Gene delivery using human cord blood–derived CD34+ cells into inflamed glomeruli in NOD/SCID mice

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Background. Bone marrow reconstitution using genetically-modified hematopoietic stem cells has been reported to confer resistance to inflammation and prevent renal injury in glomerulonephritis. Although this strategy has potentials for clinical use, taking hematopoietic stem cells from bone marrow is highly stressful for patients. In this regard, umbilical cord blood may be a useful alternative and, therefore, we focused on their suitability as a source of hematopoietic stem cells for transplantation-based therapy for glomerulonephritis.

Methods. CD34+ cells were obtained from human umbilical cord blood, retrovirally transduced with human β-glucuronidase (HBG) gene, and transplanted into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. After confirming the successful chimerism, these mice were treated with lipopolysaccharide (LPS), and local HBG expression in glomeruli was examined using immunohistochemical analysis, HBG bioassay, and Western blot analysis.

Results. Clonogenic assay showed that 88.4 ± 5.9% burst-forming unit-erythroid (BFU-E), 79.7 ± 11.4% in colony-forming unit-macrophage (CFU-M), and 81.1 ± 14.1% in colony-forming unit-granulocyte (CFU-G), respectively, possessed the transgene after transfection, suggesting that precommitted cells were susceptible to retroviral infection. Flow cytometric analysis revealed that 24.1 ± 14.5% of bone marrow cells in these chimera mice expressed human lymphocyte antigen (HLA) 8 weeks after transplantation. Also, clonogenic assay showed that a sustained engraftment of human hematopoietic cells expressed HBG. CD14-positive cells were recruited into the glomeruli upon LPS treatment and they secreted bioactive HBG, suggesting that cord blood–derived CD34+ cells may differentiate into monocyte lineage while maintaining the expression of the transgene.

Conclusion. These data indicate that umbilical cord blood cells can be utilized as a source of hematopoietic stem cells for the transplantation-based therapy of glomerulonephritis.

Recently, stem cell research has attracted considerable attention from many researchers because it could be used for organ regeneration after untreatable damage, and several stem cells (or progenitor cells), such as endothelial stem cells [1] and neural stem cells [2] have been discovered. Following the progression of this field of research, the potential for stem cell therapy has increased and several therapeutic benefits have already been reported [3, 4]. Although this approach was originally investigated for fatal or hereditary diseases, we have proposed that inflammatory diseases such as glomerulonephritis are also candidates for transplantation-based gene therapy using hematopoietic stem cells [5]. We used bone marrow–derived cells as a vehicle to deliver anti-inflammatory molecules into inflamed glomeruli [6, 7]. In the in vivo differentiation system, bone marrow cells were differentiated ex vivo to express ligands of adhesion molecules and acquire the potential to be recruited to the inflamed site, and were adenovirally transfected with a foreign gene followed by transfusion to the affected subjects. These cells may deliver anti-inflammatory cytokines into inflamed glomeruli [8]. In the in vivo differentiation system, bone marrow cells were genetically modified using retrovirus and transplanted to the affected subjects before differentiation so that they may retain the potential for self-renewal, as well as differentiation, in vivo [7]. Both systems were able to prove the therapeutic benefit of anti-glomerular basement membrane (anti-GBM) nephritis in mice [6, 7]. These strategies have several advantages over the previous glomerulus-targeted gene delivery systems (i.e., use of peripheral vessels for administration, longer therapeutic time window, and possibly no immunoreaction due to autologous transfer) and suggest that bone marrow–derived cells can be utilized for therapeutic intervention to treat local inflammation.

As a next step toward clinical application, we sought a different source of hematopoietic stem cells other than bone marrow, since taking stem cells from bone marrow is highly inconvenient for patients, especially when it is for the treatment of nonlethal diseases like inflammation.

Key words: stem cell, CD34, NOD/SCID mouse, gene delivery, glomerulonephritis.

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In this regard, umbilical cord blood cells may be a useful alternative because: (1) a substantial amount of blood (about 100 mL from each delivery [9]) may be corrected without pain and risk to mother or infant; (2) they contain a significantly higher number of hematopoietic progenitor cells when compared with adult peripheral blood [10]; (3) lower incidence and severity of graft versus host disease (GVHD) after transplantation occurs due to the immature immune system [10]; and (4) recently, large-scale banks of cord blood have been developed or are being considered throughout the world. We, therefore, examined their suitability as a source of hematopoietic stem cells for transplantation-based gene therapy for inflammatory diseases.

