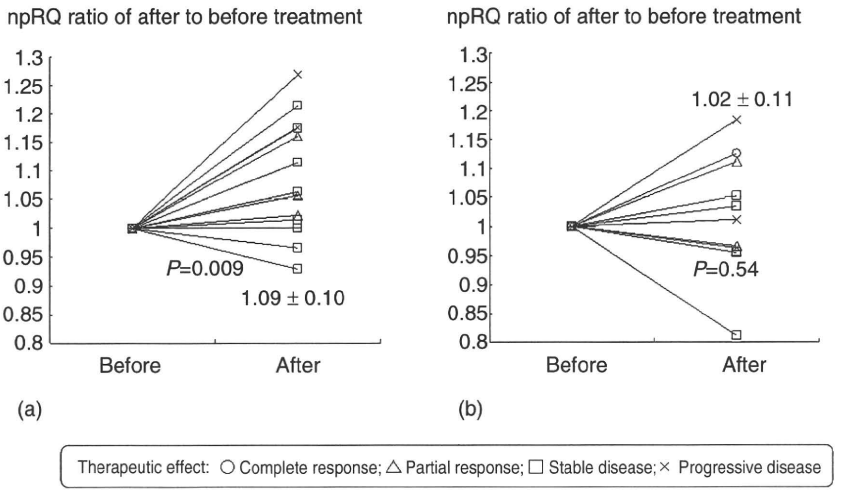

Figure 3 The npRQ ratio after compared to before 1 cycle of HAIC. In the LES group, npRQ improved in 10 patients, was stable in 1 patient, and worsened in 2 patients, regardless of response to therapy ($P = 0.009$) (a). In the control group, npRQ improved in 6 patients and worsened in 4 patients ($P = 0.54$) (b).

$P = 0.69$). Figure 3 shows the npRQ ratio of after compared to before 1 cycle of HAIC. In the LES group, npRQ improved in 10 patients, was stable in 1 patient, and worsened in 2 patients, regardless of response to therapy ($P = 0.009$). In the control group, npRQ improved in 6 patients and worsened in 4 patients ($P = 0.54$).

Blood biochemistry

Significant improvements in BTR, prealbumin and ALT levels were observed after 1 cycle of treatment in the LES group, but not in the control group. Cholinesterase levels were significantly decreased after 1 cycle of treatment in the control group, but did not differ in the LES group (Table 3). Figure 4 shows the BTR ratio of after to before 1 cycle of HAIC. In the LES group, BTR improved in 10 patients and worsened in 3 patients ($P = 0.005$). Conversely, BTR worsened in 7 patients in the control group ($P = 0.46$).

Anthropometry

No significant differences in anthropometric measurements (weight; skeletal muscle mass; body fat mass; fat-free mass; mid-upper arm muscle circumference (AMC); midarm circumference (AC); and body cell mass (BCM)) as measured using InBody were observed between groups (data not shown).

Changes in glucose tolerance

We examined the effects of LES using a BCAA-enriched nutrient on glucose tolerance using the 75-g OGTT in 21

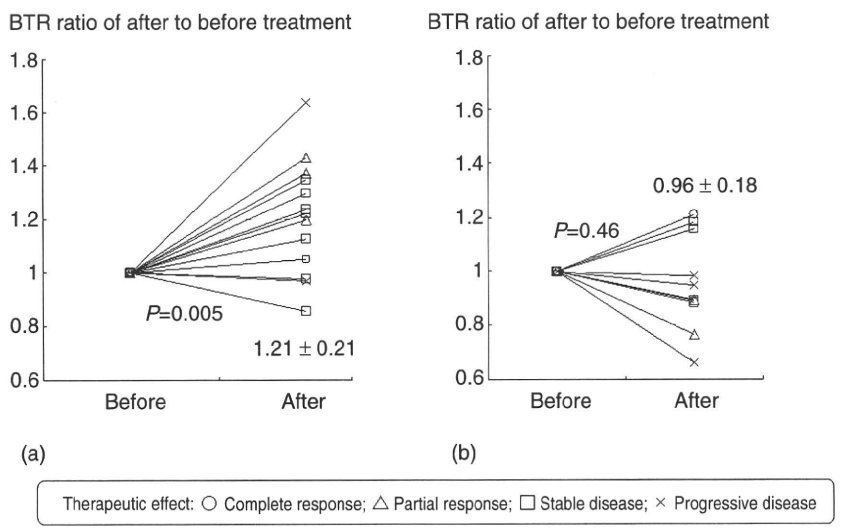

Figure 4 Branched-chain amino acid/tyrosine ratio (BTR) after compared to before 1 cycle of HAIC. In the LES group, BTR improved in 10 patients and worsened in 3 patients ($P = 0.005$) (a). Conversely, BTR worsened in 7 patients in the control group ($P = 0.46$) (b).

