Autologous Bone Marrow Cells in the Treatment of Cirrhosis of the Liver

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The data characterizing tolerance and efficiency of autologous bone marrow cells in the treatment of patients with cirrhosis of the liver are presented. Injection of autologous bone marrow cells was not associated with the development of adverse reactions. Cell therapy of patients with compensated cirrhosis arrested asthenic syndrome, reduced cytolysis, increased the level of serum albumin and platelet count. Ultrasonic examination revealed reduction of portal hypertension (the area of the spleen and the portal vein lumen decreased). In patients with decompensated cirrhosis, a positive response presenting as reduction of the disease severity (by 1.9 points) was observed in 48.6% cases. Positive shifts in these patients were associated with a decrease of ALT and AST levels, reduction of laboratory signs of cirrhosis, increase in platelet count, and reduction of the asthenic syndrome. Hence, therapy with autologous bone marrow cells is safe and, according to preliminary results, can be regarded as a new approach to the treatment of patients with cirrhosis of the liver.

Key Words: *liver cirrhosis; stem cells; bone marrow*

Cirrhosis of the liver (CL) resulting from chronic diffuse liver diseases of different etiology is a serious problem. The only effective method for the treatment of decompensated CL is liver transplantation. However, shortage of donor organs, need in long-term immunosuppression, and high costs of this treatment limit the use of this approach. Studies of the stem cell (SC) biology attract special interest. SC are retained during the postnatal period, are present in virtually all organs and tissues, are characterized by plasticity, and can migrate, thus maintaining an essential regeneration reserve. If the pool of tissue SC is exhausted, it can be replenished at the expense of circulating SC from the bone marrow or from other sources [12].

Differentiation of bone marrow SC into hepatocytes was demonstrated *in vitro* and *in vivo* [1,6, 14,16]. Injection of bone marrow to experimental animals with CL arrested hepatic cell insufficiency, induced regression of fibrosis, and increased animal survival [13]. Differentiation of bone marrow SC into hepatocytes in humans is proven by the appearance of Y chromosome in the female hepatocytes after allogenic transplantation of SC from male donors [10]. This phenomenon can be partially due to SC fusion with hepatocytes [17]. The capacity of bone marrow cells to repopulate the liver and stimulate regeneration processes (at the expense of direct differentiation of SC into hepatocytes, production of growth factors, or fusion with hepatocytes) opens new vistas for the use of SC in the treatment of CL.

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The results of our previous studies in a group of 28 patients with chronic hepatitis and CL showed that the use of autologous bone marrow cells is safe and is associated with improvement of the subjective status and biochemical values [2]. Treatment with peripheral stem hemopoietic cells obtained by their mobilization from the bone marrow also produced stable clinical effect [18].

We carried out a dynamic complex analysis of clinical laboratory values of CL patients after injection of autologous bone marrow cells.

MATERIALS AND METHODS

The study was carried out in 47 patients (26 men and 21 women) aged 15-64 years. Clild—Pugh class A CL was diagnosed in 12 (25.5%), class B in 28 (59.6%), and class C in 7 (14.9%) patients. Cirrhosis was a result of chronic viral hepatitis (B, C, or combined variants: B+C, B+D) in 53% alcoholic hepatitis in 19%, of hepatitis of mixed etiology (viral and alcoholic) in 15%, and other etiology (autoimmune, cryptogenic) in 13% patients.

The patients were selected into the study from November 2003 to January 2006 in accordance with the protocol of clinical studies, approved by the Academic Council of Institute of Clinical Immunology and local Ethic Committee. The treatment and examinations were carried out after the patients signed informed consent to participation in the study. Inclusion criteria were histologically verified CL, age no more than 65 years, and informed consent. Patients with hemorrhages from varicose esophageal veins or spontaneous peritonitis during hospitalization, with hepatocellular carcinoma or other tumor diseases, acute infections, thrombocytopenia (below 50×10^9/liter), and unable to sign informed consent were excluded from the study. Autologous bone marrow cells were injected simultaneously in two portions: intravenously and into the liver parenchyma under ultrasonic navigation. The patients were examined before and 1-3, 6, and 12 months after injection of bone marrow cells. The patients received no antiviral drugs (lamivudin, ribaverin) and interferons during the observation period.

