complained less about fatigue, although we could not completely rule out a placebo effect. In this study, among various clinical parameters, because serum albumin and ascites were improved remarkably, Child-Pugh score had improved significantly, but not MELD score. As factors constituting Child-Pugh score include albumin and ascites, not MELD score but Child-Pugh score had improved. Although there was no remarkable change in liver pathology except progenitor cell compartment, functional improvement was noted. Activation of progenitor cell compartment after ABMI seen in liver pathology might result in increase of functional hepatocytes. And we can guess these hepatocytes could contribute clinical improvements, such as increase in albumin.

All of the patients had hepatitis B virus-related cirrhosis. Eight patients had been receiving antiviral treatment before the procedure, and two patients started antiviral therapy after the procedure. Under antiviral treatment, the viral level was well controlled in most patients. Because there was some doubt that antiviral



therapy contributed to the clinical improvement, we compared serum albumin levels during the 6 months prior to ABMI and 6 months after ABMI. There was no improvement in serum albumin levels during the 6 months prior to ABMI, whereas levels rather prominently increased in the same period after ABMI. Furthermore, two patients who started antiviral therapy after ABMI did not contribute more significantly to the changes relative to the remainders who had previously received antiviral therapy. Thus, ABMI seemed to have a major effect on these changes. However, we cannot exclude additive desirable effects of antiviral therapy on these clinical and histological changes, because antiviral treatment has been shown to improve clinical state and liver fibrosis. If there is synergistic or additive effect of ABMI and antiviral therapy, ABMI would be more effective especially in HBV-related cirrhosis.

We found that the HPC compartment, including HPC activation (ductular reaction) and HPC differentiation (intermediate hepatocytes), showed a gradual increase after ABMI in serially biopsied livers. The ductular reaction represents activated progenitor cells, which are thought to correspond to oval cells or oval-like cells in rodents. These cells can differentiate toward functioning hepatocytes through intermediate cells, which are phenotypically between hepatocytes and cholangiocytes (20,21,29). Activation of the HPC compartment has been well documented in acute, subacute, and chronic severe liver injury, when the regeneration of mature hepatocytes is impaired and lost (11). All cirrhotic patients in this study showed a low level of activation of the HPC compartment in the baseline liver biopsy that clearly increased after ABMI. Activation of the HPC compartment peaked at 3 months after ABMI, with a 1.4–6.8-fold increase compared to the baseline liver biopsy. After 6 months, they returned to baseline levels.

Another important cell in liver regeneration is the proliferating hepatocyte. The PCNA labeling index of

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Figure 4. Serial changes in (A) progenitor cell compartment activation, (B) hepatocyte proliferation, and (C) portion of senescent hepatocytes. (A) Progenitor cell compartment increased significantly to a peak at 3 months after ABMI and decreased at 6 months, with a consistent pattern in all patients. *$p < 0.05$, Wilcoxon signed ranks test with Bonferroni correction. (B) Mean proliferating hepatocytes, using PCNA labeling index, tended to increase, but there was no statistical significance. (C) p21 labeling indexes were counted to evaluate hepatocytes in replicative arrest. Indexes were maintained at a very low level without significant change. CK, cytokeratin; HPF, high-power field; PCNA, proliferating cell nuclear antigen; LI, labeling index.

mature hepatocytes showed a gradual increase, with a peak at 3 months after ABMI. The maximal value was five times higher than the baseline biopsy. These proliferating mature hepatocytes are considered to contribute to clinical improvement because mature hepatocytes are functional and synthesize albumin. These proliferating hepatocytes could be from the activation of quiescent mature hepatocytes or could have originated from the HPC. There were increases in both HPC activation (ductular reaction) and HPC differentiation (intermediate hepatocytes) in all cases of serially biopsied liver tissues after ABMI; thus, at least some portion of the proliferating hepatocytes apparently originated from the HPC. Future studies using cellular tracers would be helpful in identifying the origin of the activated progenitor cells, to establish whether they come from bone marrow. In contrast, the p21 labeling index was very low in all of the biopsied livers, and the maximum p21 labeling index was less than 2.5% after ABMI. Thus, there seemed to be little effect of replicative arrest in liver regeneration in these patients.

The reversibility of liver fibrosis is another interesting issue in ABMI; improvements in liver fibrosis have been reported after bone marrow transplantation in animal experiments (25). Unfortunately, the pathological feature(s) supporting fibrosis reversal was unclear in the biopsied livers. There is a possibility of liver fibrosis reversal after ABMI, because the small biopsied sample may not sufficiently represent the subtle changes in fibrosis, considering sampling error and the uneven distribution of liver fibrosis and large cirrhotic nodules. In addition, 6 months were not sufficient to show the change of fibrosis. However, there was no significant change in stellate cell activation in the serially biopsied liver tissues.

In this study, the effect of ABMI was demonstrated, which can be used in future general clinical application, and there were no serious adverse events. The administration route and cellular processing method were chosen to minimize the risk of complications. Direct injection into the liver or infusion through the hepatic artery, portal vein, or splenic vein may be other options for cellular administration. Other modifications such as clonal selection, cellular tracing, expansion by culture, and repeated injection are important issues for cell therapy. The results of this study can be a basis for further advances and modification.

