Accumulation of Xenotransplanted Canine Bone Marrow Cells in NOD/SCID/γcnull Mice with Acute Hepatitis Induced by CCl4

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ABSTRACT. Bone marrow cell infusion (BMI) has recently been suggested as an effective therapy for refractory liver disease; however, the efficiency of BMI using canine bone marrow cells (cBMCs) has not been reported. We evaluated the accumulation potential of cBMCs in a mouse model of acute liver failure. Acute hepatitis was induced by carbon tetrachloride (CCl4) treatment in NOD/SCID/γcnull (NOG) mice and wild-type (WT) C57BL mice, and the characteristics of liver dysfunction and the degree of hepatic injury and regeneration were compared between the two mouse models. Next, female CCl4-treated NOG mice were xenotransplanted with male PKH26-labeled cBMCs, and the potential of cBMCs to accumulate in injured liver tissue compartments was examined. Fluorescence microscopy was performed to histologically detect the infused cBMCs, and DNA polymerase chain reaction was performed for detection of the male Y chromosome (SRY gene) in the recipient female NOG mice. The number of PKH26-positive cBMCs transplanted in the liver tissue gradually increased in the NOG mice. The infused cBMCs were located in the necrotic area of the liver at an early stage after transplantation, and most had accumulated a week after transplantation. However, the therapeutic efficacy of the xenotransplantation remained unclear, because no significant differences were observed concerning the extent liver injury and regeneration between the cBMC-transplanted and saline control mice. These results suggest that cBMCs will specifically accumulate in injured liver tissue and that BMC transplantation may have the potential to repair liver deficiency.

KEYWORDS: canine bone marrow cell, carbon tetrachloride (CCl4), cell therapy, liver regeneration, NOD/SCID/γcnull (NOG) mouse.


Chronic hepatitis (CH) and cirrhosis have long been recognized in many dog breeds by both primary and referral veterinary practices [18, 21]. These disorders are often perceived as extremely difficult to treat, and there are no cures. Therefore, more effective therapies are needed. In human medicine, liver transplantation is the only effective cure for CH and cirrhosis, but the limitations of transplantation, such as a lack of donors, surgical complications, rejection and high cost, have led to proposals for less invasive regenerative therapy procedures.

Recent reports demonstrated that murine bone marrow cells (BMCs) transplanted via peripheral vein populated and differentiated into albumin-producing hepatocytes via hepatoblast intermediates [1, 26, 29]. Interestingly, BMC infusion improves hepatic function, including elevation of serum albumin levels, and in mice or rats, it reduces liver fibrosis, corrects liver dysfunction and improves survival rate in human cirrhosis patient [10, 12]. On the basis of these results, a clinical trial of autologous BMC infusion was conducted, wherein it was shown that BMCs administered through a peripheral vein improved liver function in patients with liver cirrhosis [13, 22]. Therefore, BMC infusion may prove to be a curative therapy for liver cirrhosis in the future [27].

Recent studies have shown that the effects of BMC therapy on liver failure depend on many factors such as specific accumulation ability, immunomodulation, trophic support, differentiation (plasticity), revascularization and tissue regeneration [16, 17]. In particular, specific accumulation ability is one of the most important factors in cell transplant therapy [4, 28]. In murine models, accumulation ability has been established for specific disorders of the liver and pancreas [24]. In another study of liver cirrhosis patients with splenomegaly, splenectomy enhanced the repopulation of BMCs into the cirrhotic liver microenvironment and tended to result in greater improvement of liver function [12]. These studies suggest that the specific accumulation of BMCs in the disordered liver is important for effective results.

