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Immunomodulatory effect of bone marrow mesenchymal stem cells on T lymphocytes in patients with decompensated liver cirrhosis

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ABSTRACT. We explored the immunomodulatory effects of bone marrow mesenchymal stem cells (BMSCs) on peripheral blood T lymphocytes in patients with decompensation stage, hepatitis B-associated cirrhosis. MSCs from nine patients were analyzed by flow cytometry. Peripheral blood lymphocytes were isolated for fluorescent staining. Following stimulation by phytohemagglutinin (PHA), peripheral blood lymphocytes were co-cultured with BMSCs in serum and divided into four groups: (1) BMSC + lymphocyte + PHA contact culture group; (2) BMSC + lymphocyte + PHA non-contact culture group; (3) lymphocyte + PHA positive control group; and (4) lymphocyte-only negative control group. Lymphocyte proliferation and frequencies of $CD4^+CD25^+CD127^-$ Tregs and $CD4^+CD8^-IL\text{-}17^+$ (Th17) cells were detected. Cell proliferation in groups 1 and 2 declined compared with group 3 ($P < 0.01$), and was notably higher than in group 4 ($P < 0.01$). $CD4^+CD25^+CD127^-$ Tregs frequencies in groups 1 and 2 were higher than in groups 3 and 4. In an intra-group comparison before and after culture, Th17 cell frequencies in groups 1 and 2 were higher than in group 4 ($P < 0.01$), but lower than in group 3 ($P < 0.01$). The Treg/Th17

ratio in groups 1 and 2 increased ($P < 0.01$), but did not change significantly in groups 3 and 4 ($P > 0.05$). In a comparison between groups after culture, the Treg/Th17 ratio in groups 1 and 2 increased more than in groups 3 and 4 ($P < 0.01$). BMSCs from cirrhotic patients can inhibit the proliferation of peripheral blood T lymphocytes, upregulate the expression of $CD4^+CD25^+CD127^-$ Tregs, and improve Treg/Th17 imbalance. The mechanism by which this takes place may be associated with immunomodulatory effects induced by the secretion of soluble factors.

Key words: Hepatitis B; Hepatic cirrhosis; Th17; Treg/Th17; Bone marrow mesenchymal stem cells; Treg

INTRODUCTION

In the past, researchers thought that in liver disease, bone marrow mesenchymal stem cells (BMSCs) migrated and established themselves in the area of hepatic damage, then differentiated into liver cells or liver oval cells to repair damaged tissue. However, more and more researchers now think that the differentiation of BMSCs into target cells may not be the decisive step (Zhou et al., 2009). The inflammatory and immune responses are involved in the progress of hepatic cirrhosis following hepatitis B infection, and through immunomodulation, BMSCs can produce anti-inflammatory effects, suppress excessive immune response, and prevent liver cell necrosis, thereby improving liver function. However, whether the immunoregulatory function of BMSCs is affected by morbidity is still not clear. In the present study, we harvested marrow MSCs from patients with decompensated cirrhosis. We then mimicked *in vivo* environments and employed two different methods of co-culture to observe the *in vitro* inhibitory effects of BMSCs on the proliferation of autologous peripheral blood lymphocytes. We also investigated the variation in regulatory T cells (Tregs) and T helper 17 (Th17) cell subgroups. Our objective was to explore the possible mechanism involved and to provide experimental evidence for the clinical application of autologous BMSCs in the future.

MATERIAL AND METHODS

Reagents and instruments

Mesenchymal stem cell culture medium (MSCM) was purchased from Sciencell, USA. Lymphocyte separation medium (Ficoll, 1.077 g/mL) was purchased from GE Healthcare Life Sciences, USA. Phytohemagglutinin (PHA) was purchased from Sigma, USA. Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) fluorescent dye was purchased from the VICMED Company, Xuzhou, Jiangsu, China. Mouse anti-human CD34-PE, CD29-PE, CD44-FITC, CD45-FITC, CD127-PE, CD4-FITC, CD25-PC5, CD3-FITC, CD8-PC5, and IL-17A-PE monoclonal antibodies, and the flow cytometer were acquired from Beckman-Coulter, USA.

Investigated subjects

Cases were selected from the inpatients who received autologous bone marrow stem cell transplantation via the hepatic artery in our department from January 2013 to March 2014.