Our results suggest that autologous BMCs infused via a peripheral vein contribute to regeneration of the liver and improve hepatic function via progenitor cell compartment activation and hepatocyte proliferation, which lasts for 6 months or less. Because of the small number of patients, we cannot generalize these findings. However, if further studies reveal benefits, ABMI may be helpful in patients with advanced LC as a bridge therapy before liver transplantation. Further comparative studies are warranted.

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A multicenter, open-label, dose-ranging study to exploratively evaluate the efficacy, safety, and dose–response of tolvaptan in patients with decompensated liver cirrhosis

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Abstract
Objectives We examined the efficacy of tolvaptan, an orally effective nonpeptide vasopressin V_2 receptor antagonist, in a Japanese clinical study in patients with intractable ascites and/or lower limb edema associated with decompensated liver cirrhosis.
Methods Tolvaptan was orally administrated at titrated doses of 15, 30, and 60 mg once daily after breakfast for 3 days at each dose to 18 liver cirrhosis patients with persistent ascites and/or lower limb edema despite receiving oral furosemide at 40 mg/day or higher.
Results Decreased body weight and abdominal circumference and improvement of ascites and edema were observed following tolvaptan administration beginning from 15 mg. Composite ascites/edema improvement rate was 88.2% at individual maximum doses and 64.7, 80.0, and 90.9%, respectively, after 3-day administration at 15, 30, and 60 mg. Changes in body weight after 3-day administration at 15, 30, and 60 mg were -1.6 ± 0.9, -2.6 ± 1.2, and -3.4 ± 2.1 kg (mean ± SD), respectively, and decreases of 1 kg or more were seen from day 2 (24 h after first dosing). Changes in abdominal circumference ranged from −2.8 to −6.0 cm. Cumulative 24-h urine

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volumes after 3-day administration at 15, 30, and 60 mg were, respectively, 3240.3 ± 1014.5, 3943.3 ± 1060.6, and 4537.4 ± 1621.3 mL/day (mean ± SD). Urine osmolarity was markedly decreased and remained decreased until the end of treatment.

Conclusion Tolvaptan dose-dependently decreased body weight and abdominal circumference and improved ascites and edema beginning from 15 mg, demonstrating a potent aquaretic effect.

Keywords Tolvaptan (OPC-41061) · Vasopressin V_2 receptor antagonist · Decompensated liver cirrhosis · Intractable ascites · Leg edema

Introduction

Arginine vasopressin (AVP) is a neuropeptide synthesized in the paraventricular and supraoptic nuclei of the hypothalamus, transported to the posterior pituitary gland, and released into the bloodstream [1]. AVP causes vasoconstriction via V_{1a} receptors and promotes water reabsorption in the kidney via V_2 receptors, the latter of which are primarily responsible for AVP's antidiuretic effects. Patients with various disorders, including liver cirrhosis and CHF, are at risk of excess water retention or inadequate water disposal due to increased AVP secretion.

Tolvaptan is a novel, orally effective, nonpeptide vasopressin V_2 receptor antagonist developed by Otsuka Pharmaceutical Company [2, 3]. Tolvaptan selectively blocks the binding of vasopressin to V_2 receptors, thus inhibiting water reabsorption in the renal collecting ducts [2] and promoting the excretion of urine with no increase in electrolyte excretion (i.e., aquaresis) and no negative impact on renal function [4]. Unlike peptide V_2 receptor antagonists, tolvaptan possesses no intrinsic agonist activity [2]. Tolvaptan was approved by the United States Food and Drug Administration (US FDA) on 19 May 2009 for the treatment of clinically significant hypervolemic and euvolemic hyponatremia (serum sodium <125 mEq/L, or less marked hyponatremia that is symptomatic and has resisted correction with fluid restriction), including in patients with SIADH, heart failure, and cirrhosis.

Ascites and lower limb edema are commonly associated with decompensated liver cirrhosis. Ascites is caused by obstruction of hepatic lymph drainage, portal hypertension, and hypoproteinemia, and is often intractable. While there are various hypotheses regarding the mechanism of occurrence of ascites secondary to liver cirrhosis [5–8], they are all characterized by a relative decrease in circulating blood volume despite an increase in body fluid associated with water and sodium retention and by hypoalbuminemia due to impaired albumin synthesis. Treatment for intractable edema often involves the use of loop diuretics such as furosemide. However, administration of loop diuretics can lead to impaired renal function, decreased glucose tolerance, and electrolyte abnormalities such as hyponatremia and hypokalemia. Unlike the loop diuretic furosemide, tolvaptan exerts its aquaretic effect by acting on the V_2 receptors in the blood vessels rather than those in the renal tubules, and it is therefore unaffected by decreased tubular secretion and urine albumin level. When administered in combination with furosemide, tolvaptan further increases urine volume and serum osmolarity due to its aquaretic effect [9], thus enabling excess fluid to be removed from intercellular gaps. Furthermore, because tolvaptan does not increase electrolyte excretion [4], it can be administered to patients with low serum electrolyte levels.