We previously demonstrated the capacity of canine BMCs (cBMCs) for differentiation into albumin-producing hepatocyte-like cells [15]. It has also been shown that bone marrow stromal cells (BMSCs), which are one of the components of BMCs, have various therapeutic potentials such as matrix metalloproteinase activity [7]. Furthermore, xenotransplantation using canine BMSCs may be effective in resolving inflammatory fibrotic liver in CCl4-treated immuno-deficient mice [8]. Although some reports described...
autologous transplantation of BMCs in liver injured models, studies demonstrating the ability of cBMCs to accumulate in the disordered liver have been limited. In addition, the systemic effects of infused BMC populations are not well known in both dogs and rodents. Haraguchi et al. reported that the donor genomic DNAs of xenotransplanted canine BMSCs were detectable in recipient nude mice administered cyclosporin A (CSA) [8]. Cho et al. described that infused bMCs have a specific homing capacity to the liver in CCl₄-injected recipient mice [4]. However, these studies have not elucidated the localization of transplanted cells in injured liver tissue. To analyze the accumulation of BMCs in the liver, we used NOD/SCID/γcnull (NOG) mice in this study because this model accepts heterologous cells much more readily compared with any other immunodeficient model [11].

Therefore, the present study used an NOG mouse model with acute hepatitis induced by CCl₄ treatment to investigate the ability of xenotransplanted cBMCs to accumulate in injured murine liver tissue and estimate the specificity of this accumulation in the liver.

MATERIALS AND METHODS

Animals: Six-week-old NOG mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). Same age C57BL6J Jcl mice were purchased from CLEA Japan, Inc. (Kawasaki, Japan) to serve as the wild-type (WT) murine control. All mice were shipped to the Research Institute of Biosciences of Azabu University and handled with humane care under pathogen-free conditions. The mice were housed in a room under controlled temperature (25°C), humidity and lighting (12-hr light/dark cycle). Access to food and tap water was ad libitum throughout the study period. All of male NOG and C57BL mice were used in research for an experimental model of liver injury, and female NOG mice were used for BMC transplant experimentation. A 2-year-old male beagle dog that was clinically healthy was used as a donor of cBMCs for transplantation into NOG mice. All experiments in this study were performed in accordance with the Animal Protection Guidelines of Azabu University (authorization no. 10–79).

Experimental model of liver injury: Forty male NOG mice and 20 male WT mice were divided into three groups: NOG + CCl₄ (n=20), NOG + oil (n=20) and WT + CCl₄ (n=20). CCl₄ (Wako Pure Chemical Industries, Osaka, Japan) dissolved in olive oil (1 ml/kg) was administered via intraperitoneal injection in the NOG + CCl₄ and WT + CCl₄ groups, and the extent of liver injury and regeneration was investigated in these mice. The mice were sacrificed at 0 hr, 24 hr, 48 hr, 72 hr and 1 week after CCl₄ injection, and the livers were sectioned and fixed in phosphate-buffered saline (PBS) to remove proteins from the culture medium for optimal staining. The staining reaction was stopped with addition of 1% bovine serum albumin, followed by washing with PBS. Labeling and viability were verified by cultivation in hepatocyte growth medium as previously described [15].

Transplantation: Fifteen female NOG mice were grouped as follows: CCl₄ + cBMC (n=5), oil + cBMC (n=5) and CCl₄ + saline (n=5). At 1.5–2 hr after BMC harvesting, prepared MNCs (1 × 10⁶) or saline was administered to the tail veins of CCl₄- or oil-treated NOG mice. The mice were sacrificed after 1 week, and the livers, lungs and spleens were removed to evaluate the systemic distribution of the administered cBMCs (Fig. 1A). To investigate the time course of BMC accumulation in the injured liver, 32 female NOG mice were grouped into CCl₄ + cBMC (n=16) and CCl₄ + saline (n=16) groups and injected as above. These mice were sacrificed, and the livers were removed at 0 hr, 24 hr, 48 hr and 1 week after BMC transplantation (Fig. 1B).

Laboratory analysis: The mice were anesthetized with pentobarbital (Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan), and their blood was collected from the heart and centrifuged at 15,000G for 15 min at 4°C. Albumin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were analyzed with a Hitachi 9,000 series automatic analyzer.

Histological examination: Liver samples were fixed in a 10% neutral-buffered formaldehyde solution, embedded in paraffin and sectioned. The 3-µm-thick sections were stained with hematoxylin and eosin (HE). The relative necrotic area, expressed as a percentage of the total liver area, was determined in the HE-stained liver sections. Each field was acquired as 5 nonoverlapping random fields (×200 magnification) and analyzed using ImageJ software version 1.41 (http://rsb.info.nih.gov/ij).