The Child—Pugh score, including evaluation of albumin and total bilirubin levels, prothrombin index, the presence of ascites, and severity of encephalopathy, served as the integral indicator of clinical status. Common and biochemical analysis of the blood and ultrasonic examination (USE) of the abdominal organs were carried out in all patients. The severity of the asthenic syndrome was evaluated by the score characterizing weakness and fatigue (weakness: 0 (none), 1 (slight), 2 (moderate), 3 (severe), 4 (requiring rest), and 5 (at rest); fatigue: 0 (none), 1 (during excessive exercise), 2 (after walking at least 500 m or going upstairs higher than to the ground floor), 3 (after walking longer than 100 m or going upstairs to the ground floor), 4 (after minimum exercise), and 5 (at rest)).

Bone marrow was aspirated from the ileac crest under general anesthesia. Mononuclear cells (MNC) were isolated by centrifugation of aspirated cells in Ficoll-verograffin density gradient (1.078 g/liter). The following characteristics of cells were evaluated: total count and viability of MNC, percentage of $CD34^+$ and $CD34^+CD38^-$ stem cells. Proliferative activity of SC was evaluated by the count of $CD34^+$ cells in the S/G_2M cell cycle phases. Phenotypic studies and evaluation of the cell cycle were carried out by standard flow cytofluorometry (FACS-Calibur, Becton Dickinson) with the corresponding monoclonal antibodies and propidium iodide.

RESULTS

The count of MNC isolated from the bone marrow aspirate of CL patients varied from 0.5 to 4.5×10^9 (mean count $1.81\pm0.17\times10^9$). This cell population contained 4.60±0.46% $CD34^+$ and 0.60±0.08% $CD34^+CD38^-$ cells. The mean absolute count of injected $CD34^+$ cells was $(78\pm1)\times10^7$, of $CD34^+CD38^-$ cells $(12.0\pm2.0)\times10^6$ for each patient. The viability of isolated cells was at least 90%. Injection of autologous bone marrow cells was not associated with the development of manifest adverse reactions. Subfebrile temperature and fever were observed in only 5 patients. Treatment efficiency was evaluated separately in a subgroups of patients with compensated (class A; n=12) and decompensated (class B+C; n=35) CL.

Compensated CL was caused by viral (B and C; n=5), alcoholic (n=5), autoimmune (n=1), and cryptogenic (n=1) hepatites. The severity of CL before therapy was 5.4 points on average and virtually did not change during 12-month observation. By the retrospective analysis (February 2007), all patients were alive after a period of observation of 15-36 months (mean period 24.3±2.1 months). Injection of bone marrow cells induced positive changes in clinical laboratory parameters (Table 1). A decrease in transaminases (AST and ALT) levels and an increase in serum albumin level, normalization of γ-glutamyl transpeptidase (GGTP), and a trend to reduction of alkaline phosphatase were observed as soon as 1-3 months after cell therapy. Hence, injection of bone marrow cells promoted a reduction of laboratory signs of hepatocyte insuffi-

TABLE 1. Dynamics of Clinical and Laboratory Parameters after Injection of Autologous Bone Marrow Cells to Patients with Compensated CL (n=12; $M \pm S.E.$)

Parameter	Before treatment	After treatment		
		after 1-3 months	after 6 months	after 12 months
Child—Pugh, score	5.40±0.15	5.30±0.16	5.5±0.16	5.30±0.15
ALT, mmol/h×liter	2.00±0.48	0.78±0.26*	1.13±0.31*	0.98±0.21*
AST, mmol/h×liter	1.2±0.2	0.60±0.23*	0.86±0.23*	0.85±0.33*
Albumin, g/liter	41.9±1.2	47.4±2.3*	50.6±3.5*	48.7±3.1*
GGTP, U/liter	88±10	53±10*	48±9*	55±18
Alkaline phosphatase, U/liter	170±56	108±21	107±20	120±41
Platelets, 10^9/liter	145±11	198±26*	189±18*	164±15
Asthenic syndrome, score	4.80±0.22	2.3±1.0*	1.2±0.8**	1.25±0.45**
Spleen area, cm^2	65.4±5.0	N.d.	N.d.	55.8±5.2*
Portal vein lumen, mm	13.8±0.7	N.d.	N.d.	12.0±0.44*

Note. Here and in Table 2: N.d.: no data; *p_U<0.05, **p_U<0.01 compared to the status before treatment (Mann—Whitney U test).

ciency and cholestasis. Platelet count increased, which manifested most clearly after 1-3 and 6 months, but was less significant after 1 year. The most pronounced clinical effect was reduction of the asthenic syndrome, due to which working capacity of 7 workers and 2 students was retained. Ultrasonic studies 12 months after therapy showed reduction of signs of portal hypertension (reduction of the splenic area and portal vein lumen). Hence, evaluation of the clinical status and liver function after injection of autologous bone marrow cells showed positive shifts in all patients with compensated CL (Child—Pugh class A).