Thus, tolvaptan in combination with conventional diuretic therapy is expected to promote further diuretic effect and to improve ascites and edema without inducing electrolyte imbalance in patients whose ascites or edema is not improved by treatment with conventional diuretic therapy alone, or in whom conventional diuretics cannot be administered at higher doses or in combination due to the risk of decreased serum electrolyte levels.

In this study, in order to examine the efficacy of tolvaptan in improving ascites and edema by concomitant use with conventional diuretics, we administered tolvaptan to patients who had persistent ascites (diagnosed by ultrasonography) and/or lower limb edema secondary to liver disease despite their use of the conventional diuretic furosemide.

Methods

Subjects

Over a period from 2004 to 2005, 8 study sites and 18 subjects participated in this study conducted in Japan. Of the 18 subjects who received tolvaptan, 17 were included in efficacy analysis, and one subject was excluded due to a deviation from the study protocol (violation of concomitant medication use). Of the 17 subjects included in the efficacy analysis, 17 received 3-day administration of tolvaptan at 15 mg, 15 received 3-day administration at the titrated dose of 30 mg, and 11 received 3-day administration at the titrated dose of 60 mg. All 18 subjects who received tolvaptan were included in the safety analysis.

Demographics and other baseline characteristics are shown in Table 1.

Table 1 Baseline characteristics of subjects included in efficacy analysis

Number of subjects	17
Age (years)[a]	57.6 ± 7.1
Sex (male/female) (*n*)	14/3
Body weight (kg)[b]	60.69 ± 9.99
Liver disease (cirrhosis) (*n*)	
Hepatitis B	4
Hepatitis C	6
Alcoholic cirrhosis	5
Primary biliary cirrhosis	1
Other	1
Food restriction [*n* (%)]	10 (58.8)
Encephalopathy [*n* (%)]	0 (0.0)
Liver cancer [*n* (%)]	6 (35.3)
Diabetes mellitus [*n* (%)]	8 (47.1)
Varicose veins [*n* (%)]	15 (88.2)

[a] Mean ± SD

[b] At time of screening examination, mean ± SD

Criteria for eligibility

Patients between 20 and 69 years of age who had persistent ascites and/or lower limb edema despite their use of oral furosemide at a dose of 40 mg/day or higher were eligible for enrollment in the study. Subjects were either hospitalized patients or patients who could be hospitalized for the entire study period from the start of the pretreatment observation period until completion of the end-of-treatment examination.

The main exclusion criteria were: (1) complication of: (a) hepatic encephalopathy (Inuyama classification [10] grade II or higher), (b) poorly controlled hepatocellular carcinoma (with imaging-based diagnosis of vascular infiltration of main portal vein, first branch of portal vein, inferior vena cava, or main hepatic vein), (c) esophageal or gastric varices (endoscopic findings within 1 month prior to screening indicating the need for therapy), (d) diabetes mellitus with poorly controlled blood glucose, (e) heart failure (NYHA class III or IV), (f) anuria (urine volume of 100 mL/day or less), (g) impaired urination, or (h) hyponatremia (serum Na <120 mEq/L); (2) body mass index exceeding 35; (3) clinical laboratory values of: (a) hemoglobin <9.0 g/dL, (b) total bilirubin >3.0 mg/dL, (c) serum creatinine >2.0 mg/dL, (d) serum sodium >147 mEq/L, (e) serum potassium >5.5 mEq/L, (f) or prothrombin time <30% (if not using activity ratio, prolonged by 5 s or more above upper limit of normal range or vs. the control value); and (4) patients who had used blood products, including albumins, within 7 days prior to the start of tolvaptan administration.

All patients gave written informed consent to participate in the study, and the study was approved by the institutional review board (IRB) of each study site.

Treatment protocol

The study consisted of a pretreatment observation period and a treatment period. The pretreatment observation period began 3 days prior to the start of tolvaptan administration, and from that time the dosage regimen of the conventional diuretics being used was fixed until completion of the end-of-treatment examination. Of 19 enrolled subjects, 18 subjects whose fluid volume expansion status (change in body weight) showed no or little change (change of within ±1.0 kg in pre-breakfast body weight from day 2 to day 3 of pretreatment observation period) during the pretreatment observation period were eligible to advance to the treatment period. One subject was judged to be ineligible to advance to the treatment period due to excessive weight decrease during the pretreatment observation period. During the treatment period, tolvaptan was orally administered once daily after breakfast in combination with the fixed regimen of conventional diuretics, including furosemide, and administration of tolvaptan was continued in a dose-titration manner until either ascites (as verified by ultrasonography) and lower limb edema symptoms disappeared or administration at the highest dose was completed.

Administration of tolvaptan was initiated at 15 mg/day and assessment was performed after 3 days to determine whether or not to titrate. If ascites and edema disappeared after 3-day repeated administration at 15 mg/day, administration was stopped. If ascites or edema persisted, the dose of tolvaptan was titrated to 30 mg/day and administration was continued for another 3 days, after which the same procedure was repeated for titration to 60 mg/day. The maximum dose was set at 60 mg/day and each dose was administered for 3 days only, for a maximum total administration period of 9 days.

