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An Assay for Human Hematopoietic Stem Cells Based on Transplantation into Nonobese Diabetic Recombination Activating Gene–Null Perforin-Null Mice

Hitoshi Minamiguchi,[1] John R. Wingard,[2] Joseph H. Laver,[3] Elsie S. Mainali,[3] Leonard D. Shultz,[4] Makio Ogawa[1]

[1]Department of Veterans Affairs Medical Center and Department of Medicine, Medical University of South Carolina, Charleston, South Carolina; [2]Department of Medicine, University of Florida, Gainesville, Florida; [3]Department of Pediatrics, Virginia Commonwealth University, Richmond, Virginia; [4]The Jackson Laboratory, Bar Harbor, Maine

Correspondence and reprint requests: Makio Ogawa, MD, PhD, Department of Veterans Affairs Medical Center, 109 Bee St., Charleston, SC 29401-5799 (e-mail: ogawam@musc.edu).

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ABSTRACT

Nonobese diabetic recombination activating gene–null perforin-null (NOD-*Rag1^{null}Prf1^{null}*) mice, which totally lack mature T and B cells and natural killer cell cytotoxic function, survive longer and are easier to breed than NOD-severe combined immunodeficiency (*scid*) or NOD-*scid*/β_2-microglobulin^{null} mice. We have tested the use of NOD-*Rag1^{null}Prf1^{null}* mice as recipients in a long-term xenograft assay for human hematopoietic stem cells (HSCs) by adopting Yoder and colleagues' method of conditioned newborn mice, with minor modifications. Pregnant NOD-*Rag1^{null}Prf1^{null}* dams were treated with busulfan 22.5 mg/kg. On the day of delivery, the busulfan-exposed pups underwent transplantation with 4 to 5 million T cell–depleted human cord blood mononuclear cells via the facial vein. At 2 months after transplantation, all 11 transplanted mice showed human hematopoietic engraftment in the peripheral blood. At 6 months after transplantation, human cells were detected in 5 mice, which showed higher than 0.9% human cell engraftment at 2 months. The mean percentage of human CD45$^+$ cells in the bone marrow of engrafted mice was 43.9% ± 36.5% (range, 2.0%-79.9%). Next, we tested the usefulness of conditioned newborn NOD-*Rag1^{null}Prf1^{null}* mice for applications to characterize the dye efflux capability and phenotypic features of human HSCs. Given that cord blood HSCs have the ability to efflux rhodamine 123 (Rho), we attempted transplantations of sorted cells that retained a low level (Rho^{low}) or high level (Rho^{high}) of Rho. Six-month engraftment was found only with the Rho^{low} cells, which contained high percentages of CD34$^+$CD38$^-$ cells and side population cells with Hoechst 33324 efflux activity. These observations suggest that Rho^{low} cells are highly enriched for primitive hematopoietic cells. Accordingly, conditioned newborn NOD-*Rag1^{null}Prf1^{null}* mice provide a desirable model for an assay of long-term transplantable human HSCs.
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KEY WORDS

NOD-*Rag1^{null}Prf1^{null}* mice • Newborn mice • Transplantation • Cord blood

INTRODUCTION

Several transplantation models based on immune-deficient mice have been developed as assays for human hematopoietic stem cells (HSCs). In 1988, McCune et al. [1] first described the scid-hu model, in which long-term engraftment of human lymphomyeloid cells was established by transplanting fetal human tissues, such as liver, bone, thymus, and lymph nodes, into C.B-17-severe combined immunodeficiency (*scid*) mice. C.B-17-*scid* mice are homozygous for a mutation (*Prkdc^{scid}*) in the catalytic subunit of a DNA-dependent protein kinase (*Prkdc*), which functions in DNA double-strand break repair and V(D)J recombination in immunoglobulin and T-cell recep-