Immunohistochemistry: Tissue sections were subjected to immunohistochemistry using rat monoclonal antibody to the Ki-67 antigen (1:200; Dako, Tokyo, Japan), which detects proliferating cells. Microwave antigen retrieval was performed in citrate buffer (pH 6.0) for 10–20 min. The sections were treated with 0.3% H₂O₂ in methanol for 20 min. For the primary antibody reaction, slides were incubated in a wet chamber overnight at 4°C. The immunoreactive materials were visualized using a peroxidase staining kit (Histofine Simple Stain MAX PO; Nichirei, Tokyo, Japan) and diamino benzidine (Histofine Simple Stain DAB; Nichirei). The sections were counterstained with hematoxylin. The number of cells and the number of Ki-67-positive cells per field in each mouse liver were measured at ×400 magnification, and...
the ratio of Ki-67-positive cells was calculated.

**Double fluorescence analysis:** For fluorescence analysis, freshly isolated lungs, spleens and livers were embedded in optimal cutting temperature medium and frozen. Frozen sections (5 µm) were obtained using a cryostat (model HM505; Microm) equipped with a tungsten carbide knife. After fixation with 3.7% paraformaldehyde, polymerized actin was stained with phalloidin (Alexa Fluor 488 phalloidin, Invitrogen, Carlsbad, CA, U.S.A.). Vectashield mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, U.S.A.) was used for nuclear staining. The lung and spleen sections were nuclear stained with DAPI alone. Images were observed and captured by fluorescence microscopy (FSX100; Olympus, Tokyo, Japan). Computer-assisted image analysis was performed using ImageJ software version 1.41.

**Canine Y chromosome (SRY)-specific PCR:** Genomic DNA was isolated from NOG mouse liver tissue homogenates using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The presence or absence of the sex-determination region of the male Y chromosome (SRY gene) in the recipient female NOG mice was assessed by PCR as previously described [25]. Primer sequences for the SRY gene were obtained from published sequences (forward primer, 5'-CAAGATGGCTCTAGAGATATCC-3'; reverse primer, 5'-AGCTGTCCGTGTAGGTTGTA-3'), which amplified a 284-bp product. The PCR conditions were as follows: incubation at 94°C for 2 min; 40 cycles of incubation at 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. PCR products were separated using 2% agarose gel electrophoresis and stained with ethidium bromide. Positive (male canine genomic DNA) and negative (female canine genomic DNA) controls were included in each assay.

**Statistical analysis:** Data are expressed as means ± standard error of the mean (SEM). The Mann–Whitney U test was used to compare the different groups as appropriate. P values of <0.05 were considered statistically significant. Analyses were performed using GraphPad Prism 5.

**RESULTS**

**Characterization of CCl4-induced acute liver dysfunction in NOG mice:** The degree of liver injury was assessed by measuring serum liver enzymes (Fig. 2A). Serum albumin levels were significantly decreased at 48 hr after CCl4 injection, after which the levels improved to within the normal range in both NOG and WT mice. Both serum ALT and AST
increased markedly 24 hr after CCl₄ injection; however, they decreased to within the normal range 48 hr after CCl₄ injection, with no differences between the NOG and WT mice. To evaluate liver damage and regeneration, the necrotic areas of the liver were measured in NOG and WT mice injected with CCl₄. There were no significant differences in the extent of liver necrosis between the NOG and WT mice (Fig 2B). Immunohistochemistry using Ki-67, a marker for cell proliferation, was performed to assess hepatocyte regeneration (Fig. 2C). Ki-67-positive hepatocytes were limited 24 hr after CCl₄ injection; however, there were many positive cells 48 hr after CCl₄ injection, with no differences between the NOG and WT mice.