According to case history, physical signs, hepatitis B virus markers, liver biochemistry, B ultrasound, or computed tomography scan results, nine patients were diagnosed as having decompensated cirrhosis. Among them, there were seven males and two females aged 43-68 years, with an average age of 56.1 ± 10.5 years. Child-Pugh scores of all patients were ≥9 points. No corticosteroids or immunosuppressive agents had been used previously.

Methods

Isolation, culture, and purity identification of BMSCs

Bone marrow (5-10 mL) from patients with decompensated cirrhosis was collected. After dilution with an equal volume of phosphate-buffer saline (PBS), the samples were slowly added to tubes with lymphocyte separation medium at a density of 1.077 g/cm. The samples were centrifuged at 1800 *g* for 20 min, and the white membrane layer containing mononuclear cells was then carefully aspirated and washed twice with PBS. After counting, the cells were seeded into culture flasks that contained MSCM. Cells were cultured at 37°C in 5% CO_2 and conditions of saturated humidity. After 48 h, the culture fluid was refreshed, the non-adherent cells were removed, and the fluid was changed every 3 or 4 days thereafter. Cell morphology was observed under a microscope to record primary culture time. When the adherent cells covered 80% of the culture surface, they were digested by pancreatin for passage (1:2). The third generation of MSCs was co-incubated with fluorescently labeled CD34, CD45, CD29, and CD44 monoclonal antibodies for 20 min. After washing with PBS, cell morphology was observed under a microscope and cell immunophenotype was determined by flow cytometer.

Separation of lymphocytes and CFDA-SE staining

Heparin-anticoagulated fresh peripheral blood (10 mL) was drawn aseptically from patients with decompensated cirrhosis. Mononuclear cells were abstracted using Ficoll solution, and monocytes were then excluded by the adherence method. The lymphocytes were collected and the concentration was adjusted to 1 x 10^6/mL after resuspension. The CFDA-SE working solution was then added to a final concentration of 5 M. After thorough mixing, the lymphocytes were incubated at 37°C for 20 min. Autologous serum was used to terminate the reaction and lymphocyte CFDA-SE staining was observed under a fluorescence microscope.

Co-culture of BMSCs and peripheral blood lymphocytes

After digestion and counting, the third generation of BMSCs was seeded on a 24-well plate with 2 x 10^4 cells/well. After complete adherence, BMSCs received 20 Gy radiation produced by a linear accelerator, then the supernatant was aspirated and discarded. Lymphocytes were seeded on BMSCs at a density of 2 x 10^5 cells/well, PHA (final concentration 2 μg/mL) was added to stimulate the proliferation of lymphocytes, and autologous serum was used to make a total volume of 1 mL. The experimental groupings were: (1) BMSC + lymphocytes + PHA contact culture group; (2) BMSC + lymphocytes + PHA non-contact culture group; (3) lymphocytes + PHA positive control group; and (4) lymphocytes-only negative control group. Samples from each group were made in triplicate and co-cultured for 72 h. A Transwell system was used for non-contact culture.

Proliferation of lymphocytes detected by flow cytometry

The lymphocytes from each well were collected and washed twice in PBS. Proliferation of lymphocytes in each well was detected using the green channel of the flow cytometer.

Treg cell frequency detection

The lymphocytes were washed in PBS and resuspended, and CD25-PC5, CD4-FITC, and CD127-PE monoclonal antibodies were then added. The mixture was incubated in the dark for 20 min before detection. CD4 was used for gating T lymphocytes, and the frequency of $CD4^+CD25^+CD127^-$ Tregs was detected.

Detection of Th17 cell frequency

After harvesting and washing in PBS, the cells were seeded on a 24-well plate. Phorbol ester (25 ng/mL), ionomycin (1 μg/mL), and monensin (1.7 μg/mL) were then added and the cells were incubated in the dark at 37°C for 5 h. The cells were divided into 100-μL detecting and control tubes, and 10 μL CD3-FITC and CD8-PC5 monoclonal antibodies were added to the tubes, respectively, followed by incubation in the dark at room temperature for 15 min. Fixation and permeabilization buffers were added stepwise. IL-17A-PE monoclonal antibody was added to the detecting tube and the isotype control was added to the control tube. The tubes were then incubated at room temperature in the dark for 20 min, 3 mL PBS was added, and the tubes were kept standing for 10 min, centrifuged, and the supernatant was discarded. The cells were resuspended in 0.5 mL PBS, analyzed with a flow cytometer, and T lymphocytes were gated and delineated using CD3 and CD8 to detect the frequency of Th17 cells.