The end-of-treatment examination in each subject was performed on the day following the final tolvaptan administration. A follow-up assessment was performed at 7–10 days after the final tolvaptan administration.

The primary efficacy endpoint for this study was composite ascites/edema improvement rate. Secondary efficacy endpoints were change in body weight, change in abdominal circumference, ascites improvement rate, lower limb edema improvement rate, and ascites/edema resolution rate.

Improvement of ascites was defined as a decrease of at least 2 cm in abdominal circumference. Improvement of lower limb edema was determined based on the difference in edema severity assessment between before and after administration according to 4 grades: (1) "none"—no observable pitting; (2) "mild"—barely visible pitting; (3) "moderate"—observable pitting; and (4) "severe"—obvious edema at first sight.

The grading criteria for determining improvement of ascites were: "improved" abdominal circumference decreased by 2 cm or more; "unchanged" change in abdominal circumference of less than 2 cm; "worsened" abdominal circumference increased by 2 cm or more or emergence of ascites. The grading criteria for determining improvement of lower limb edema were: "markedly improved"—resolution or improvement by 2 grades or more; "improved"—improvement by one grade; "unchanged"—symptoms unchanged or no symptoms at baseline; "worsened"—worsened by one grade or more.

The improvement rate for each symptom was calculated as: (number of subjects with a grading of "improved" or "markedly improved")/(number of subjects with corresponding symptom) × 100. The composite ascites/edema improvement rate combined the ascites improvement rate and the lower limb edema improvement rate.

The ascites/edema resolution rate was defined as the percentage of subjects who had ascites and/or edema at baseline and whose ascites and edema were confirmed by the investigator to be resolved at the time of physical examination. Ascites/edema resolution rate was calculated as: (number of subjects who showed resolution of ascites and edema)/(number of subjects included in efficacy analysis) × 100 (Table 3).

Pharmacodynamic endpoints were cumulative 24-h urine volume, urine osmolarity, serum sodium level, and plasma AVP level.

Statistical analysis

In the analysis of the primary efficacy endpoint, for subjects with ascites and/or lower limb edema at baseline (day −1), the point estimate and two-sided 95% confidence interval for the composite ascites/edema improvement rate at individual maximum doses were determined.

In the analysis of secondary efficacy endpoints, for change in body weight and abdominal circumference, the mean and standard deviation of the amount of change and of the percent change from baseline (day 1) at each postdose time point were calculated and a *t* test was performed at baseline and at each postdose time point. For subjects with ascites/edema at baseline (day −1), the point estimate and two-sided 95% confidence interval for the ascites/edema resolution rate were determined.

For analysis of pharmacodynamic endpoints, for cumulative 24-h urine volume, urine osmolarity, and plasma AVP level, the mean and standard deviation of the percent change from baseline (day −1 or day 1) at each postdose time point were calculated and a *t* test was performed at baseline and at each postdose time point.

Results

Pharmacodynamic assessment

The aquaretic action of tolvaptan significantly increased urine volume at all postdose measurement time points compared with the baseline (1445 ± 420 mL/day), with cumulative 24-h urine volumes of 3240.3 ± 1014.5, 3943.3 ± 1060.6, and 4537.4 ± 1621.3 mL/day (mean ± SD) following 3-day repeated administration at 15, 30, and 60 mg, respectively, indicating a dose-dependent diuretic effect throughout the treatment period (Fig. 1).

Administration of tolvaptan also significantly decreased urine osmolarity (Fig. 2) and increased serum sodium level (Fig. 3) and plasma AVP level (Fig. 4) at all postdose measurement time points compared with the baseline.

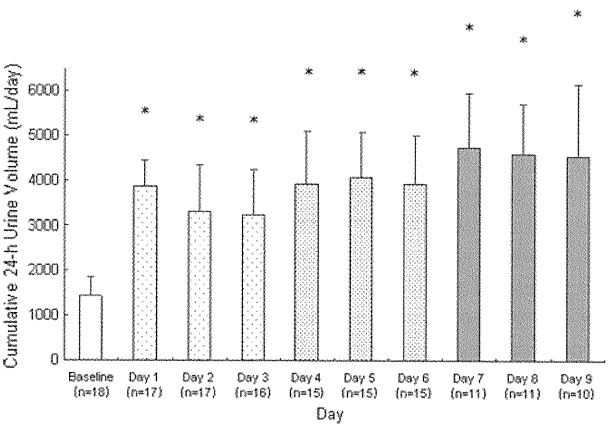

Fig. 1 Cumulative 24-h urine volume (mean ± SD). Urine was cumulatively collected during the 24-h period after each dosing (15 mg on days 1 through 3, 30 mg on days 4 through 6, and 60 mg on days 7 through 9). Statistically significant (*$P < 0.05$) increases in urine volume compared with the baseline were observed at all measurement time points following the start of tolvaptan administration. Urine volume was markedly increased from the first day of administration at 15 mg, and remained increased until the final administration at that dose. In addition, further increases in urine volume were observed with dose titration from 15 to 30 mg and again with dose titration from 30 to 60 mg

