Humanizing NOD/SCID/IL-2Rγnull (NSG) mice using busulfan and retro-orbital injection of umbilical cord blood-derived CD34+ cells

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Background

Humanized mouse models are still under development, and various protocols exist to improve human cell engraftment and function.

Methods

Fourteen NOD/SCID/IL-2Rγnull (NSG) mice (4–5 wk old) were conditioned with busulfan and injected with human umbilical cord blood (hUCB)-derived CD34+ hematopoietic stem cells (HSC) via retro-orbital sinuses. The bone marrow (BM), spleen, and peripheral blood (PB) were analyzed 8 and 12 weeks after HSC transplantation.

Results

Most of the NSG mice tolerated the regimen well. The percentage of hCD45+ and CD19+ cells rose significantly in a time-dependent manner. The median percentage of hCD45+ cells in the BM was 55.5% at week 8, and 67.2% at week 12. The median percentage of hCD45+ cells in the spleen at weeks 8 and 12 was 42% and 51%, respectively. The median percentage of hCD19+ cells in BM at weeks 8 and 12 was 21.5% and 39%, respectively (P = 0.04). Similarly, the median percentage of hCD19+ cells in the spleen at weeks 8 and 12 was 10% and 24%, respectively (P = 0.04). The percentage of hCD19+ B cells in PB was 23% at week 12. At week 8, hCD3+ T cells were barely detectable, while hCD7+ was detected in the BM and spleen. The percentage of hCD3+ T cells was 2–3% at week 12 in the BM, spleen, and PB of humanized NSG mice.

Conclusion

We adopted a simplified protocol for establishing humanized NSG mice. We observed a higher engraftment rate of human CD45+ cells than earlier studies without any significant toxicity. And human CD45+ cell engraftment at week 8 was comparable to that of week 12.

Key Words Humanized mice, Busulfan, Retro-orbital sinus, Hematopoietic stem cell

INTRODUCTION

Several human diseases do not have appropriate animal models [1], or the available animal models have significant differences from the human counterpart [1]. Non-human primates have been regarded as ideal models for translating basic research findings into clinical applications due to their similarities to humans [1, 2]. Recently, the European governing bodies and the United States National Institutes of Health have significantly reduced the use of chimpanzees in research due to cost and ethical concerns [3]. Humanized mice carrying human hematopoietic and immune systems are considered as ideal tools for studying hematopoiesis, infectious disease, and immunology [3]. For example, dengue and human immunodeficiency virus infect humanized mice, while they do not replicate in rodents [4]. Still, humanized mouse models need further development in order to more closely recapitulate human biological systems.

Humanized mouse models are still under development, and various protocols exist to improve human cell engraftment and function. The source of stem cells can vary depending on the research objectives. Usually, CD34+ cells yield long-term engraftment and are chosen to study hematopoietic stem cells. The available animal models have significant differences from the human counterpart [1]. Non-human primates have been regarded as ideal models for translating basic research findings into clinical applications due to their similarities to humans [1, 2]. Recently, the European governing bodies and the United States National Institutes of Health have significantly reduced the use of chimpanzees in research due to cost and ethical concerns [3]. Humanized mice carrying human hematopoietic and immune systems are considered as ideal tools for studying hematopoiesis, infectious disease, and immunology [3]. For example, dengue and human immunodeficiency virus infect humanized mice, while they do not replicate in rodents [4]. Still, humanized mouse models need further development in order to more closely recapitulate human biological systems.

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Peripheral blood mononuclear cells are used to study transplantation immunology [5, 6]. Total body irradiation (TBI) has been a standard conditioning regimen to achieve high levels of human cell engraftment in xenograft animal models [3, 7] because it triggers the secretion of stem cell factor (SCF), which is critical for hematopoietic stem cell engraftment, proliferation, and survival [7]. However, TBI is a tedious procedure to implement, because it requires strict regulation for the use of irradiators, remote location from animal housing, and special animal care [8]. Recently, researchers have used chemotherapeutic agents such as busulfan to induce similar hematopoietic effects as TBI [8-12]. The route of donor cell administration is another factor to consider for successful transplantation [13]. Donor cells can be administered via vein, liver, or bone [3]. The tail vein is the most frequently used intravascular access [13, 14], but it is technically challenging and often requires the heating of mice to enhance peripheral vasodilation [13]. The retro-orbital sinus is an alternative administration route that causes less pain and distress for animals [15, 16]. However, uncertainty exists about the time and duration of engraftment after human cell transplantation into the NOD/SCID/IL-2Rγnull (NSG) mice. Human CD45+ cells can be detected in the peripheral blood as early as 3 weeks after human HSC injection [8]. In other studies, high engraftment level was observed 22-24 weeks after human HSC transplantation [17, 18]. It was reported that NSG mice survived up to 300 days after human HSC transplantation [8].

