Hypoxic Treated Bone Marrow Cells Enhanced Foxp3 and IL-10 Expression in a Murine Liver Injury Model

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Purpose: It has been reported that bone marrow (BM)-derived stem cells retain the capability of regenerating organs, as well as functioning as an immune modulator. We previously reported that liver function recovered with bone marrow cells (BMCs) transplantation in a liver failure mouse by CCl4 injection and after hypoxic conditioned BMCs transplantation, cell migration and engraftment had been improved. We investigated immune modulation after hypoxic conditioned BMCs transplantation. Methods: To induce the acute liver failure, C57BL/6 mice were injected intra-peritoneally once a day with CCl4 (10 μL/g) for two consecutive days, and were used as liver injury recipients. Donor BMCs were incubated under the desired level of O2 (0.1% and 20%) for 24 hours. We then injected the conditioned BMCs through the tail vein into the CCl4-injected mice. For further analyzing immunological changes after transferring BMexposed to hypoxia to CCl4 treated mice, we separated spleen cells after BMCs transplantation and analyzed phenotype changes of Foxp3, IL-10, IL-4, IL-12 and IFN-γ of CD4+ T cells. Results: The proportion of CD4+CD25+Foxp3+ cells, IL-10 secreting cells increased in CCl4 damaged mice after the BMCs transfers after hypoxic treatment. Although the proportion of IL-4 expressing CD4+ cells increased under the hypoxic conditions, did not have statistical significance. The percentage IFN-γ and IL-12 expressing CD4+ cells did not show statistical significance. Conclusion: It is suggested that hypoxic conditioned BMCs transplantation affected the immune modulation via inducing Foxp3+ and IL-10 secreting CD4+ cells in CCl4 damaged mice. (Clin Pediatr Hematol Oncol 2010;17:51~58)

Key Words: Carbon tetrachloride, Bone marrow cells, Hypoxia, Regulatory T cell, Foxp3, IL-10, CD4+CD25+ cells

Introduction

The most effective treatment for patients with end stage liver disease is liver transplantation. However, small pool of available organ donors, graft intol-
rance, and technical difficulties have emerged as the problems of liver transplantation, thus stem cell-based therapies have been suggested as the supplemental treatment\(^1\). Under the severe liver injury, oval (murine) or hepatic (human) progenitor cells located within the intrahepatic biliary tree were activated. In such case, some antigens traditionally associated with hematopoietic cells (c-kit, flt-3, CD34) can be expressed on oval or hepatic progenitor cells and this observation could lead to the notion that some hepatic oval cells are possibly derived from a precursor cells from bone marrow (BM). Therefore, in addition to the potential replacing stem cells in the liver, another population of stem cells resides in the BM and contribute to restoring liver cell function, as we had shown in our previous experiment\(^2\). Especially, mesenchymal stem cell from BM and adipose tissue appear to be the most suitable extra-hepatic candidate for hepatic differentiation.

In case of transferring BMCs to the liver failure patients, the mechanism of cell-to-cell interactions on the basis of immune modulation should be considered for improving survival and engraftment after the cell therapy. Several factors attribute to the enhancing cell growth or engraftment, such as hypoxia and immune response\(^3\). We previously reported that hypoxic treatment of bone marrow cells (BMCs) enhanced the engraftment of transferred cell after CCl\(_4\) damaged liver mouse models due to increase of cell survival and increased the expression of CXCR4 of BMCs\(^4\). We hypothesized that transferring hypoxic conditioned BMCs to the CCl\(_4\) injected mice might affect overall systemic immune reaction, especially on the differentiation of Foxp3\(^+\) or IL-10 secreting T cells playing regulatory roles. In this study, we analyzed the proportion of T cells expressing Foxp3, IL-10, IL-4, IL-12 or IFN-\(\gamma\) after transferring BMCs incubating under the hypoxic conditions (0.1% of oxygen).