The distribution of disease forms in the group with decompensated CL was as follows: class B cirrhosis in 28 patients and class C in 7. The disease was caused by chronic viral hepatitis (n=20), alcoholic hepatitis (n=4), or combination of both (n=7). Secondary biliary cirrhosis, drug-induced, cryptogenic, and autoimmune CL were diagnosed in few cases. Six (17%) patients died during the period of observation from acute bleeding from varicose esophageal veins developing 4-6 months after the start of therapy. In the remaining 29 patients, a significant reduction of the Child—Pugh score was observed after 12 months: from 8.30±0.25 to 7.40±0.24 (p_U<0.001). The severity of CL decreased in 17, did not change in 10, and increased in 2 patients. Hence, the positive response (reduction of Child—Pugh score) was observed in 17 (48.6%) patients, stabilization of the process in 10 (28.6%), and a negative response (deterioration of the status or lethal outcome) in 8 (22.8%) cases.

Clinical laboratory parameters in the subgroup of patients with positive response (n=17), indicating alleviation of CL, were recorded 1-3 months after therapy and were most pronounced after 12 months (Table 2). By this period, the severity of patients' condition decreased by 1 point in 10 patients, by 2 points in 6, by 4 points in 1, the mean for group value being 1.9 points. In 13 patients, positive changes were due to arrest of ascites; ascites was initially detected in 16 patients, while after treatment it persisted in only 3. According to USE data, disappearance of the fluid from the abdominal cavity was not paralleled by appreciable changes in other sonographic signs of portal hypertension (area of the spleen, portal vein lumen). However, despite significant subsequent reduction of the diuretic doses, no ascites was detected in any of the patients after 6 and 12 months. Moreover, this effect was attained in 3 patients with class C cirrhosis with strained ascites resistant to diuretics.

In addition to disappearance of ascites, the reduction of the Child—Pugh score was due to reduction of total bilirubin in 7 cases, increase in serum albumin level in 5, decrease in the severity of encephalopathy in 2, and increase of the prothrombin index attesting to alleviation of hepatocyte insufficiency in 2 cases.

The mean level of serum albumin virtually did not change in the group in general. On the other hand, stable reduction of total bilirubin was recorded in the patients over the entire period of observation. The decrease in the level of transaminases was less stable: significant reduction was recorded 1-3 months after cell therapy, while by the end of 1 year this reduction was significant only for ALT. The concentration of alkaline phosphatase virtually did not change after treatment; just a trend to its

TABLE 2. Dynamics of Clinical and Laboratory Parameters in Patients with Decompensated CL with Positive Response to Cell Therapy (n=17; $M \pm S.E.$)

Parameter	Before treatment	After treatment		
		after 1-3 months	after 6 months	after 12 months
Child—Pugh, score	8.6±0.4	7.3±0.4*	7.2±0.4*	6.7±0.3**
ALT, mmol/h×liter	1.80±0.24	1.17±0.25**	1.50±0.44	1.20±0.22*
AST, mmol/h×liter	1.20±0.14	0.78±0.12*	1.20±0.19	0.90±0.13
Albumin, g/liter	34.1±1.4	32.0±1.8	35.0±2.7	34.5±1.5
Total bilirubin, μmol/liter	37.0±3.6	24.5±3.1**	27.3±4.5*	24.5±4.0*
GGTP, U/liter	161±40	115±36*	114±48*	78±27*
Alkaline phosphatase, U/liter	150±24	151±23	137±26	129±19
Platelets, 10^9/liter	101±11	148±18*	147±16*	106±14
Asthenic syndrome, score	5.9±0.5	3.2±1.2*	2.1±0.5**	2.3±0.5**
Spleen area, cm^2	132±19	N.d.	N.d.	126±17
Portal vein lumen, mm	13.1±0.6	N.d.	N.d.	13.5±0.6

reduction was recorded. On the other hand, GGTP level decreased significantly and 12 months after therapy reached the minimum. These shifts indicated reduction of cytolysis and cholestasis syndromes. Similarly as in patients with compensated CL, cell therapy was associated with a transitory increase in platelet count. A reduction of the asthenic syndrome was observed in the patients with positive response to treatment over the entire period of observation, being the most pronounced 6 and 12 months after cell therapy.