Migration of PKH26-positive cBMCs into injured liver lobules: PKH26-positive cBMCs (1 × 10⁶) were injected into liver-damaged and control (undamaged) female NOG mice. Infused cBMCs could be visualized by fluorescence microscopy a week after transplantation (Fig. 3). A few PKH26-positive cells were detected in sections of the liver from undamaged controls (Fig. 3A), and there were no PKH26-positive cells in CCl₄-damaged mice without cBMC transplantation. In contrast, in the transplanted CCl₄-treated mice, the number of PKH26-positive cells gradually increased, spreading into the liver lobules (Fig. 3A, 3B). Some PKH26-positive cells were observed in the lung tissue of both liver-damaged and control mice (Fig. 3A, 3B), while no PKH26-positive cells were detected in spleen tissue from any of the groups (Fig. 3A).

Detection of the SRY gene in transplanted NOG mice: PCR amplification of the canine SRY gene, located on the Y
chromosome, demonstrated that transplanted cBMCs were present in the livers of the transplanted female mice, indicating that male cBMCs accumulated in the liver tissue of the female mice (Fig. 3C).

**Localization of infused cBMCs in the injured Liver**: Phalloidin staining of frozen liver sections was performed to further elucidate the localization of cBMCs in the mouse liver. The actin cytoskeleton was disordered in necrotic tissue, and PKH26-positive cells were slightly accumulated in the necrotic areas 24 and 48 hr after transplantation (Fig. 4A). One week after transplantation, the number of accumulated cBMCs (PKH26-positive cells) gradually increased and spread into the liver lobules (Fig. 4A, 4B).

**Effects of cBMC transplantation on CCl4-induced acute liver injury**

**Fig. 3.** Engraftment of donor cBMCs in liver, lung, and spleen tissues was examined 1 week after transplantation. (A) Detection of PKH26-labeled infused cells in liver tissues by fluorescence microscopy. A number of PKH26-positive cBMCs were detected in livers of the CCl4 + cBMC group, but only a few cBMCs were detected in livers of the oil + cBMC group. In both the CCl4 + cBMC and oil + cBMC groups, some PKH26-positive cells were detected in the lung tissue, but not in the spleen tissue. Scale bar=200 µm (B) Quantitative comparison of PKH26-positive cBMCs in liver and lung tissues. PKH26-positive cells were significantly increased in the CCl4 + cBMC group compared with the oil + cBMC group, but the number of PKH26-positive cells in the lung tissue did not differ between the groups. *Significant difference compared with the Oil+cBMC group (n=4 per group; P<0.05). (C) The canine male-specific SRY gene (284 bp) was amplified exclusively in the livers of the female xenotransplanted mice. Lane 1, molecular weight standard; lane 2, cBMC-infused liver tissue; lane 3, saline-treated liver tissue; lane 4, male dog liver tissue (positive control); lane 5, female dog liver tissue (negative control).
liver dysfunction in the NOG mice: To clarify the therapeutic efficacy of cBMC transplantation in an acute liver hepatitis model, the degrees of hepatic injury and regeneration were compared between the cBMC-transplantation and saline (control) groups (Fig. 5). As parameters of hepatic injury, serum albumin and hepatic enzyme levels (Fig. 5A) and necrotic areas (Fig. 5B) were measured. As a measure of hepatocyte regeneration, the Ki-67-positive ratios were measured in each group (Fig. 5C). However, none of these parameters were significantly different between the control and transplantation groups (P=0.872).