To monitor the behavior of human cells in vivo, a bone marrow repopulating system using the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse was applied. NOD/SCID mice are characterized by a functional deficit of natural killer cells, absence of circulating complement, and defects in the differentiation and function of antigen-presenting cells (APC), as well as an absence of T and B cell functions, facilitating reconstitution with human hematopoietic cells [11, 12]. Using transplantation techniques, chimera mice, which have a mouse body with a human hematopoietic system, may be established.

In this study, we report that umbilical cord blood cells may be a useful alternative as a source of hematopoietic stem cells for transplantation-based gene therapy to treat inflammatory diseases such as glomerulonephritis.

METHODS

Experimental Design

CD34+ cells were obtained from human cord blood and retrovirally transfected with human β-glucuronidase (HBG) as a marker. Successful transfection into predifferentiated cells was confirmed by clonogenic assay. These cells were transplanted into NOD/SCID mice, and 8 weeks later successful chimerism was again confirmed by clonogenic assay and flow cytometric analysis. Since we found that LPS induced adhesion molecules even in the glomeruli of NOD/SCID mice (data not shown), these chimera mice received 25 μg LPS purified from Klebsiella O3 [13] or saline as a control for 7 days. Tissue specimens were subjected to immunohistochemical analysis on human CD14 and HBG to confirm that the transplanted premature cells may differentiate into monocyte lineage cells at the recruited site and those cells still express transgene. HBG bioactivity and Western blot analysis of isolated glomeruli were also compared between LPS-treated and saline-treated mice.

Animals

A breeding colony of NOD/SCID mice was established at the laboratory animal center of the Jikei University School of Medicine (Tokyo, Japan) from breeding pairs purchased from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in a positive airflow ventilated rack and bred and maintained under specific pathogen-free conditions.

Establishment of chimera mice

Human umbilical cord blood samples were obtained, with informed consent, from placentas of full-term normal newborn infants. After isolation of mononuclear cells from cord blood by density gradient centrifugation with Nicoprep™ 1.077 (Nycomed Pharma AS, Oslo, Norway), CD34+ cells were obtained from mononuclear cells by magnetic bead separation (Dynal CD34 Progenitor Cell Selection System; Dynal, Lake Success, NY, USA) according to the manufacturer’s instructions. These cells were suspended in MyeloCult H5100 (StemCell Technologies, Inc., Vancouver, British Columbia, Canada) with penicillin G (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) supplemented with 200 units/mL human interleukin-6 (IL-6) (Kirin Brewery Co., Ltd., Tokyo, Japan), and 100 ng/mL human stem cell factor (mSCF) (Kirin Brewery Co., Ltd.), Fli-t-3/Flik-2 ligand (100 ng/mL; R & D Systems, Minneapolis, MN, USA), human thrombopoietin (10 ng/mL; Kirin Brewery Co., Ltd.). After cultivation in a humidified atmosphere of 5% CO2 at a concentration of 1 × 106 cells/mL for 24 hours, the bone marrow cells were transplanted with the human HBG gene via a retrovirus as described later. NOD/SCID mice were prepared for transplantation with a sublethal dose of irradiation (3.5 Gy). Genetically-modified CD34+ cells (5 × 106/20 g body weight) were infused into recipient mice via the tail vein once daily for 4 consecutive days. Bone marrow cells were freshly prepared for each injection.

Recombinant retrovirus preparation and infection

CD34+ cells were transduced with HBG via a retrovirus using a centrifuge method [14], ψCRIP packaging cells [15] producing an MFG-GC vector (1 × 109 cfu/mL) expression of HBG were constructed as described before [16]. A retroviral vector–containing supernatant was collected from confluent monolayers of these packaging cells grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% calf serum (CS) (Gibco/BRL, Grand Island, NY, USA). These supernatants were filtered (0.45 μm) and centrifuged at 14,000 g for 2 hours at 4°C to concentrate the virus [17], followed by resuspension in one-tenth the volume of culture medium. After pre-stimulation with IL-6, hSCF, thrombopoietin, and Fli-t-3/Flik-2 ligand for 24 hours, CD34+ cells (5.0 × 105/mL culture medium) were transferred into a round-bottomed tube (Falcon #2051, Franklin Lakes, NJ, USA), which was coated with a recombinant human fibronectin fragment CH-296 (Takara Shuzo Co., Ltd., Shiga, Japan).
Clonogenic assay for transduced CD34+ cells