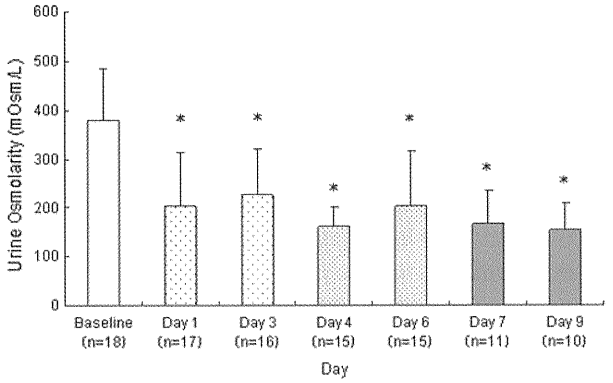

Fig. 2 Urine osmolarity (mean ± SD). Urine osmolarity was measured at 24 h after each dosing (15 mg on days 1 through 3, 30 mg on days 4 through 6, and 60 mg on days 7 through 9). Statistically significant ($*P < 0.05$) decreases in urine osmolarity compared with the baseline were observed at all measurement time points following the start of tolvaptan administration. Urine osmolarity was markedly decreased from the first day of administration at 15 mg, and remained decreased until completion of treatment

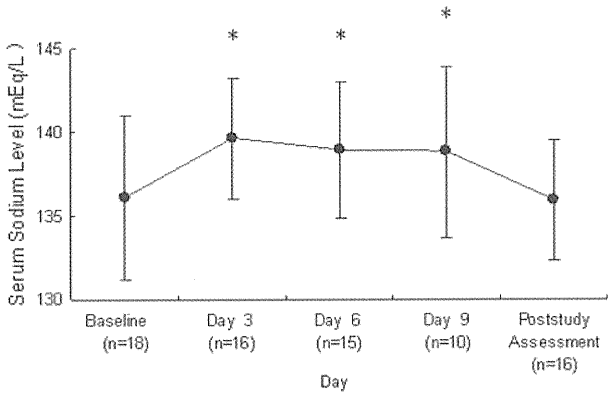

Fig. 3 Serum sodium level (mean ± SD). Serum sodium level was measured at 24 h after the final dosing of 3-day repeated oral administration at each dose (15 mg on days 1 through 3, 30 mg on days 4 through 6, and 60 mg on days 7 through 9). Statistically significant ($*P < 0.05$) increases in serum sodium level compared with the baseline were observed at all measurement time points following the start of tolvaptan administration at 15 mg on day 1. The increased serum sodium level showed a tendency to return to the baseline level after completion of treatment

Efficacy of tolvaptan against intractable ascites and edema

Decreases in body weight and abdominal circumference and improvement of ascites and lower limb edema were observed following administration of tolvaptan beginning from 15 mg. Decreases in mean body weight of 1 kg or more were seen from Day 2 (24 h after first dosing). Changes in body weight following 3-day repeated administration at 15, 30, and 60 mg were, respectively, −1.6 ± 0.9, −2.6 ± 1.2, and −3.4 ± 2.1 kg (mean ± SD),

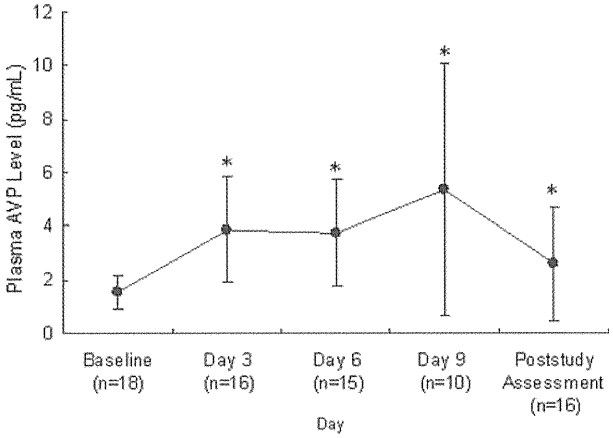

Fig. 4 Plasma AVP level (mean ± SD). Plasma AVP level was measured at 24 h after the final dosing of 3-day repeated oral administration at each dose (15 mg on days 1 through 3, 30 mg on days 4 through 6, and 60 mg on days 7 through 9). Statistically significant ($*P < 0.05$) increases in plasma AVP level compared with the baseline were observed at all measurement time points following the start of tolvaptan administration at 15 mg on day 1. The increased plasma AVP level showed a tendency to return to the baseline level after completion of treatment

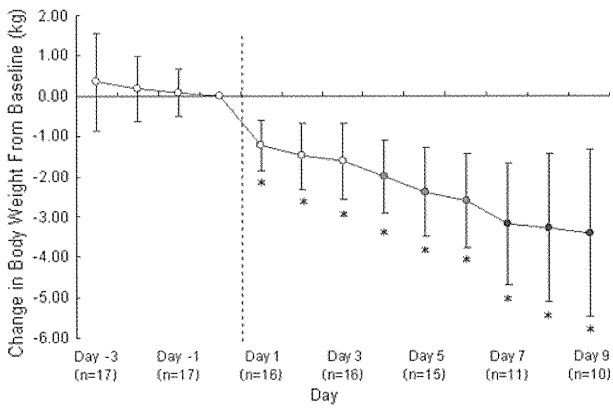

Fig. 5 Change in body weight from baseline (mean ± SD). Body weight was measured at 24 h after each dosing (15 mg on days 1 through 3, 30 mg on days 4 through 6, and 60 mg on days 7 through 9), and changes from the baseline were calculated. Although body weight showed almost no decrease during the pretreatment observation period, statistically significant ($*P < 0.05$) decreases compared with the baseline were observed from the start of tolvaptan administration at 15 mg on day 1. Body weight continued to gradually decrease until the final administration, at which time a statistically significant ($*P < 0.05$) difference from the baseline was observed