Table 4 Changes in glucose tolerance

	LES group (n = 12)			Control group (n = 9)		
	Before	After	*P*-value	Before	After	*P*-value
AUC glucose	396.1 ± 117.0	363.2 ± 135.6	0.055	355.3 ± 62.9	321.1 ± 108.6	0.17
AUC insulin	181.8 ± 123.3	223.2 ± 169.0	0.10	155.4 ± 85.3	117.7 ± 71.4	0.15
Fasting glucose (mg/dL)	99.5 ± 27.9	98.5 ± 32.6	0.71	100.7 ± 13.9	99.6 ± 18.7	0.89
Fasting insulin (μ U/mL)	11.3 ± 10.2	12.8 ± 7.1	0.38	14.5 ± 15.6	8.2 ± 1.8	0.28
HOMA-IR	2.9 ± 2.7	3.3 ± 2.5	0.47	4.0 ± 5.2	2.1 ± 0.8	0.32

AUC, area under the concentration curve; HOMA-IR, homeostasis model assessment method for insulin resistance; LES, late evening snack.

of 23 patients. One patient with DM in the LES group did not undergo the 75-g OGTT after treatment due to markedly high glucose levels, while the remaining patient in the control group declined to undergo the 75-g OGTT after treatment.

Table 4 shows changes in glucose tolerance before and after 1 cycle of treatment. In the LES group ($n = 12$), 1, 2, and 9 patients exhibited NGT, IGT, and DM, respectively. In the control group, 3, 2, and 4 patients exhibited NGT, IGT, and DM, respectively. No significant differences at baseline were seen between groups with regard to NGT, IGT, or DM using the 75-g OGTT, fasting glucose, fasting insulin, homeostasis model assessment method for insulin resistance (HOMA-IR), AUC glucose, and AUC insulin. AUC glucose tended to improve after 1 cycle of treatment in the LES group ($P = 0.055$). However, no significant differences in other parameters were apparent.

Prognosis

No significant differences in survival rates were seen between groups ($P = 0.667$; log-rank test) (Fig. 5a). On the other hand, survival in patients assessed as SD or PD according to the response criteria[30] tended to improve in the LES group ($n = 10$ in the LES group, $n = 7$ in the control group; $P = 0.156$; log-rank test) (Fig. 5b). In addition, no significant differences between groups assessed as SD or PD were seen with relation to background (data not shown).

By final follow-up, 2 patients remained alive (LES group, $n = 1$; control group, $n = 1$), while the other 21 patients had died. In the LES group, cause of death was cancer progression in 12 patients. In the control group, cause of death was cancer progression in 8 patients and hepatic failure in 1 patient.

Case presentation

Figure 6 shows a patient from the LES group. This 49-year-old man showed multiple HCCs in both lobes (stage III).[27,28] Mild ascites was identified, but no hepatic encephalopathy was present. On admission, hepatic reserve function was defined as Child-Pugh B (9 points). Prior to starting LES, npRQ was 0.71, and BTR value was low, at 2.2. After 1 cycle of HAIC, laboratory investigations were improved, and no ascites was apparent. Hepatic reserve function had improved to Child-Pugh A (6 points). Values of npRQ and BTR increased to 0.75 and 2.68, respectively, after 1 cycle of treatment. The patient exhibited DM on the 75-g OGTT. AUC glucose improved after 1 cycle of treatment (before, 414.75;