In this study, young NSG mice (4-5 wk old) were conditioned with busulfan and injected with HSCs via their retro-orbital sinuses. They were maintained in pathogen-free sterile conditions, but without individualized ventilating cages (IVC). Most of the NSG mice did well after busulfan and HSC injection. We analyzed the bone marrow, spleen, and peripheral blood of NSG mice at weeks 8 and 12 after human HSC transplantation and examined human cell engraftment.

**MATERIALS AND METHODS**

**Mice**

NOD.Cg-Prkdcrenull IL2rγm1Wv/Sj (NOD-Scid IL2rγnull, NSG) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). For all experiments, mice were bred as a homozygous line and maintained under specific pathogen-free conditions at Laboratory of Animal Research Center in Korea Institute of Radiological Medical Sciences (Seoul, Korea). Experiments were conducted according to the guidelines for ethical use of animals of our institution under an approved protocol (KIRAMS 2015-0018).

**Isolation of human CD34+ cells from human umbilical cord blood**

Human umbilical cord blood (hUCB) was provided by the Seoul Metropolitan Government Public Cord Blood Bank (Allcord, Korea). Mononuclear cells (MNCs) were enriched using a RosetteSep Human Progenitor Enrichment kit (StemCell Technologies, Canada) and isolated from hUCB using Ficoll-Paque PREMIUM (GE Healthcare Bio-Sciences AB, Sweden) density gradient centrifugation. MNCs were enriched for hCD34+ cells using a human CD34 MicroBead Kit UltraPure (Miltenyi Biotec, Spain) according to the manufacturer’s instructions. The purity of hCD34+ cells was 87.6% as determined using a FACSCount II flow cytometer (BD Biosciences, USA), and these hCD34+ cells were preserved in STEM-CELLBANKER medium (Zenoaq, Japan) at -80°C until use.

**Transplantation of hCD34+ cells**

NSG mice were conditioned with busulfan (Korea Otsuka Pharmaceutical, Korea). Busulfan was dissolved in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO, USA) and diluted with 0.9% saline. The liquid busulfan solution was intraperitoneally (i.p.) injected into NSG mice (25 mg/kg body weight, 500-625 μg per dose) 48 and 24 hours prior to transplantation. The next day, 1×10^5 hCD34+ cells in 100 μL phosphate-buffered saline (PBS) were transplanted into the NSG mice via retro-orbital sinus injection. To prevent urinary tract infections, we used enrofloxacin (0.27 mg/mL) as a prophylactic antibiotic in drinking water of mice.

**Analysis of engraftment**

Eight and 12 weeks after transplantation, mice were sacrificed and mononuclear cells were isolated from bone marrow, spleen, and peripheral blood. Single-cell suspensions were prepared by standard procedures and were stained with the following antibodies: hCD34-fluorescein isothiocyanate (FITC), hCD45-allophycocyanin (APC) (Miltenyi Biotec, Spain), hCD3-fluorescein isothiocyanate (FITC), and hCD19-phycocerythrin (PE) (BD Biosciences, USA). Flow cytometry was performed using a FACSCount II (BD Biosciences, USA). Ten thousand to one million events were acquired per sample and analyzed with FACSCount software (BD Biosciences, USA). Cell lysates were prepared from bone marrow and spleen, and western blotting was performed by standard procedures using hCD45 and hCD7 antibodies (BD Biosciences, USA).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., CA) and SPSS (IBM Corp., USA) and P<0.05 was considered statistically significant.