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software. Data are reported as means ± SD. A paired *t*-test was applied to analyze intra-group variable variation before and after co-culture. An independent-sample *t*-test was used for comparison between groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Identification of marrow MSC

Primary MSCs were successfully cultivated from the marrow of nine patients with decompensated cirrhosis. The purity of the three generations of BMSCs reached 95%, and cells had a similar spindle-like morphology (Figure 1). There was high expression of CD44 (95.01%) and CD29 (92.79%), and low expression of CD34 (1.31%) and CD45 (1.08%), in accordance with the immunological characteristics of MSC.

Figure 1. Morphology of bone marrow mesenchymal stem cells (100X).

Effects of BMSCs on proliferative response induced *in vitro* by peripheral blood lymphocytes

Labeled cells were excited by blue laser (488 nm) and observed under a fluorescent microscope. The cells exhibited green fluorescence (Figure 2). Flow cytometry analysis revealed that cell proliferation in the contact culture and non-contact culture groups was significantly lower than in the positive control group ($P < 0.01$), and the negative control group ($P < 0.01$). No significant difference was found between the contact and non-contact culture groups ($P > 0.05$) (Table 1).

Table 1. Comparison of lymphocyte proliferation, regulatory T cell (Treg) and T helper 17 (Th17) frequencies, and Treg/Th17 ratios in each group (means ± SD, N = 9).

Groups	Lymphocyte proliferation	Tregs (%)	Th17 (%)	Treg/Th17
Negative control	5.76 ± 1.61	4.41 ± 0.81	1.08 ± 0.63	5.17 ± 2.42
Positive control	61.34 ± 7.42	22.37 ± 5.52	4.25 ± 1.49	6.11 ± 3.36
Non-contact culture	33.79 ± 3.28[ab]	42.47 ± 7.62[ab]	2.29 ± 1.07[ab]	20.05 ± 11.59[ab]
Contact culture	29.31 ± 2.89[ab]	47.69 ± 8.90[ab]	2.31 ± 1.04[ab]	22.58 ± 12.39[ab]

[a]Compared with positive control group $P < 0.01$. [b]Compared with negative control group $P < 0.01$.

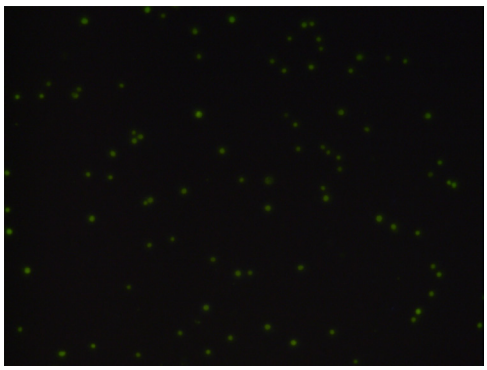

Figure 2. Lymphocytes labeled with carboxyfluorescein diacetate succinimidyl ester (200X).

Effects of BMSCs on Treg cell subsets in peripheral blood

In intra-group comparisons before and after culture, the frequency of $CD4^+CD25^+CD127^-$ Tregs was 3.28 ± 0.71% before culture; except for the negative control group, the frequency increased significantly following culture ($P < 0.01$). In between-group comparisons after culture, both contact and non-contact culture groups had a higher frequency of $CD4^+CD25^+CD127^-$ Tregs compared with the positive and negative control groups ($P < 0.01$), although there was no significant difference between the two groups ($P > 0.05$) (Table 1).

Effects of BMSCs on Th17 cell subsets in peripheral blood

In intra-group comparisons before and after culture, the frequency of Th17 was 0.80 ± 0.49% before culture; except for the negative control group, the frequency increased significantly following culture ($P < 0.01$). In between-group comparisons after culture, both contact and non-contact culture groups had higher frequencies of Th17 than the negative control group ($P < 0.01$), but lower frequencies than the positive control group ($P < 0.01$), and there was no significant difference between the two groups ($P > 0.05$) (Table 1).