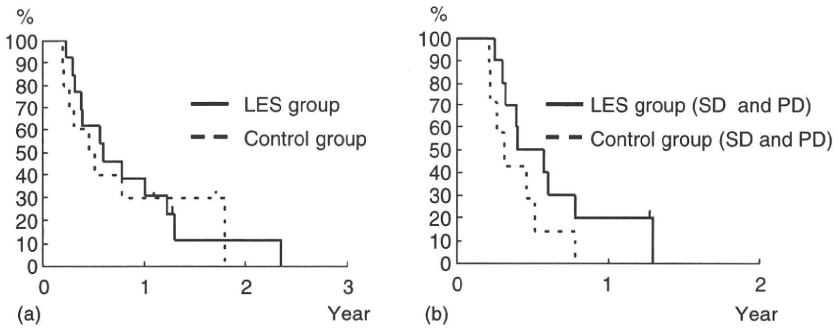

Figure 5 No significant differences in survival rates were seen between groups ($P = 0.667$; log-rank test) (a). On the other hand, survival in patients assessed as stable disease (SD) or progressive disease (PD) according to the response criteria tended to improve in the LES group ($n = 10$ in the LES group, $n = 7$ in the control group; $P = 0.156$; log-rank test) (b).

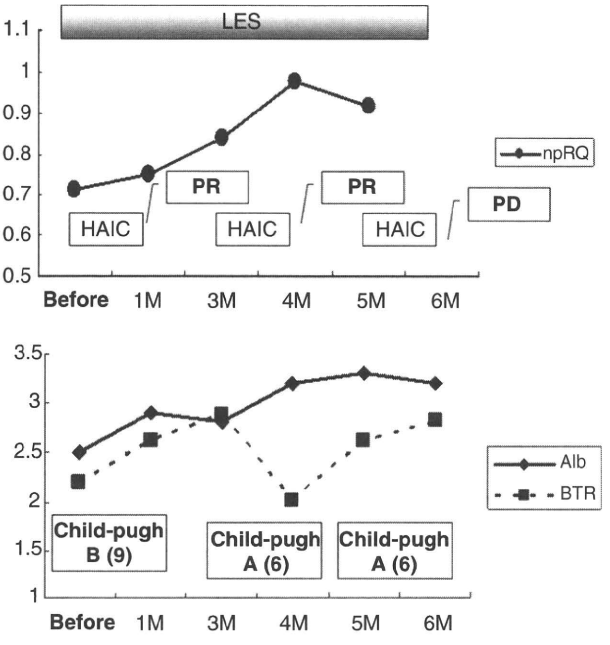

Figure 6 A case in the LES group. This 49-year-old man had multiple HCCs in both lobes (stage III). On admission, hepatic reserve function was Child-Pugh B (9 points). Prior to starting LES, npRQ was 0.71, and BTR was low at 2.2. After 1 cycle of HAIC, hepatic reserve function was improved to Child-Pugh A (6 points) from Child-Pugh B (9 points). Values of npRQ and BTR increased to 0.75 and 2.68, respectively, after 1 cycle of the treatment. The patient exhibited PR according to the response criteria. Thereafter, he received 3 courses of HAIC, and npRQ, serum albumin and BTR values improved. After the third course of HAIC, he exhibited PD. One year after the first course of HAIC, he died of tumor progression.

after, 369.75). PR was exhibited according to the response criteria.[30] Thereafter, the patient received 3 courses of HAIC, and npRQ, serum albumin and BTR value improved. After the third course of HAIC, he exhibited PD. One year after the first course of HAIC, he died of tumor progression.

Figure 7 shows a patient from the control group. This 73-year-old woman presented with massive HCC in the right lobe with tumor thrombus in the main trunk of the portal vein (Vp4) (stage IV A).[27,28] No ascites or hepatic encephalopathy was identified. On admission, hepatic reserve function was defined as Child-Pugh A (6 points). Prior to starting HAIC, npRQ was 0.91. However, BTR was low, at 2.34. After 1 cycle of HAIC, laboratory investigations showed slight deterioration. Values for npRQ and BTR decreased to 0.87 and 2.06, respectively, after 1 treatment cycle. The patient exhibited DM on the 75-g OGTT. AUC glucose remained almost unchanged (before, 446.25; after, 437.75). She exhibited SD according to the response criteria.[30] Although she received a second course of HAIC, npRQ, serum albumin and BTR values worsened. In addition, she showed moderate ascites during the second course of treatment. After the second course of treatment, hepatic reserve function was classified as Child-Pugh C (10 points). Thereafter, she developed hepatic encephalopathy and died of hepatic failure 6 months after the first course of HAIC.