Repeated histological study of liver biopsy specimens was carried out 12 months after cell therapy in 9 of 47 patients. The group consisted of 3 patients with compensated CL and 6 with decompensated CL with positive response to therapy. A significant reduction of the histological activity index (HAI) according to Knodel's score (from 7.6±0.9 to 5.1±1.2; p_U<0.05) was noted. The decrease of HAI was detected in all patients with class A cirrhosis and in 4 of 6 patients with decompensated CL. The index increased from 9 to 13 in 1 patients and did not change in another.

Comparative analysis of clinical laboratory parameters and the characteristics of injected cells was carried out in groups of patients with positive shifts (n=17), process stabilization (n=10), and negative response (n=8) in order to clear out the cause of treatment efficiency in decompensated cirrhosis. The patients of these groups initially did not differ by the severity of their status (the Child—Pugh score), HAI, biochemical activity (ALT and AST levels), severity of anemia and thrombocytopenia, and incidence of hypersplenism syndrome (Table 3). However, the patients with negative response were older (p<0.05). The counts of isolated/injected MNC were similar in the analyzed groups, but the percentage of $CD34^+$ cells in the injected dose was significantly lower in patients with negative response in comparison with other groups. Evaluation of the absolute counts of $CD34^+$ cells in the groups with stabilization and negative response showed that these counts were lower than in the group with positive response, the difference however being statistically negligible. No significant differences in the absolute counts of $CD34^+CD38^-$ cells (a more primitive SC fraction) or proliferative activity of $CD34^+$ cells were detected in the analyzed groups, though these parameters were lower in patients with the negative response.

Our results indicate the safety of isolation of bone marrow cells from patients with CL and the possibility of derivation of their sufficient amount, including stem hemopoietic cells. Realization of the procedure under total anesthesia had no negative impact for patients' status and did not lead to the development of complications. Injection of autologous bone marrow cells intravenously and into the liver parenchyma caused no manifest adverse reactions or complications, except transitory fever in some patients. Hence, therapy including isolation and injection of bone marrow cells was in general well tolerated by CL patients.

Cell therapy of patients with compensated CL (n=12; class A according to Child—Pugh) promoted arrest of the asthenic syndrome, reduction of cytolysis (AST, ALT), and increase in serum albumin level and platelet count. In addition, USE showed reduction of portal hypertension (decrease in the splenic area and portal vein lumen). Positive

effect in the group of patients with decompensated CL (class B+C; *n*=35) presented by reduction (by 1.9 points on average) of CL severity in 48.6% cases. The class of disease severity chsnged in 9 patients: class A instead of class B was diagnozed in 6 cases and class B instead of class C in 3. Reduction of the Child—Pugh score was paralleled by a reduction of the asthenic syndrome, increase in platelet count, reduction of total bilirubin, GGTP, and transaminase levels, though the effect on transaminases was less stable and did not manifest at all terms of observation.

The reduction of hepatocyte insufficiency can be due to the substitution effect of SC and to the effects of growth/trophic factors suppressing hepatocyte apoptosis, stimulating their proliferation, and activating liver SC [1]. Reduction of portal hypertension seemed to be largely due to the anti-inflammatory effect of the transplanted bone marrow cells. The development of fibrosis was caused by activation of astrocytes producing the extracellular matrix proteins and possessing contractile activity. Activation of these cells is initiated and maintained by an inflammatory process [7]. Our previous data showed that the level of cytokine production, particularly production of IFN-γ and TNF-α, was significantly higher in CL than in patients with the initial stages of fibrosis. In addition, the pro- to antiinflammatory cytokines (TNF-α/IL-10) ratio directly correlated with the CL severity score and cytolysis intensity [3]. We hypothesized that natural suppressors present in the bone marrow can suppress inflammatory activity, including activity of astrocytes, by reducing the severity of portal hypertension. A serious argument in favor of this hypothesis is the detected reduction of HAI after cell therapy. The reduction of laboratory signs of the cytolytic and cholestatic syndromes seems also to be linked with the antiinflammatory effect.