and body weight at all postdose measurement time points was significantly decreased compared with the baseline (Fig. 5). As was observed for body weight, decreases in mean abdominal circumference were also observed following tolvaptan administration, with statistically significant differences from the baseline seen at all postdose measurement time points (Fig. 6).

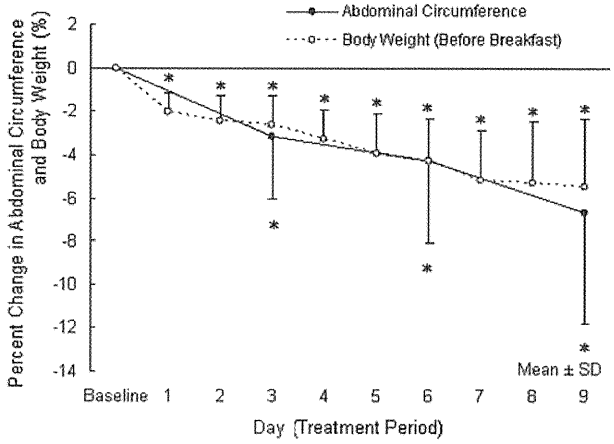

Fig. 6 Time courses of percent change in body weight and abdominal circumference from baseline (mean ± SD). Body weight and abdominal circumference were measured at 24 h after each dosing (15 mg on days 1 through 3, 30 mg on days 4 through 6, and 60 mg on days 7 through 9), and percent change from the baseline was calculated. Both body weight and abdominal circumference showed dose-dependent percent decreases from the baseline with administration of tolvaptan, and statistically significant (*$P < 0.05$) percent decreases from the baseline were observed at all measurement time points

At individual maximum doses, the ascites improvement rate was 87.5% (14 of 16 subjects) and the lower limb edema improvement rate was 83.3% (5 of 6 subjects). The composite ascites/edema improvement rate (primary efficacy endpoint) was 88.2% (15 of 17 subjects) at individual maximum doses or at discontinuation of administration (Table 2a), and 64.7, 80.0, and 90.9%, respectively, after 3-day administration at 15, 30, and 60 mg (Table 2b). In addition, the ascites/edema resolution rate at individual maximum doses was 41.2% (7 of 17 subjects) (Table 3).

Dose–response in this study was evaluated based on the results for changes in cumulative 24-h urine volume, body weight, and abdominal circumference and for improvement of ascites/edema. Regarding body weight, a further body weight decrease of 500 g or more was seen in 8 of 15 subjects following dose titration from 15 to 30 mg/day and in 6 of 11 subjects following dose titration from 30 to 60 mg/day. Decreases in body weight were greater on the first day of administration after dose titration than on the second and third days of administration at the same dose. Regarding abdominal circumference, a further abdominal circumference decrease of 2 cm or more was seen in 7 of 15 subjects following dose titration from 15 to 30 mg/day and in 4 of 11 subjects following dose titration from 30 to 60 mg/day. Of the 7 subjects in whom ascites was not improved (abdominal circumference decrease of less than 2 cm) by 3-day repeated administration at 15 mg/day, 5 subjects showed improvement or resolution of ascites following dose titration to 30 mg/day. Regarding improvement of ascites/edema, although 6 of 17 subjects were assessed as "unchanged" at 15 mg/day, 4 of those 6 subjects showed improvement following dose titration to 30 mg/day. In addition, 2 of 3 subjects assessed as "unchanged" at 30 mg/day showed improvement of ascites/edema following dose titration to 60 mg/day (Fig. 7).

Safety evaluation

Following administration of tolvaptan in a dose-titration manner (15–60 mg/day) to subjects with ascites and/or lower limb edema associated with decompensated liver cirrhosis, adverse events were observed in all 18 subjects who received tolvaptan, for a total of 69 episodes. However, most of the events were considered to have been due either to the pharmacological action of tolvaptan or to the underlying disease. Adverse events observed in 2 or more subjects during the study are summarized in Table 4. Most of the adverse events were observed following administration at 15 mg, and the incidence of adverse events did not increase with dose titration. The most frequently reported adverse events, occurring in 3 subjects or more, were thirst, pollakiuria, insomnia, and increased blood uric acid. No noteworthy changes in clinical laboratory values (hematocrit and hemoglobin), blood pressure, or ECG were observed. In particular, as shown in Table 5, no increases in blood pressure were observed.