DISCUSSION

PEM IS OFTEN observed in cirrhotic patients,[2,3] and this malnutrition adversely affects prognosis.[3] When energy metabolism of cirrhotic patients is measured using indirect calorimetry, npRQ decreases as the severity of liver cirrhosis increases.[3] However, no clinical studies have evaluated energy metabolism in patients with HCC. We therefore investigated energy metabolism

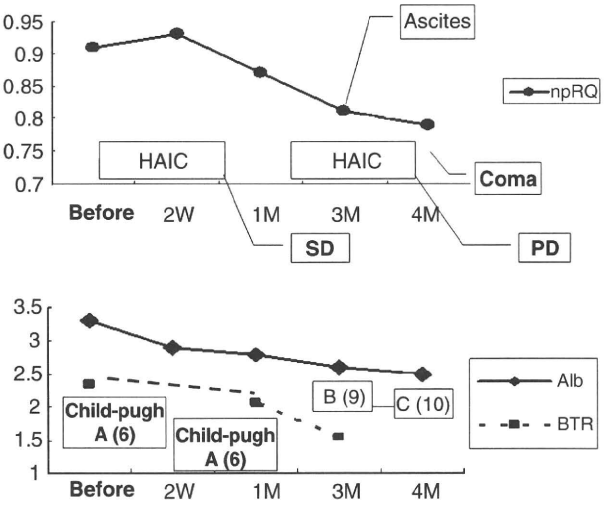

Figure 7 A case in the control group. This 73-year-old woman presented with massive HCC in the right lobe with tumor thrombus in the main trunk of the portal vein (Vp4) (stage IV A). On admission, hepatic reserve function was Child-Pugh A (6 points). Prior to starting HAIC, npRQ was 0.91. However, BTR was low at 2.34. After 1 cycle of HAIC, npRQ and the BTR value decreased to 0.87 and 2.06, respectively. She exhibited SD according to the response criteria. Although she received a second course of HAIC, npRQ, serum albumin and BTR values all worsened. After the second course of treatment, hepatic reserve function was Child-Pugh C (10 points). Thereafter, she developed hepatic encephalopathy and died of hepatic failure 6 months after the first course of HAIC.

using indirect calorimetry in cirrhotic patients without HCC and with various stages of HCC under the same conditions of liver capacity, namely Child-Pugh A. In our study, no significant differences were seen among 3 groups (LC group, HCC stage I/II group, and HCC stage III group), but npRQ was significantly lower in patients with stage IV HCC than in cirrhotic patients without HCC, or in patients with stage I/II or stage III HCC. Although there was a significant difference in age between the HCC stage III group and the HCC stage IV group, there was no significant correlation between the value of npRQ before treatment and age (data not shown). These findings suggest that patients with advanced HCC remained under conditions of severe energy malnutrition even if liver capacity was good. A hypermetabolic rate in patients with gastrointestinal malignancy is reportedly usually associated with the most advanced stage of the disease.[24] The p53 tumor suppressor gene regulates glucose metabolism, and loss of p53 upregulates energy metabolism.[35] Mutation of the p53 gene is associated with poor tumor differentiation and advanced stage of HCC.[36] We speculate that reductions in npRQ among patients with advanced HCC may be related to the upregulation of glucose metabolism in cancer cells, but the underlying mechanisms remain unclear. Our results suggest that nutritional support is warranted for cirrhotic patients with advanced HCC, even if liver capacity is good.

In an attempt to improve the state of energy malnutrition, LES has been developed and improved energy metabolism has been reported.[5–8,11–13] However, those studies focused on the effects of LES in patients with cirrhosis.

Poon *et al.* reported that nutritional supplementation with oral BCAAs is beneficial for increasing serum albumin level, reducing morbidity and improving quality of life in patients undergoing transarterial chemoembolization for HCC,[25] but the nutritional supplementation did not use LES. Takeshita *et al.* only reported that LES using BCAA-enriched nutrients prevents suppression of liver function in patients with HCC undergoing transarterial chemoembolization.[26] That study did not evaluate energy metabolism before and after LES. We therefore investigated the efficacy of nutritional support using LES in patients with advanced HCC undergoing HAIC using indirect calorimetry.