Clonogenic assay was performed as previously described [19]. In brief, transduced CD34+ cells were plated at a concentration of 1 × 10^3 cells/mL in MethoCult GF H4434V (methylcellulose-containing medium with erythropoietin, stem cell factor, GM-CSF, and IL-3) (StemCell Technologies, Inc.). After 14 days, CFU-G, CFU-M, and BFU-E were selected and DNA was extracted following a previously published method [20]. The yield of DNA was monitored by human-specific β-globin microgel polymerase chain reaction (PCR) [21]. To amplify the HBG cDNA sequence, PCR was carried out on genomic DNA samples in a final volume of 100 μL. The primers used for PCR were 5′-GAT GGT GAT CGC TCA CAC CA-3′ and 5′-CGG TTA CTG TTC AGT CAT GA-3′, which amplify part of the HBG cDNA sequence. The reaction mixture contained 250 μmol/L of each deoxynucleoside triphosphate (dNTP), 2.5 units of AmpliTaq Gold (Perkin Elmer, Foster City, CA, USA), 2.5 mmol/L MgCl2, and 0.5 μmol/L of each primer in AmpliTaq buffer. Thermal cycling was performed on Takara PCR Thermal cycler MP (Takara Shuzo Co., Ltd.) as follows: 95°C for 5 minutes for initial denaturing, followed by 63 cycles of 94°C for 1 minute, 60°C for 1 minute, and 60°C for 5 minutes for the final extension cycle. After electrophoresis in a 2% agarose gel, the amplified products were visualized with ethidium bromide staining. Experiments were performed in quadruplicate and representative pictures are shown.

Two-color immunofluorescent staining

Each specimen was embedded in optimal cutting temperature (OCT) compound (Miles Scientific, Naperville, IL, USA) and quickly frozen in liquid nitrogen. Cryostat sections (6 μm) were dried and fixed with acetone for 15 minutes. After blocking the endogenous biotin with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA), the sections were incubated with sheep anti-human CD14 (Chemicon, Temecula, CA, USA) for 2 hours at 37°C. After rinsing in PBS, the sections were incubated with Alexa Fluor® rabbit anti-goat IgG (H+L) conjugate (Molecular Probes, Leiden, The Netherlands) and FITC-streptavidin (Becton Dickinson, Franklin Lakes, NJ, USA). The sections were mounted in p-phenylenediamine (PD)-PBS-glycine and observed under a fluorescence photomicroscope. Pictures were taken using an autoexposure camera (Zeiss, Thornwood, NY, USA).

HBG enzymatic activity of isolated glomeruli

HBG bioactivity of isolated glomeruli was assayed as described [22]. Briefly, glomeruli from chimera mice transplanted with LPS or saline treatment were isolated and homogenized in distilled water using a glass homogenizer. The homogenates were spun at 14,000g for 10 minutes at 4°C. The clear supernatant was assayed fluorometrically for HBG activity with the synthetic fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronidase (Sigma). After incubation of each sample with the substrate for 30 minutes at 37°C, reactions were terminated by the addition of 0.17mol/L glycine-carbonate buffer (pH 10.4). The 4-methylumbelliferone (4-MU) that had formed was measured fluorometrically. Units of HBG activity are defined as nanomoles 4-MU produced/hr/mg protein. Protein concentration was determined with a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA).

Immunoprecipitation of human HBG

Since measurements of HBG enzymatic activity were made both of total HBG activity representing the endogenous murine HBG activity and any human HBG activity resulting from gene transfer, immunoprecipitation of human HBG was performed to estimate the human HBG activity only. Each sample was divided into two identical aliquots and the human HBG was immunoprecipitated from one of each pair. Ten micrograms of protein of each homogenate were mixed with either 10 μL of anti-human HBG or distilled water, and shaken at 4°C for 1 hour. Twenty five microliters of protein A sepharose 4 fast flow® (Amersham Pharmacia Biotech, Buckinghamshire, UK) was then added to each of the samples, which were inverted overnight at 4°C. Samples were spun at
12,000g for 2 minutes and supernatant removed for measurement of HBG enzymatic activity.