**Materials and Methods**

1) Mice

The animal care committee at Ewha Womans University College of Medicine (Seoul, Korea) approved all of the procedures and protocols used in this study. Six- to eight-week-old C57BL/6 mice (Koatec, Pyeong Taek, Korea) were housed in an animal care facility, with food and water available *ad libitum* under specific pathogen-free condition. The mice were exposed to a 12:12 hour light dark cycle in room air at room temperature. For BMCs donor mice, enhanced green fluorescent protein (EGFP)-transgenic mice, which ubiquitous expression GFP (Okabe et al., 1997) were obtained from RIKEN BRC (BRC No. C57BL/6-Tg (CAG-EGFP) C14-Y01-FM131Osbg). To induce the acute liver failure, six-week-old female C57BL/6 mice were injected intraperitoneally once a day with CCl\(_4\) (10 \(\mu\)L/g) in a 10% solutions mixed in mineral oil for two consecutive days, and were used as liver injury recipients.

2) Cell isolation and transplantation

For donor BMCs isolation, six- to eight-week-old female EGFP transgenic C57BL/6 mice were killed by cervical dislocation, and their limbs were subsequently removed. BM was flushed from the medullary cavities of both the femurs and tibiae with RPMI-1640 medium (Gibco BRL, Carlsbad, CA, USA) using a 25-gauge needle. BMCs were plated on 10-cm dishes in \(a\)-modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) using a 25-gauge needle. BMCs were plated on 10-cm dishes in \(a\)-modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies), and were incubated under normoxia (20% O\(_2\)) or hypoxia (0.1% O\(_2\)) in 5% CO\(_2\) at 37°C for 24 hours. Hypoxic conditioning
of cells was performed in an enclosed chamber (Billups-Rothenberg, Del Mar, CA, USA) flushed with premixed gas mixture (0.1% O2, 5% CO2, and 94% N2) for the indicated duration of time. For experiments with various levels of O2, BMCs were incubated under the desired level of O2 (0.1% and 20%) for 24 hours. We then injected the conditioned BMCs through the tail vein into the CCl4-injected mice. One day after the injection of CCl4, 1×10^7 of hypoxia or normoxia-conditioned BMCs (resuspended in 200 μL RPMI-1640 medium) from transgenic EGFP mice were injected into recipient mice via the tail vein. The same volume of suspension medium (RPMI-1640) was used in the control group instead of BMCs. The mice were sacrificed 7 days after BMC transplantation. The spleen cells were isolated for flow cytometric analysis. The number of subjects in each of the individual groups was three to four.

3) Cell staining for flow cytometry

For preparation of spleen cell suspensions, spleens were removed and minced with a nylon mesh. After the cells were pelleted, the erythrocytes were lysed using hypotonic buffer containing 0.75% NH4Cl. The prepared cells were stained for CD4 (clone RM4-5, eBioscience, San Diego, CA, USA) and/or CD25 (clone PC61.5, eBioscience) followed by fixation in 2% paraformaldehyde and permeabilization in 0.5% saponin. This was followed by staining for intracellular IL-4 (clone 11B11, eBioscience), IFN-γ (clone XMG1.2, BD Pharmingen, San Jose, CA, USA), and IL-12 (clone C15.6, BD Pharmingen), or appropriate isotype controls (clones R3-34, BD Pharmingen). For IL-10 and Foxp3 staining, cells were fixed in Fix/Perm buffer from eBioscience. This was followed by permeabilization in 0.5% saponin and staining for IL-10 (clone JES5-16E3, BD Pharmingen) as indicated above. Finally, the cells were permeabilized in buffer and stained for Foxp3 (clone FJK-16s, eBioscience) according to manufacturer’s instructions. Samples were acquired on a FACSCalibur (BD) and were analyzed using CellQuest software (BD).

Results

1) Hypoxic conditioned donor BMCs enhanced the proportion of Foxp3+ cells

To investigate the effect on Foxp3-expressing T cells after transferring BMCs to CCL4 damaged mice, we isolated the spleen and stained the cells for the detection of CD4^+CD25^+Foxp3^+ cells (Fig. 1). Control group of mice showed the 0.8±0.6%, normoxic conditioned BMCs showed 2.2±0.8%, and hypoxic conditioned groups showed 2.8±0.7% of Foxp3^+ cells (Fig. 1A). Under the gate of CD4^+ cells, the proportion of CD25^+Foxp3^+ cells showed 52.9±17.2%, normoxic conditioned BMCs showed 63.3±8.3%, and hypoxic conditioned groups showed 72.2±15.5% of Foxp3^+ cells (Fig. 1C).