**RESULTS**

**Reconstitution of human cells in NSG mice transplanted with hUCB-derived CD34+ cells**

Fourteen NSG mice received i.p. busulfan and retro-orbital hCD34+ cell injection, and most of them tolerated the regimen well (Fig. 1). However, 1 mouse showed decreased activity and hunched posture and died 20 days after hCD34+ cell injection (Fig. 2). We analyzed the bone marrow, spleen,
Humanization of NSG mice using busulfan

Fig. 1. Scheme for generating the humanized NSG mice. MNCs isolated from hUCB were enriched using a RosetteSep kit and human CD34 MicroBead Kit. NSG mice were conditioned by busulfan and CD34⁺ cells were injected via retro-orbital sinus.

Fig. 2. Survival and weight changes of the NSG mice after hCD34⁺ cell injection. Humanized NSG mice were monitored daily after transplantation. Most of the NSG mice did well, but one mice (depicted with an arrow) showed features suggesting GVHD (weight loss, hunched posture and diminished activity as shown in the photo) 10 days after transplantation.

and peripheral blood of the NSG mice at 8 and 12 weeks after transplantation and found that the percentage of human CD45⁺ cells rose significantly in a time-dependent manner (Fig. 3). The median percentages of human CD45⁺ cells in the bone marrow were 55.5% at week 8 and 67.2% at week 12. Similarly, the median percentages of human CD45⁺ cells...
in the spleen at weeks 8 and 12 were 42% and 51%, respectively.

**Reconstitution of human B- and T-cells in humanized NSG mice**

The percentage of hCD19⁺ cells rose significantly in a time-dependent manner (Fig. 4A). The median percentages of human CD19⁺ cells in bone marrow at weeks 8 and 12 were 21.5% and 39%, respectively (P=0.04). Similarly, the median percentages of human CD19⁺ cells in spleen at weeks 8 and 12 were 10% and 24%, respectively (P=0.04). The percentage of hCD19⁺ B-cells in peripheral blood was 23% at week 12. The percentage of human CD3⁺ T cells in the bone marrow, spleen, and peripheral blood of humanized NSG mice was 2-3% at week 12 (Fig. 4B). However, human CD3⁺ cells were barely detectable at week 8 (Fig. 4B). Therefore, we performed western blotting using anti-hCD7, a marker of early T cell lineage. Because of the limited sample volume that remained after hCD45, hCD19, and hCD3 analysis, samples from only 4 mice could be analyzed. We observed that hCD7⁺ cells were detectable in the bone marrow and spleen of all 4 mice at 8 weeks after hUCB-derived CD34⁺ cell injection (Fig. 5).

**DISCUSSION**

Humanized mice are gaining attention as animal models for translating basic research findings into clinical applications [3]. Currently, the NOD/SCID/IL-2Rγc null (NSG) mice, which lack T-, B-, and NK cell activity, are considered as ideal candidates to establish humanized mice [19]. Many preliminary reports have demonstrated the utility of humanized mice in infectious disease and immunology [3, 4]. Humanized mouse model protocols are still under development to improve human cell engraftment and function [3].

With a simplified protocol, we observed a higher engraftment rate of human CD45⁺ cells than earlier studies. The NSG mice were conditioned with busulfan, injected with

![Fig. 3](image3.png)

**Fig. 3.** Human cell reconstitution of NSG mice transplanted with hUCB-derived CD34⁺ cells. Levels of human CD45⁺ cells in mouse tissues at different times after transplantation are shown. Bone marrow, spleen, and blood were isolated from the humanized NSG mice and MNCs isolated from each organ were stained and analyzed. The percentages are represented as mean±SEM in humanized mice.

![Fig. 4](image4.png)

**Fig. 4.** Human CD19⁺ and CD3⁺ cell reconstitution from NSG mice injected with hUCB-derived CD34⁺ cells. The percentages are represented by mean±SEM in humanized mice.