Effects of BMSCs on Treg/Th17 ratio

In intra-group comparisons before and after culture, the Treg/Th17 ratio in peripheral blood was 4.87 ± 2.16 before culture; the Treg/Th17 ratio in the contact and non-contact culture groups increased significantly following culture ($P < 0.01$). No significant difference was found before and after culture in the positive and negative groups ($P > 0.05$). In between-group comparisons after culture, both contact and non-contact culture groups had higher ratios than the positive and negative control groups ($P < 0.01$), and there was no significant difference between the two groups ($P > 0.05$) (Table 1).

DISCUSSION

Based on the results of clinical trials, it is currently thought that autologous transplantation of BMSCs for treating liver cirrhosis and liver failure is feasible and safe (Peng et al., 2011). However, the differentiation potential and immunomodulatory mechanism of transplanted BMSCs in patients with liver cirrhosis remain unclear. *In vitro* experiments have verified that BMSCs have negative regulatory effects on allogeneic immune response, yet there are very few reports about the regulatory effects of BMSCs on autologous immunocompetent cells. In the research reporting inhibition of T lymphocyte proliferation by MSCs, there is still controversy surrounding the differences between contact and non-contact culture in suppressing T lymphocytes (Krampera et al., 2003; Tse et al., 2003). Autologous serum medium was applied in the present study. The *in vitro* inhibition of autologous lymphocyte proliferation by BMSCs was observed in both contact culture and non-contact culture, which suggests that direct cell contact is not necessary for inhibition of lymphocyte proliferation by BMSCs. In addition to contact inhibition, BMSCs may play a suppressive role by secreting soluble cytokines. Zhao et al. (2010) added anti-TGF-β1 antibody to the culture system of MSCs + T lymphocytes and found that the ability of MSCs to inhibit T lymphocyte proliferation was reversed.

Immune injury induced by the hepatitis B virus is crucial in the progress of post-hepatitis B cirrhosis from the compensation to the decompensation stage (Xue-Song et al., 2012). As an important part of the immune response, effective $CD4^+$ T lymphocytes can be divided into Th1, Th2, Th17, Treg, and T9 according to cell differentiation and function, and current research focuses on the dysfunctional Th17 and Treg cells. Th17 is a newly discovered effective $CD4^+$ T lymphocyte subset, which is characterized by the secretion of IL-17. Th17 acts on different target cells, induces the production of other cytokines, becomes involved in regulating the cytokine network, triggers the release of inflammatory mediators, and thereby mediates the occurrence and development of the inflammatory response (Muranski and Restifo, 2013). Being widely accepted as negative immunomodulatory cells, Treg cells play an immune role through the release of soluble cytokines, such as TGF-β1 and IL-10. Data have shown that the progress from chronic hepatitis B to cirrhosis and liver failure is associated with abnormal changes in Tregs (Li et al., 2012a). Our research found that, when compared with healthy people, patients with decompensated cirrhosis have a lower incidence of peripheral Tregs, a higher incidence of Th17, and a Treg/Th17 imbalance. As a result, some researchers consider that Treg and Th17 cells inhibit and promote inflammatory reactions, respectively, and disruptions in the balance between the two contribute greatly to the progression of chronic hepatitis B to cirrhosis and liver failure (Li et al., 2012b). Some researchers have indicated that MSCs can change the composition of T cell subsets, secrete transforming growth factor TGF-β1 and prostaglandin E2, induce the proliferation of Treg cells (De Miguel et al., 2012; Soleymaninejadian et al., 2012), and inhibit the expression of Th17 cells through the CCL2 pathway (Rafei et al., 2009). The results of the present study showed that, after co-culture of BMSCs and autologous peripheral blood lymphocytes, Treg cell frequency was significantly upregulated, and the Treg/Th17 ratio was notably higher than in the positive control group without BMSCs. However, no significant differences were found in Treg frequency and Treg/Th17 ratio in comparisons between the contact and non-contact culture groups, from which we infer that immunosuppression of BMSCs is mainly led by the mediation of soluble cytokines.

In summary, both contact and non-contact cultures of MSCs obtained from patients with hepatitis B-associated cirrhosis in the decompensation stage inhibited the proliferation of autologous lymphocytes, significantly upregulated the expression of $CD4^+CD25^+CD127^-$ Tregs, and improved the Treg/Th17 imbalance. We will arrange experimental animal models of liver cirrhosis according to the results of the *in vitro* tests to learn more about the immunosuppressive mechanism of BMSCs and provide experimental evidence for the clinical application of autologous BMSCs in treating liver cirrhosis and liver failure in the future.

Conflicts of interest

The authors declare no conflict of interest.

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