2) Hypoxic conditioned donor BMCs enhanced the proportion of CD4^+CD25^+IL-10^+ cells

To investigate the effect on IL-10 secreting T cells after transferring BMCs to CCL4 damaged mice, we isolated the spleen and stained the cells for the detection of CD4^+CD25^+IL-10^+ cells (Fig. 2). Control group of mice showed the 1.2±0.4%, normoxic conditioned BMCs showed 2.5±1.0%, and hypoxic conditioned groups showed 4.9±0.2% of Foxp3^+ cells (Fig. 2A). Under the gate of CD4^+ cells, the proportion of CD25^+IL-10^+ cells showed 49.0±19.8%, normoxic conditioned BMCs showed 54.6±6.4%, and hypoxic conditioned groups showed 61.3±0.8% of Foxp3^+ cells (Fig. 2C).
Fig. 1. Percentage of CD4⁺CD25⁺ Foxp3⁺ T cells increased after transferring culture medium only (control), normoxia (20% BM) or hypoxia-conditioned BMCs (0.1% BM). (A) Percentage of Foxp3⁺CD25⁺ cells from spleen cells was analyzed. Each is one representative flow cytometric analysis of one mouse. (B) Cells from five mice were stained with anti-CD25 and anti-Foxp3 antibodies and analyzed. Data expressed as the mean ± SE (*P<0.05 using one way ANOVA). (C) CD4⁺ cells were gated and analyzed the percentage of CD25⁺Foxp3⁺ cells. Each is one representative flow cytometric analysis of one mouse. (D) Proportion of Foxp3⁺CD25⁺ cells out of CD4⁺ cells from 5 mice were analyzed using One way ANOVA. Data are expressed as the mean ± SE.
Fig. 2. Percentage of IL-10 secreting CD4⁺CD25⁺ T cells increased after transferring culture medium only (control), normoxia (20% BM) or hypoxia-conditioned BMCs (0.1% BM). (A) Percentage of IL-10⁺ CD25⁺ cells from spleen cells was analyzed. Each is one representative flow cytometric analysis of one mouse. (B) Cells from five mice were stained with anti-CD25 and anti-IL-10 antibodies and analyzed. Data expressed as the mean±SE (**P<0.01 and ***P<0.001 using one way ANOVA). (C) CD4⁺ cells were gated and analyzed the percentage of CD25⁺IL-10⁺ cells. Each is one representative flow cytometric analysis of one mouse. (D) Proportion of IL-10⁺ CD25⁺ cells out of CD4⁺ cells from 5 mice were analyzed using one way ANOVA. Data are expressed as the mean±SE.
Fig. 3. Percentage of IL-4, IFN-γ, and IL-12 secreting CD4⁺ T cells were analyzed after transferring culture medium only (control), normoxia (20% BM) or hypoxia-treated BMCs (0.1% BM). (A) Percentage of IL-4, IFN-γ, and IL-12 secreting CD4⁺ T cells were analyzed. Each is one representative flow cytometric analysis of one mouse. (B) Cells from five mice were stained with anti-IL-4, anti-IFN-γ and anti-IL-12 antibodies and analyzed using one way ANOVA. Data are expressed as the mean±SE.
3) Cytokine expression of BMCs after hypoxic conditioning

To comparison of the cytokine expression of IL-4, IFN-γ, and IL-12, cells were stained with corresponding antibodies and analyzed with flow cytometer (Fig. 3). For the CD4⁺IL-4⁺ cells, control group of mice showed the 0.4±0.2%, normoxic conditioned BMCs showed 0.8±0.7%, and hypoxic conditioned groups showed 2.0±2.2% of cells. For the CD4⁺IFN-γ⁺ cells, control group of mice showed the 0.9±0.7%, normoxic conditioned BMCs showed 1.2±1.3%, and hypoxic conditioned groups showed 1.4±0.9% of cells. For the CD4⁺IL-12⁺ cells, control group of mice showed the 1.6±1.4%, normoxic conditioned BMCs showed 1.6±1.4%, and hypoxic conditioned groups showed 1.7±1.3% of cells. Although the proportion of IL-4 expressing CD4⁺ cells increased under the hypoxic conditions, did not have statistical significance. The percentage IFN-γ and IL-12 expressing CD4⁺ cells did not show statistical significance (Fig. 3B).