Adverse events judged to be adverse drug reactions (i.e., potentially study-related) were also observed in all 18 subjects who received tolvaptan, for a total of 53 episodes. Four serious adverse events (anal fistula, esophageal varices, hepatic neoplasm malignant, and hepatic encephalopathy) were observed in one subject each, and relationship to tolvaptan could not be denied for the anal fistula observed in one subject during 3-day repeated administration at 60 mg. It was confirmed at the poststudy follow-up assessment that all adverse drug reactions were either recovered or ameliorated. Discontinuation of tolvaptan administration or dose reduction was not required in any subject. No adverse events were attributable to aggravation of the underlying disease by administration of tolvaptan in patients with decompensated liver cirrhosis.

Discussion

Although tolvaptan had previously been shown to improve volume expansion in patients with heart failure [11–13] and to raise serum sodium level in cases of hyponatremia [14], its effects on ascites and edema of the extremities

Table 2 Improvement rates at (a) individual maximum doses and (b) each dose

	Grading				Total	Improvement rate (%)[a]	Two-sided 95% CI
	Markedly Improved	Improved	Unchanged	Worsened			
(a)							
Ascites[b]	–	14	2	0	16	87.5	61.7–98.4
Lower limb edema[c]	5	0	1	0	6	83.3	35.9–99.6
Composite ascites/edema[d]	4	11	2	0	17	88.2	63.6–98.5
(b)							
Ascites[b]							
15 mg Day 3	–	9	7	0	16	56.3	
30 mg Day 6	–	12	3	0	15	80.0	
60 mg Day 9	–	10	1	0	11	90.9	
Lower limb edema[c]							
15 mg Day 3	3	0	3	0	6	50.0	
30 mg Day 6	4	1	0	0	5	100.0	
60 mg Day 9	2	0	1	0	3	66.7	
Composite ascites/edema[d]							
15 mg Day 3	1	10	6	0	17	64.7	38.3–85.8
30 mg Day 6	5	7	3	0	15	80.0	51.9–95.7
60 mg Day 9	2	8	1	0	11	90.9	58.7–99.8

[a] Improvement rate = (number of subjects with grading of improved or markedly improved)/(total number of subjects with corresponding symptom) × 100

[b] Grading criteria for ascites: improved = abdominal circumference decreased by 2 cm or more; unchanged = change in abdominal circumference of less than 2 cm; worsened = abdominal circumference increased by 2 cm or more or emergence of ascites

[c] Grading criteria for lower limb edema: markedly improved = resolution or improvement by 2 grades or more; improved = improvement by one grade; unchanged = symptoms unchanged or no symptoms at baseline; worsened = worsened by one grade or more [severity grades: (1) none = no observable pitting; (2) mild = barely visible pitting; (3) moderate = observable pitting; (4) severe = obvious edema at first sight]

[d] Grading criteria for composite ascites/edema: markedly improved = improved for ascites and markedly improved or improved for lower limb edema; improved = unchanged for ascites and markedly improved or improved for lower limb edema; unchanged = unchanged for both ascites and lower limb edema; worsened = worsened for either ascites or lower limb edema

Table 3 Ascites/edema resolution rate at individual maximum doses

Resolved	Not resolved	Total	Resolution rate (%)	Two-sided 95% CI
7	10	17	41.2	18.4–67.1

Ascites/edema resolution rate = (number of subjects resolved)/(total number of subjects) × 100

associated with decompensated liver cirrhosis remained to be explored.

In the present study, we administered tolvaptan as add-on therapy to decompensated liver cirrhosis patients with ascites and/or lower limb edema that was resistant to conventional diuretics. We evaluated efficacy based on changes in body weight, abdominal circumference, daily urine volume, and the severity of lower leg edema, all of which are commonly used parameters for assessing hypervolemia associated with decompensated liver cirrhosis.

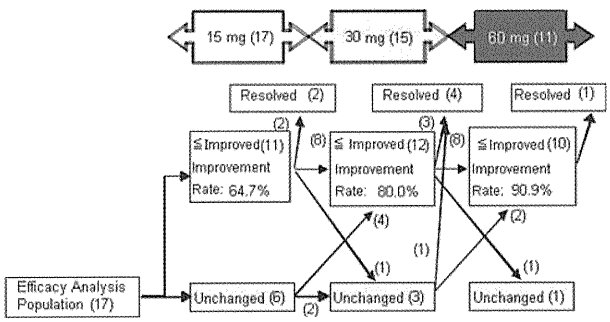

Fig. 7 Changes in composite ascites/edema improvement gradings. Composite ascites/edema improvement was assessed by the investigator after the final dosing of 3-day repeated oral administration at each dose (15 mg on days 1 through 3, 30 mg on days 4 through 6, and 60 mg on days 7 through 9). Four of six subjects assessed as "unchanged" at 15 mg/day were assessed as "improved" or "markedly improved" following dose titration to 30 mg/day, and 2 of 3 subjects assessed as "unchanged" at 30 mg/day were assessed as "improved" or "markedly improved" following dose titration to 60 mg/day, with some subjects showing further improvement with each dose titration. *Numbers in parentheses* indicate number of subjects