The present findings showed that LES using BCAA-enriched nutrients improves npRQ, BTR, ALT, and prealbumin significantly before and after 1 cycle of HAIC compared with the control group. Nakaya *et al.* reported that LES using BCAA-enriched nutrients improved npRQ, BTR, and serum albumin before and 3 months after in cirrhotic patients compared with LES using ordinary food.[8] Although no significant difference in serum albumin was identified in our study, prealbumin (a rapid turnover protein with a half-life in plasma of 2 days) was significantly increased by LES. Prealbumin is more sensitive to changes in protein-energy status than albumin.[37] We thus consider that improvement of npRQ and prealbumin reflects energy metabolism in cirrhotic patients with advanced HCC undergoing HAIC. Unfortunately, we could not evaluate nutritional parameters in the long term, as some patients died in the short term. Despite the small sample size, of the 12 patients for whom npRQ was evaluated at 3 months after HAIC, patients treated with LES ($n = 6$) tended to show improved npRQ ($P = 0.10$), and npRQ was not significantly difference before and 3 months after HAIC in control patients ($n = 6$; $P = 0.91$; data not shown).

BCAAs reportedly improve glucose intolerance.[11–13,38] The present study evaluated glucose tolerance before and at the end of 1 cycle of treatment. AUC glucose in the LES group tended to improve ($P = 0.055$). In addition, AUC glucose in patients who showed glucose intolerance (IGT and DM; $n = 11$) in the LES group tended to improve (before, 412.3 ± 107.7; after, 376.1 ± 134.3; $P = 0.052$) and AUC glucose in patients who had glucose intolerance ($n = 6$) in the control group showed no significant difference (before, 376.5 ± 67.8; after, 338.3 ± 132.7; $P = 0.30$). One reason might be the effect of LES itself. A LES improves postprandial hyperglycemia, because the glucose load per meal is decreased by fractionated meals including a LES, and glucose is properly oxidized in the tissues. Another reason might be the effects of the leucine and isoleucine contained among the BCAAs. Leucine and isoleucine promote glucose uptake in skeletal muscle under insulin-free conditions.[39] Leucine also increases the activity of p70S6 kinase via the mammalian target of rapamycine pathway, and the ability to synthesize glycogen is improved.[39] However, we have previously reported that glucose tolerance worsened after 3 months of LES administration in cirrhotic patients with DM according to the 75-g OGTT.[40] In 12 patients (LES group, $n = 6$; control group, $n = 6$) for whom glucose tolerance was evaluated using the 75-g OGTT at 3 months after HAIC, AUC glucose was significantly worsened in both groups in this study (data not shown). We reported that LES combined with an alpha-glucosidase inhibitor to slow glucose absorption into the blood and ameliorate postprandial hyperglycemia improved glucose tolerance (AUC glucose) over the long term (3 months) in

patients with liver cirrhosis,[41] and concomitant use of an alpha-glucosidase inhibitor with LES might be a useful nutritional therapy in patients with advanced HCC who show glucose intolerance.

Unfortunately, no significant differences in survival rate were identified between groups ($P = 0.667$; log-rank test). Poon *et al.* also reported no difference in survival between patients who received BCAAs and those receiving ordinary food.[25] However, survival in patients assessed as SD or PD tended to improve in the LES group ($P = 0.156$; log-rank test), although no significant differences between groups assessed as SD or PD were seen with relation to background. Significant improvement in npRQ was observed in the LES group, and significant reductions in cholinesterase and natural killer cell activity were observed in the control group, for groups assessed as SD or PD (data not shown). In addition, the frequency of HCC treatment tended to be increased in the LES group (data not shown). We speculate that life prolongation in patients assessed as SD or PD may be related to improvements in energy metabolism and immune defense,[32] and the continuation of HCC treatment, by means of LES using BCAA-enriched nutrients. Our previous study identified therapeutic effect as an independent prognostic factor in cirrhotic patients with advanced HCC treated using HAIC.[20] In this study, patients exhibited CR or PR showed good prognosis without relation to LES (data not shown). Therefore, we consider that patients exhibited SD or PD may be suitable candidates for LES using BCAA-enriched nutrients because of life prolongation. As our study examined only a small population, further investigations are necessary.