![Fig. 5](image5.png)

**Fig. 5.** hCD45 and hCD7 expression levels in spleen (SPL) and bone marrow (BM) of NSG mice 8 weeks after hUCB-derived CD34⁺ cell injection.
hUCB-CD34+ cells via retro-orbital sinus, and housed without IVCs. Traditionally, total body irradiation (TBI) has been used as conditioning regimen [7]. However, the administration of TBI requires strict facility regulations and results in substantial mortality [8]. It is suggested that animals receiving TBI need to be maintained in strictly controlled pathogen-free conditions [8]. Busulfan (1,4-butanediol dimethanesulfonate) is an alkylating agent that has long been used in human HSC transplantation [20]. Because it has predominantly myelosuppressive and minimally immunosuppressive properties, recent studies suggested that busulfan induces similar hematopoietic effects to TBI while being easier and less expensive for animal transplantation models [9-12]. Some studies used the same busulfan dose and cell dose as our study [8, 10, 12, 17, 18]. The engraftment rate varied depending on the dose of busulfan, CD34+ cells and the time point of analysis [8, 10, 12, 17, 18]. Choi et al. conditioned NSG mice with busulfan and infused 1×10⁵ hUCB-derived CD34+ cells and reported that human CD45+ cells comprised 76% of bone marrow CD45+ cells at the 24th week [10]. At the 12th week, the percentage of human CD45+ cells in the NSG mice injected with 1×10⁵ hUCB-derived CD34+ cells after a single dose of busulfan conditioning (20 mg/kg) was 25.33% [17]. Our median percentages of human CD45+ cells in the bone marrow of NSG mice at 8 and 12 weeks after transplantation were 59.3% and 72.3%, respectively. We adopted split dose busulfan conditioning (2 doses of 20-30 mg/kg) and assumed that this might have contributed to a higher engraftment rate. Moreover, there were no infectious complications after HSC transplantation. Our NSG mice were maintained in less strict conditions and all mice did well after HSC transplantation.

In the current study, the percentage of human CD3+ cells was in the range of 2.3% at week 12 after transplantation. Hayakawa et al. reported that their percentage of human CD3+ cells in the peripheral blood of NSG mice was 0.6% at 8 weeks after transplantation [8]. Meanwhile, the percentage of T cells was 21.15% in the mesenteric lymph node at week 12 after HSC transplantation [17]. T cell engraftment in NSG mice gradually increases after HSC transplantation [17]. The percentages of human CD3+ cells in mouse bone marrow at weeks 12 and 22 after transplantation were 3.33% and 40.6%, respectively [17]. Still, it is uncertain to what extent the transplanted human cells could reconstitute a hematopoietic system in NSG mice. Singh et al. observed a prolonged human cell chimera over 300 days, with increasing CD3+ T cell levels [17]. On the other hand, limited engraftment of myeloid lineage cells, especially red blood cells, was reported [8, 21]. Humans and mice differ in the growth factors and cytokines required for the development of the hematopoietic and immune systems [22]. NSG mice lack the HLA molecule for human T cell education, and have poorly organized lymphoid architecture and deficiencies in lymph node development [23]. Various studies have attempted to increase reconstitution of human hematopoietic cells. Transgenic expression of IL-3, GM-CSF, and SCF increased the percentages of human myeloid cells in the bone marrow of NSG mice engrafted with human HSC [24]. The bone marrow, liver, thymus (BLT) model showed robust and consistent engraftment of multiple human hematopoietic lineages [23, 25, 26]. Cotransplantation of fetal bone tissue facilitated the development and reconstitution of human B cells in humanized NSG mice [11]. A recent study reported that intrahepatic injection of human UCB-derived CD34+ cells can facilitate human T cell development in livers of humanized NSG mice [27]. Administration of recombinant human IL-7 also improved T cell development in humanized mice [22, 28]. To improve human hematopoietic engraftment and myeloid differentiation, new generations of immunodeficient mouse strains that express human hematopoietic growth factors are under development [24, 29].

In conclusion, we observed a high rate of hUCB-derived CD34+ cell engraftment in NSG mice using a simplified protocol. The recipient NSG mice were conditioned with busulfan, injected with CD34+ cells via retro-orbital sinus, and maintained without IVCs. Most of them tolerated the regimen, and human cell engraftment after 8 weeks was comparable to the 12th week. Further studies are necessary to increase engraftment rate and function of human hematopoietic cells.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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