Table 4 Summary of adverse events

Item	Tolvaptan 15–60 mg ($N = 18$[a]) n (%)
Adverse events occurring during study (all causes)	18 (100)
Serious adverse events[b]	4 (22.2)
Adverse drug reactions occurring during study	18 (100)
Serious adverse events judged to be adverse drug reactions[c]	1 (5.6)
Adverse events (all causes) by body system and MedDRA preferred term[d]	
Gastrointestinal disorders	
Constipation	2 (11.1)
Esophageal varices	2 (11.1)
General disorders and administration site conditions	
Thirst	15 (83.3)
Malaise	2 (11.1)
Pyrexia	2 (11.1)
Investigations	
Blood uric acid increased	3 (16.7)
Blood glucose increased	2 (11.1)
Metabolism and nutrition disorders	
Anorexia	2 (11.1)
Psychiatric disorders	
Insomnia	4 (22.2)
Renal and urinary disorders	
Pollakiuria	8 (44.4)
Skin and subcutaneous tissue disorders	
Dry skin	2 (11.1)

[a] All subjects who received at least one dose of the study medication (tolvaptan) were included in safety analysis

[b] All-cause serious adverse events occurring during the study were anal fistula, esophageal varices, hepatic neoplasm malignant, and hepatic encephalopathy in one subject each

[c] The only serious adverse event judged to be an adverse drug reaction (i.e., potentially study-related) was anal fistula in one subject

[d] Adverse events occurring in 2 or more subjects are listed

A marked decrease in body weight accompanying increased urine volume was observed soon after the start of administration of tolvaptan, which has a vasopressin V_2 receptor blocking action. Similar to the effects previously seen in patients with heart failure, in the present study administration of tolvaptan also improved ascites and pitting edema (signs indicating hypervolemia) in patients with decompensated liver cirrhosis.

Serum sodium level was also increased following administration of tolvaptan, as was seen in heart-failure patients and cases of hyponatremia. This increase in serum sodium level is considered to be due to an increase in urine volume induced by tolvaptan's vasopressin V_2 receptor antagonist action. As rapid elevation of serum sodium can cause central pontine myelinolysis (CPM), any increase in serum sodium level should not exceed 12 mmol/L within a 24-h period [15, 16]. No complications of CPM or hypernatremia have been observed in clinical studies of tolvaptan.

Although plasma AVP level also increased during administration of tolvaptan, it subsequently decreased after completion of treatment. This increase in plasma AVP was probably due to an increase in plasma osmotic pressure rather than to any decrease in plasma volume, since hematocrit and hemoglobin values remained unchanged after administration of tolvaptan, indicating that a decrease in plasma volume was unlikely. In addition to promoting the reabsorption of water in the kidney via vasopressin V_2 receptors, AVP also induces vasoconstriction via vasopressin V_1 receptors, resulting in an increase in blood pressure. While increased blood pressure can lead to the rupture of esophageal varices, no variceal bleeding was observed in this study, indicating that tolvaptan would be safe for the treatment of hypervolemia as a complication of decompensated liver cirrhosis. Although thirst is a common adverse drug reaction seen with tolvaptan, in the present study thirst was improved by allowing free access to drinking water.

The results of this study demonstrated that tolvaptan exerted a dose-dependent aquaretic effect in patients with furosemide-resistant intractable ascites and/or edema. Most of the adverse events reported were predictable based on tolvaptan's known pharmacological action, and dose titration to 60 mg was well tolerated, with no discontinuations due to adverse events.

In conclusion, based on current study data, tolvaptan is considered to be a safe and effective agent for the treatment of chronic liver failure patients with ascites and/or pitting

Table 5 Blood pressure values

Item (unit)	Time point	*N*	Mean	SD
Systolic blood pressure (mmHg)	Day 1 predose	18	110.5	16.6
	2–4 h postdose	17	108.2	12.4
	6–8 h postdose	18	110.2	12.1
	Day 2	17	109.4	13.7
	Day 3	17	108.9	15.2
	Day 4 predose	17	107.3	16.0
	2–4 h postdose	15	109.1	13.9
	6–8 h postdose	16	105.0	15.9
	Day 5	15	109.5	12.7
	Day 6	15	106.7	12.3
	Day 7 predose	15	108.3	14.2
	2–4 h postdose	11	106.6	10.0
	6–8 h postdose	11	105.5	8.2
	Day 8	11	104.8	11.1
	Day 9	11	104.7	14.6
	Day 10	10	105.8	12.1
Diastolic blood pressure (mmHg)	Day 1 predose	18	68.1	7.6
	2–4 h postdose	17	65.3	9.5
	6–8 h postdose	18	67.0	7.3
	Day 2	17	66.4	7.9
	Day 3	17	67.4	4.7
	Day 4 predose	17	68.1	13.0
	2–4 h postdose	15	65.3	9.0
	6–8 h postdose	16	65.6	9.7
	Day 5	15	68.2	8.7
	Day 6	15	67.1	8.1
	Day 7 predose	15	68.4	9.2
	2–4 h postdose	11	69.3	10.5
	6–8 h postdose	11	69.9	6.7
	Day 8	11	66.4	8.9
	Day 9	11	63.4	4.9
	Day 10	10	68.6	5.9

edema that is resistant to powerful diuretics such as furosemide. We also concluded that continued investigation in a parallel-group comparison study is needed to further clarify the drug's efficacy.

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