tors. Therefore, *scid* mice are radiosensitive and lack serum antibody and functional T and B cells [1-3]. Subsequently, nonobese diabetic (NOD)-*scid* mice, which lack hemolytic complement and show reduced macrophage function and natural killer (NK) cell activity [4], have been widely used as recipients in the xenograft assay for human lymphocytes [5] and HSCs from human umbilical cord blood (CB) [6-8], bone marrow (BM) [9,10], mobilized peripheral blood (PB) [11,12], and fetal liver [13,14]. One major problem with this strain of mice is the low level of engraftment except when large numbers of cells are injected. This is probably caused by the presence of residual NK cell cytotoxic activity [15]. Furthermore, NOD-*scid* mice become leaky and generate mature lymphocytes with age [4]. NOD-*scid*/β2-microglobulin *(B2m)*^{null} mice have been reported to have further reduced NK cell cytotoxic activity and to support higher levels of human cell engraftment than NOD-*scid* mice [15]. We previously reported that the use of conditioned newborn NOD-*scid/B2m*^{null} mice allows measurement of human hematopoietic engraftment up to 4 months after transplantation [16,17]. Longer-term experimentation with this strain of mice, however, is limited by their short life span, which is caused by the spontaneous development of lymphomas [15,18]. In addition, this strain of mice is highly radiosensitive and difficult to breed. Another immune-incompetent mouse model, NOD-recombination activating gene *(Rag1)*^{null} mice, which are radiation resistant and totally lack mature T and B cells because of a complete inability to initiate V(D)J recombination, seem to provide longer-term human cell engraftment and are easier to breed than NOD-*scid* mice [19]. Genetic crossing of perforin (*Prf1*) gene–targeted mutation onto a NOD-*Rag1*^{null} strain results in the absence of NK cell cytotoxic function. In these mice, NK cells are not capable of killing target cells because of the absence of perforin, the major mediator of cytotoxic activity [20]. To test the use of NOD-*Rag1*^{null}*Prf1*^{null} mice as recipients in a long-term xenograft assay for human HSCs, we adopted Yoder and colleagues' [21] method of conditioning newborn mice, with minor modifications. In this article, we describe an assay that allows high-level multilineage hematopoietic engraftment for longer than 6 months after transplantation of human CB cells.

Recently, several studies have indicated that HSCs in the BM of adult mice have the ability to efflux the dye rhodamine 123 (Rho) [22-24]. This dye has an affinity for mitochondria, and its efflux is regulated by the multidrug-resistant pump MDR1-encoded adenosine triphosphate–binding cassette transporter [25]. It has been reported that transplantable HSCs in adult human BM have a CD34^{+}Rho^{low} phenotype [26] or a CD34^{+}c-kit^{+}Rho^{low} phenotype [27]. Muench et al. [28] also demonstrated an HSC phenotype in lineage^{-}CD34^{+}Rho^{low} human fetal liver cells. Regarding primitive hematopoietic cells in human CB, Liu and Verfaillie [29] reported that long-term culture-initiating cells were highly enriched in CD34^{+}CD33^{-}CD38^{-}c-kit^{+}Rho^{low} cells. Given that the efflux activity is an important feature of HSCs in CB, we first investigated Rho efflux capability of human CB transplantable HSCs by using transplantation into conditioned newborn NOD-*Rag1*^{null}*Prf1*^{null} mice. Only CB cells with a high Rho efflux capability were capable of sustaining hematopoiesis for 6 months.

MATERIALS AND METHODS

Mice

NOD/LtSz-*Rag1*^{null}*Prf1*^{null} mice were generated by Dr. Leonard D. Shultz (Jackson Laboratory, Bar Harbor, ME) [20] and have been bred and maintained at the Veterans Affairs Medical Center of the Medical University of South Carolina under defined flora in ventilated cages with irradiated food and filtered water.

Human Umbilical CB Cells

Human CB cells were obtained from LifeSouth Cord Blood Bank of the University of Florida and Medical College of Virginia Hospitals. Mononuclear cells (MNCs) were isolated from CB by density gradient centrifugation at 350*g* for 30 minutes by using Accupaque (Accurate Chemical and Scientific Corp., Westbury, NY). For depletion of T cells, MNCs were then incubated with mouse antibodies against human CD3, CD4, and CD8 (Caltag Laboratories, Burlingame, CA) for 30 minutes at 4°C. After washing, cells were incubated with sheep anti-mouse immunomagnetic beads (Dynabeads M-450 coupled to sheep anti-mouse immunoglobulin G; Dynal, Great Neck, NY) for 30 minutes at 4°C, and unbound cells were harvested. In experiments testing Rho uptake, MNCs were incubated with mouse antibodies against human CD2, CD3, CD4, CD5, CD7, and CD8 (Caltag Laboratories), and unbound cells were magnetically harvested by using Dynabeads.

Rho Staining

Cells were suspended at 10^7/mL in Dulbecco modified Eagle medium (DMEM) containing 2% fetal bovine serum and 1 mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (DMEM^{+}) and incubated with Rho (Molecular Probes, Eugene, OR) at 0.1 μg/mL for 30 minutes at 37°C, as previously described [30]. For inhibitor experiments, reserpine (Sigma, St. Louis, MO) was added to cells at a final concentration of 5 μmol/L. The cells were washed once with warm (37°C) Hanks balanced salt solution (HBSS) containing 2% fetal bovine serum and 1

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