In conclusion, LES using BCAA-enriched nutrients offers the possibility of improving energy metabolism and glucose tolerance in cirrhotic patients with advanced HCC undergoing HAIC. Although our study design shows limitations in the comparison between ordinary food and both LES and BCAA, we speculate that these results are caused by effects from both LES and BCAA. We consider that tailored nutritional support, such as tumor staging, is required in patients with HCC.

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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

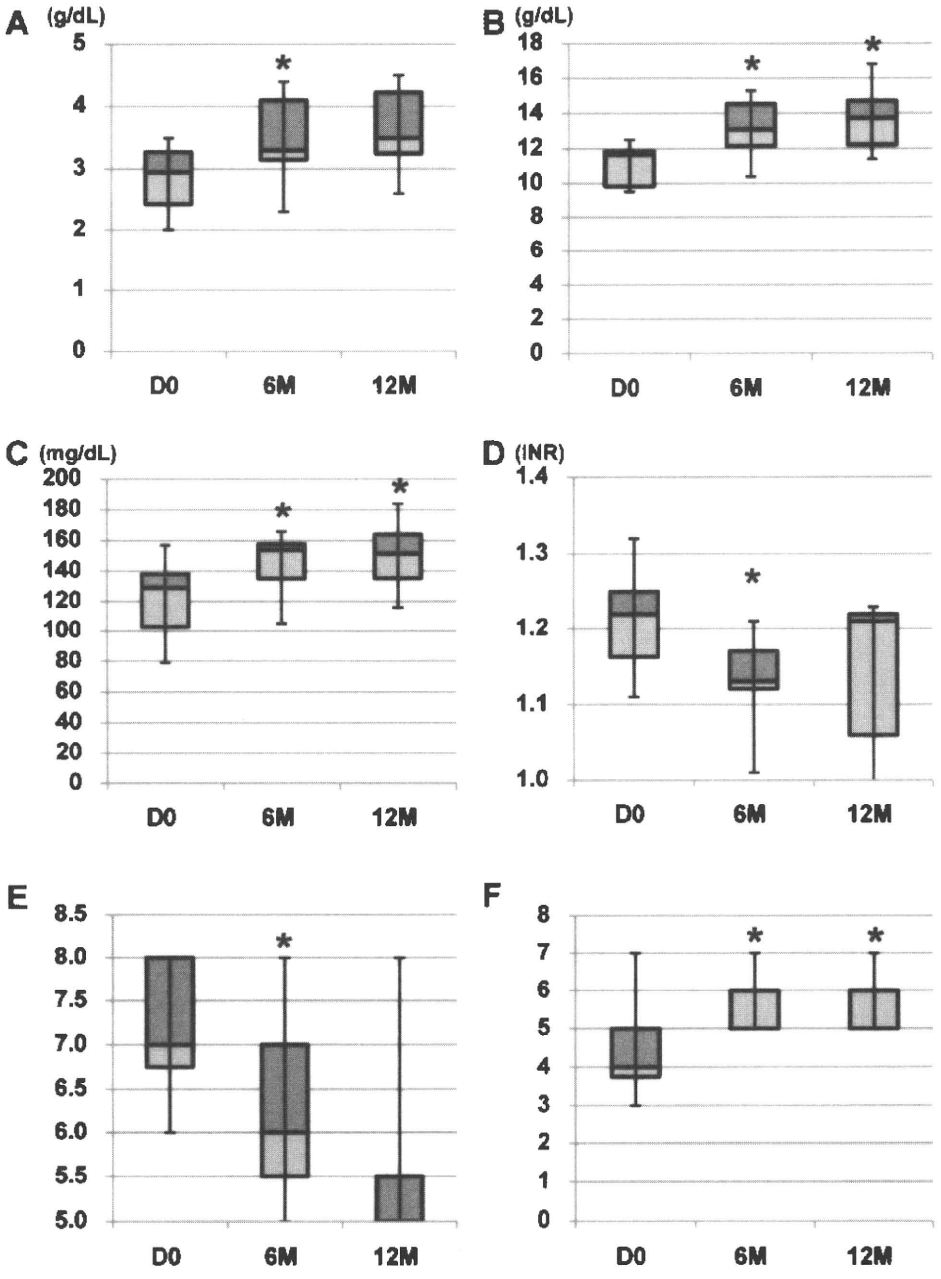


Figure 1. Serial changes in clinical parameters. Serum albumin (A), hemoglobin (B), serum cholesterol (C), prothrombin time (D), Child-Pugh score (E), and feeling of well-being (F) were serially checked according to the follow-up schedule. Gradual but significant improvements in serum albumin (A), hemoglobin (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.