**Western blot analysis**

Western blot analysis was performed to measure the secretion of HBG from recruited human CD14+ cells in the glomeruli as described [6]. Briefly, homogenates of isolated glomeruli (2 μg) from LPS-treated and saline-treated mice were subjected to electrophoresis in a 12.5% sodium dodecyl sulfate-polyacrylamide gel and then transferred to nitrocellulose membrane. The membrane was blocked with 5% dried milk, 0.1% Tween 20, PBS, and incubated with anti-human HBG antibody (×1000). It was then incubated with rabbit anti-gout immunoglobulin horseradish peroxidase conjugate (Cosmo Bio, Tokyo, Japan). Antigen-antibody complexes were visualized by chemiluminescence reagents (Amersham Pharmacia Biotech).

**Analysis of proteinuria and serum creatinine**

The concentration of albumin in urine was measured by single radial immunodiffusion (SRID) [23] using a specific antibody for mouse albumin [24]. Briefly, samples were applied to a 1% agarose gel containing anti-mouse albumin and incubated for 48 hours. The diameters of the expressed rings on the gel were measured and their concentrations were assessed by comparison with a standard curve. Concentration of plasma creatinine was measured using the Vision analyzer kit (Abbott Laboratories, North Chicago, IL, USA), which is based on the Jeffe reaction.

**Statistical analysis**

Data are expressed as mean ± SE. Statistical analysis was performed using the two-sample t test to compare data in different groups. P < 0.05 was found to be significant.

**RESULTS**

**Susceptibility of CD34+ cord blood cells to retroviral transfection**

To examine the efficacy of CD34+ cord blood cells to retroviral transfection, CD34+ cells were established from freshly obtained human cord blood cells and retrovirally transfected with the human HBG gene, using centrifugation methods with concentrated viral supernatant. These cells were cultured in MethoCult GF H4434V for 14 days and DNA from each colony was subjected to PCR on the HBG gene. As shown in Figure 1, three different lineages of hematopoietic cell possessed the transgene (88.4 ± 5.9% in BFU-E, 79.7 ± 11.4% in CFU-E, and 81.1 ± 14.1% in CFU-G, respectively), and there were no significant differences between lineages, suggesting that CD34+ cells from human cord blood cells are susceptible to retroviral transfection in the precommitted form.

**Establishment of chimera mouse that has mouse body with human hematopoietic system**

To establish the chimera mouse, the hematopoietic systems were exchanged for human cells, and HBG-transfected CD34+ cells were transplanted to NOD/SCID mice. After 8 weeks, bone marrow cells from these mice were subjected to flow cytometric analysis on human HLA ABC antigen. As shown in Figure 2A, 24.1 ± 14.5% of bone marrow cells in these chimera mice expressed human HLA ABC antigen, showing successful chimera in these mice. To confirm that these reconstituted bone marrow cells still maintained the transgene clonogenic assay was also performed on HBG. As shown in Figure 2B, the cells from these marrows still possessed the transgene (31.4 ± 10.8% in BFU-E, 38.9 ± 18.9% in CFU-E, and 33.8 ± 19.9% in CFU-G, respectively),
showing a sustained engraftment of human hematopoietic cells expressing the HBG gene.