Discussion

In this report we demonstrated that the proportion of CD4⁺CD25⁺Foxp3⁺ cells, IL-10 secreting cells increased in CCl4 damaged mice after hypoxic conditioned BMCs transplantation.

Hypoxic conditioned BMCs transplantation increased expression of pre-survival and pro-angiogenic factors and subsequently cell death of hypoxic stem cells was significantly lower than normoxic stem cells⁵. Under hypoxia, transcription factor, HIF-1, activate and regulate glycolysis, angiogenesis, erythropoiesis and cell survival⁶. HIF-1 is composed of an oxygen-sensitive HIF-1α subunit and constitutive active HIF-1α. Hypoxia induce anti-inflammatory program by inducing expression of HIF-1α and it lead to increase the number of Foxp3⁺CD4⁺CD25⁺ cells⁷.

Regulatory T cells (Tregs) are characterized by expression of CD4, CD25, and Foxp3. The mechanism of action of Treg specific transcriptional factor, Foxp3, is via cell-to-cell contact resulting in suppression of effector T cell proliferation⁸. Tregs play crucial role in the control of immune responses by limiting immune responses in transplantation, graft-versus-host disease, allergic diseases, infections and cancer⁹-¹¹. Other type of Tregs have been described such as Th3 or Tr1 cells acting by producing regulatory cytokines including IL-10 and TGF-β. IL-10 and TGF-β promote host survival by suppressing hepatic inflammation in acute inflammation¹².

BMCs contribute to hepatocyte replacement after injury, but other BM-derived cells contribute to hepatic replacement after damage as well as to contribute to the collagen-producing cells which eventually lead to fibrosis of the liver¹³. Because immune responses can determine outcome of acute liver failure¹⁴, the systemic induction of regulatory T cells and IL-10 secreting cells after transferring BMCs in acute live injury might affect outcome of regeneration process. We suggest that hypoxic conditioned BMCs transplantation modulate immune response via inducing Foxp3⁺ and IL-10 secreting CD4⁺ cells in CCl4 damaged mice.

요 약

목적: 골수유래 줄기세포가 조직 재생 능력뿐만 아니라 면역 조절 기능도 가지고 있음이 알려져 있다. 본 저자들은 CCl4 투여 마우스 간손상 모델에서 골수세포 투여로 간기능이 회복되며, 저산소 처리 시 골수세포의 이동과 생장이 증가됨을 보고한 바 있다. 이에 저산소 처리한 골수세포의 면역 조절 기능을 알아보기 위해 이에 관련된
면역조절 T-세포의 발현 및 관련 사이토카인의 분비를 비교하였다.

방법: C57BL/6 마우스에 2일 동안 CCL4 (10 µL/g)을 투여하여 급성 간손상 숙주 모델을 제작하였다. 공여 골수세포는 산소의 농도를 달리하여 (0.1% 및 20%) 24시간 배양 후 간손상마우스에 이식하였다. 이식한 마우스의 비장세포를 분리하여 CD4⁺ T 세포의 Foxp3, IL-10, IL-4, IL-12 및 IFN-γ의 발현을 비교하였다.

결과: 저산소 처리한 골수세포를 CCl4 투여한 간손상 마우스에 이식한 군에서 CD4⁺CD25⁺Foxp3⁺ IL-10 분비 세포가 증가하였다. IL-4를 발현하는 CD4⁺ T 세포의 비율은 저산소 처리한 골수세포 투여 시 증가하였으나 통계적으로 유의하지 않았다. IFN-γ와 IL-12를 발현하는 CD4⁺ T 세포의 비율은 유의한 차이가 없었다.

결론: 저산소 처리한 골수세포가 CCl4 투여한 간손상 마우스에서 Foxp3⁺ IL-10 분비 CD4⁺ 세포를 유도하여 면역 조절에 기여할 것으로 생각된다.

References

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