The possibility of using SC for stimulation of liver regeneration in CL patients attracts special interest, as a principally new approach to the treatment of this disease [1,4]. Two strategies are now discussed: the use of peripheral stem hemopoietic

TABLE 3. Characteristics of Patients with Different Response to Cell Therapy

Parameter		Response to therapy		
		positive (*n*=17)	stabilization (*n*=10)	negative (*n*=18)
Age, years		42.0±2.8	41.0±3.2	51.5±2.9*,++
Sex	male	13	2	3
	female	5	8	5
Etiology of CL	viral	9	8	3
	alcoholic	1	—	1
	mixed	6	1	1
	other	1	1	3
Child—Pugh, score		8.60±0.37	8.00±0.29	8.70±0.83
HAI after Knodel		9.5±1.3	8.10±0.96	8.5±2.1
ALT, mmol/h×liter		1.85±0.22	1.80±0.28	1.50±0.39
AST, mmol/h×liter		1.15±0.11	1.22±0.11	1.40±0.47
Hemoglobin, g/liter		118.0±4.7	114.0±6.7	122±7
Platelets, 10^9/liter		101±12	100±25	179±60
Hypersplenism syndrome	abs.	15	8	5
	%	88	80	63
MNC count, $\times 10^9$		1.80±0.26	1.74±0.37	1.89±0.27
$CD34^+$ cells, %		5.6±0.9	4.5±0.4	2.90±0.16*,++
$CD34^+$ cells, $\times 10^6$		94±20	74±14	53±7
$CD34^+CD38^-$ cells, %		0.70±0.17	0.5±0.1	0.66±0.10
$CD34^+CD38^-$ cells, $\times 10^6$		14±4	7.0±1.0	12±3
Count of $CD34^+CD38^-$ cells in the cell cycle S/G_2M phase		30.0±4.4	23±5	17±7

Note. $^*p_U$=0.045, $^{**}p_U$=0.05 compared to positive response; ^+p_U=0.03, $^{++}p_U$=0.005 compared to stabilization.

cells mobilized from the bone marrow by G-CSF and injection of SC isolated from the bone marrow. It was found that mobilization of SC in patients with CL was as affective as in donors [8]. Injection of mobilized SC to CL patients led to a lasting improvement in 2 patients with alcoholic CL. The severity of cirrhosis in these patients decreased from 12 to 9 and from 8 to 7 points, the improvement persisting during 30 months of the follow-up period [18]. Injection of separated $CD34^+$ stem cells obtained after mobilization from the peripheral blood, was tried [9]. Injection of cells in a dose of 1×10^6—2×10^8 into the portal vein (n=3) or hepatic artery (n=2) led to a reduction of total bilirubin in 3 of 5 patients and increase serum albumin in 4 of 5 patients within 2 months after therapy.

Regeneration of the liver was stimulated by portal injection of autologous bone marrow $CD133^+$ cells in patients with a history of resection of a liver fragment because of a tumor [3]. Results of clinical trials of autologous bone marrow cell injections in CL patients [2] and similar data indicated clinical improvement of CL patients' condition after injection of autologous bone marrow cells [15]. The study was carried out in 9 patients with class B cirrhosis of mainly viral etiology. Intravenous injection of bone marrow MNC containing 1.2×10^8 $CD34^+$ cells promoted a reduction of CL severity after 1 and 6 months by 1 point. Analysis of individual values showed reduction of the Child—Pugh score in 7 (77.8%) cases. In 1 patient the Child—Pugh score did not change and in another patient it increased. As patients' status was stable during 2 months before cell therapy and their basic drug therapy did not change during the subsequent follow-up period, the improvement was regarded as a result of transplantation.

Higher incidence of positive responses in these studies in comparison with ours can be explained by lesser sample, shorter follow-up period (6 months), higher dose of injected bone marrow cells, and selection of patients with stable condition. In our study negative clinical shifts were observed in the overwhelming majority of patients during 2-3 months before therapy. Moreover, CL severity was higher in some patients (class C; n=7). Reduction of the Child—Pugh score in half of patients (48.6%) in such a sample is an obvious progress. In addition, stabilization of the process in patients with decompensated CL (28.6% cases) can also be regarded as a positive result of cell therapy. Our results demonstrate the possibility of clinical effect lasting over 12 months in the most grave category of patients (class B and C) with the disease progressing before therapy. Similar data were obtained previously: positive shifts (reduction of bilirubin level, increase in albumin over 4 months) in a group of 10 patients with class B and C cirrhosis after injection of about 10^8 bone marrow cells into the hepatic artery [11].

Hence, our results indicate the safety and efficiency of injection of bone marrow cells to CL patients. Injection of autologous bone marrow cells at the initial stages of CL can be regarded as an approach preventing the disease progress, in decompensated CL as a provisional measure due to which it is possible to prolong the period before liver transplantation. However, further studies are needed, including controlled and randomized (if possible) studies with a long prospective period of observation.

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