**Delivery into inflamed site by in vivo differentiated human cells**

Since Verstegan et al [25] previously reported that human monocytes may spontaneously grow out in the SCID mice after transplantation with unfractionated human cord blood cells, we speculated that transplanted human CD34+ cells may differentiate into mononuclear cells and effectively deliver transgene into inflamed glomeruli. Chimera mice were treated with LPS or saline once daily for 7 days and subjected to immunohistochemical analysis, HBG enzymatic assay, and Western blot analysis of HBG expression. At day 7, kidneys were obtained from these mice and tissue sections were stained with human CD14 and HBG. As shown in Figure 3A, CD14+ cells were detected in the glomeruli upon LPS treatment, and 19.3 ± 4.4% of those cells secreted HBG. The total HBG enzymatic activity in isolated glomeruli was not significantly elevated by LPS treatment (716.0 ± 68.8 nmol/hr/mg in saline-treated mice vs. 1085.9 ± 137.5 nmol/hr/mg in LPS-treated mice; P = 0.0821) (Fig. 3B). However, transplanted human HBG activity, which was estimated by subtraction of endogenous murine HBG activity from total HBG, was significantly elevated by LPS treatment (148.1 ± 52.0 nmol/hr/mg in saline-treated mice vs. 574.8 ± 102.7 nmol/hr/mg in LPS-treated mice; P = 0.0041). Furthermore, Western blot analysis revealed that isolated glomeruli from LPS-treated chimera mice contained higher amount of HBG protein compared with control chimera mice (Fig. 3C). Together, these data suggested that umbilical cord blood-derived CD34+ cells might differentiate into monocyte lineage cells while maintaining the expression of the foreign gene.

Although native macrophages may influence the behavior of donor macrophages, only a negligible number
of native macrophages could be detected in the glomeruli of the NOD/SCID mice treated with LPS (data not shown), probably because the number of native macrophages in NOD/SCID mice are small [26] and are functionally immature [27], especially when the response to LPS is impaired [28]. Therefore, donor macrophages may occupy the area of intercellular adhesion molecule-1 (ICAM-1) expression in the glomeruli without competition with native macrophages, by which the significance of transgene activity might be enhanced. On the other hand, recruitment of transplanted cells into glomeruli might affect renal function and, therefore, renal function after LPS treatment was examined in chimera mice. Both urine albumin and serum creatinine levels were under detectable level (the lowest detectable concentrations of albumin and creatinine are 20 mg/mL and 0.3 mg/dL, respectively), suggesting that accumulation of exogenous human CD14 cells, per se, did not affect renal function in chimera mice.

**DISCUSSION**

Transplantation-based gene therapy has been proposed as a novel therapeutic strategy for chronic inflammatory diseases such as glomerulonephritis [5]. This approach is based on the fact that inflammatory cells such as macrophages and neutrophils are capable of being recruited to and activated at the inflamed glomeruli and, therefore, genetic manipulation of bone marrow–derived cells or bone marrow, per se, using transplantation-based technology may modify inflammation. We previously reported that bone marrow reconstitution using genetically-modified hematopoietic progenitor cells to supply anti-inflammatory mononuclear cells to inflamed glomeruli confer resistance against glomerular inflammation [7]. As the next step toward the clinical use, we investigated the suitability of human umbilical cord blood as a source of hematopoietic stem cells, which may reduce the risks to patients, and showed that CD34+ cells from human cord blood may reconstitute bone marrow; these cells may differentiate into monocyte lineage cells, which may deliver foreign genes into inflamed glomeruli. In addition to fewer risks to the mother and infant, umbilical cord blood cells have several advantages as a source of hematopoietic stem cells for clinical allogeneic transplantation. For example, umbilical cord blood cells can be cryopreserved and easily shipped and thawed for use on demand, eliminating delays and uncertainties that presently complicate marrow collection from unrelated donors [29]. The amplification of allogeneic responses by neonatal T lymphocytes has been shown to be less than that of adult T cells, which may underlie umbilical cord blood reduced graft-versus-host reactivity [30, 31]. Although these advantages strengthen the rational of its clinical use, there are several obstacles to be overcome. First, the efficacy of retroviral transfection is quite low for human stem cells compared to mouse stem cells [32]. We tried several different methods and finally concluded that the most efficient transfection was done by a centrifugation method [14] using recombinant human fibronectin fragment CH-286–coated tubes [18] with concentrated viral supernatant [17]. This modification resulted
Broxmeyer et al [40] reported that umbilical cord blood necessary to engraft larger patients. For this purpose, expansion of umbilical cord blood progenitor cells to 1/1000 of monocyte lineage cells using an IL-11 and granulocyte colony stimulating factor (G-CSF) has been reported to be able to expand in the presence of stem cell factor (SCF) and cytometric analysis. This work was supported by a grant from the Ministry of Education, Science and Culture of Japan; and Sankyo Foundation of Life Science.

REFERENCES

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REFERENCES