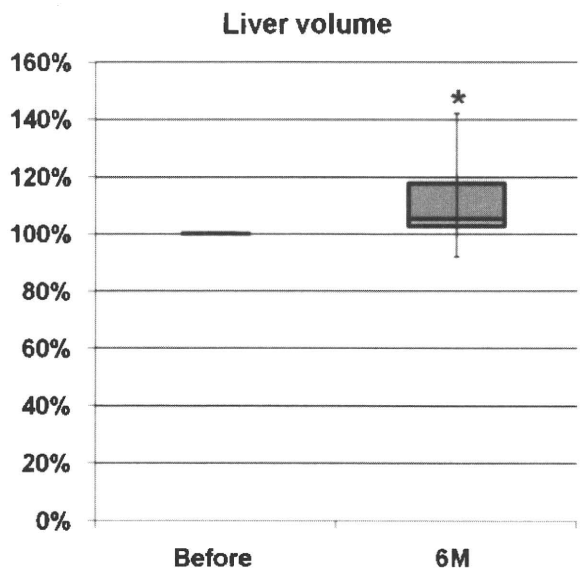


Figure 2. Changes in liver volume before and at 6 months after ABMI. MRIs taken in the sixth month were analyzed to compare liver volume. Significant liver volume enlargement was observed after 6 month of ABMI. $*p < 0.05$, Wilcoxon signed ranks test.