DISCUSSION

In the present study, the infused cBMCs not only accumulated in the injured mouse liver at an early stage after transplantation but also accumulated specifically in injured tissue. Although several investigators have reported the ability of BMCs to engraft in experimental models, including rodent models [3, 6, 14], to our knowledge, this is the first study to investigate whether canine BMCs can accumulate in the injured liver tissue of an immunodeficient mouse model. Transplantation of cBMCs into recipient mice was success-
ful, as indicated by the detection of both PKH26-labeled cBMCs and the canine SRY gene in the liver tissue of female NOG mice. In particular, infused cBMCs were located in the necrotic area of the liver an early stage after transplantation, with the largest accumulation at 1 week after transplantation. CCl4-induced acute liver injury in NOG mice peaked at 24 hr, and most liver regeneration occurred at 48 hr after CCl4 injection. However, the time course of cBMC migration to the injured liver tissue did not correspond to the time course of liver injury or regeneration, because most cBMC accumulation occurred more than 48 hr after transplantation. Previously, Thomas et al. demonstrated that macrophage therapy was effective for improving liver fibrosis in a murine hepatic fibrosis model. Their migration of infused macrophages was detected in liver tissue from 1 to 7 days after transplantation, but the number of cells was not increased [28]. Chronic hepatitis and developed hepatic fibrosis models were used for their study, which they were established by a long-term protocol of CCl4 injection on two consecutive days per week for at least 6 weeks. In the present study, NOG mice were injected with CCl4 a single time and used as an acute hepatitis model, so its pathogenesis might be the cause of the different time course of cell accumulation in chronic hepatic injury. Therefore, we suggested that transplanted cell accumulation in the injured liver might be affected by the difference in acute or chronic hepatic deficiency, or transplanted cell fraction.

Several immunodeficiency models, such as NOG mice, have been developed for heterograft experimentation [2]. NOG mice can accept heterologous cells much more readily than any other immunodeficient rodent model [9, 12]. Although the immune system plays an important role in liver
regeneration [5, 19], it is not known whether NOG mice are an appropriate model for spontaneous acute hepatitis. In the present study, we further confirmed these findings by comparing CCl4-induced hepatitis and liver regeneration between NOG and WT mouse models of acute hepatitis, which suggested that NOG mice can be used as an acute liver injury model similar to WT mice. Both serum hepatic enzyme levels and necrotic areas showed maximum increases 24 hr after CCl4 injection, and Ki-67-positive hepatocytes appeared 48 hr after CCl4 injection (Fig. 2). These findings were consistent with those of a previous study [20]. It is well known that the hepatocyte growth factor (HGF) is the most important factor for hepatic regeneration [23]. We expect that liver regeneration is not influenced by the immunodeficiency of NOG mice, because HGF may be secreted normally in these mice.

Infused cBMCs also accumulated in the liver and lung but not in the spleen. Thomas et al. [28] and Cho et al. [4] have demonstrated the efficient trafficking of rodent BMSCs to various target organs following transplantation, with a major portion of the input cells retained in the lung. In the green fluorescent protein (GFP)/CCl4 mouse model, Iwamoto et al. [12] demonstrated that splenectomy enhanced the migration of GFP-positive BMCs to the damaged liver. With the exception of migration to the lung, our findings agree with these data, although it is difficult to precisely evaluate the accumulation in the spleen in NOG mice because they have natural splenic atrophy.

In this study, cBMC transplantation did not improve hepatic function and regeneration. Serum ALT increased extremely (approximately 15,000 IU/l) 24 hr after CCl4 injection in both the saline and cBMC groups. In this study, the hepatic necrotic area 24 hr after CCl4 injection was 50% of the liver, indicating that change was the greatest value in this acute hepatitis model. We suggested that CCl4-induced acute liver injury may have been so severe that it became impossible to evaluate the therapeutic effects of cBMC transplantation. In the hepatic fibrosis model, some studies indicated that bone marrow-derived cells could decrease collagen fibers and reduce hepatic fibrosis through expression of matrix metalloproteinases (MMPs) [4, 7, 8]. Haraguchi et al. reported that transplanted cBMSCs migrated into liver regions exhibiting severe inflammation and fibrosis, and fibrosis was significantly reduced in CCl4/CSA mice transplanted with canine bone marrow stromal cells (cBMSCs) [8]. Therefore, the therapeutic effects of cBMC transplantation should be evaluated in clinical canine cases or in a hepatic fibrosis model.

In conclusion, we demonstrated that cBMCs accumulate after xenotransplantation in NOG mice with induced liver injury. These results suggest that cBMCs may have potential in relation to liver regeneration, although the precise mechanisms for regulating cBMC stimulation in the damaged liver remain uncertain. A further study of these mechanisms is required to develop cell therapies that utilize cBMCs for repair of the damaged liver.

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