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

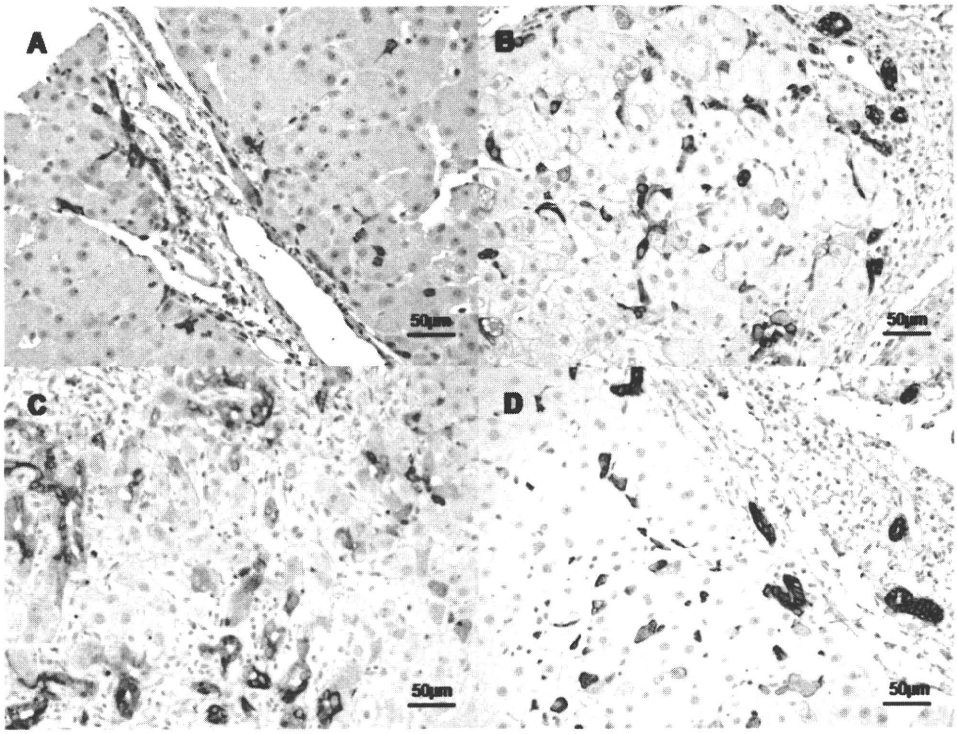
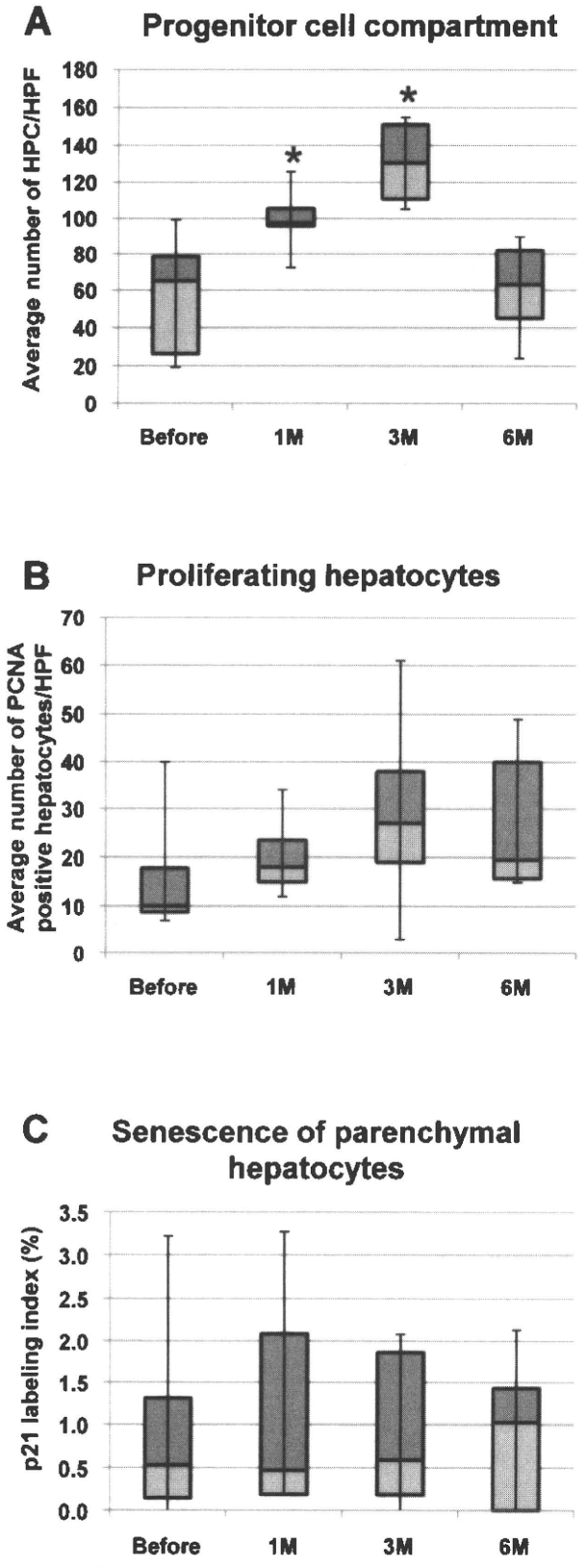


Figure 3. Progenitor cell compartment in the baseline and serially biopsied liver tissue. Ductular reactions and intermediate hepatocytes, which showed positive expression of cytokeratin 7, were counted as the progenitor cell compartment. Before ABMI, the progenitor cell compartment showed a low level 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

FACING COLUMN

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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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

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.

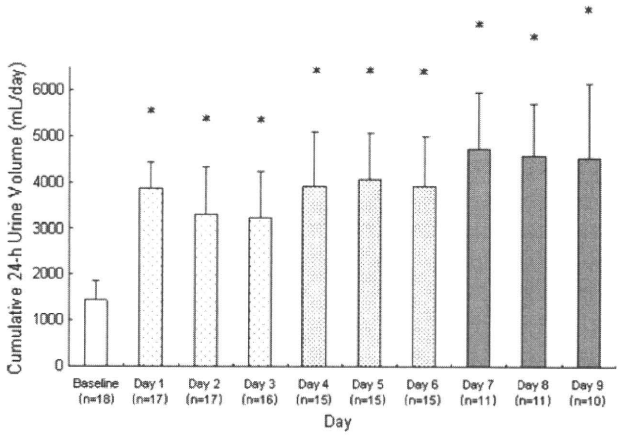

Fig. 1 Cumulative 24-h urine volume (mean ± SD). Urine was cumulatively collected during the 24-h period after each dosing (15 mg on days 1 through 3, 30 mg on days 4 through 6, and 60 mg on days 7 through 9). Statistically significant (*$P < 0.05$) increases in urine volume compared with the baseline 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