Long-Term Human Hematopoiesis in SCID-hu Mice Bearing Transplanted Fragments of Adult Bone and Bone Marrow Cells

By Yuji Heike, Tatsuo Ohira, Minako Takahashi, and Nagahiro Saijo

An attempt was made to establish an SCID-hu murine model of long-term human hematopoiesis by coimplantation of a bone fragment and bone marrow (BM) cells from an adult human. The SCID-hu mice were treated with a cytokine mixture (recombinant human stem cell factor, interleukin-3, granulocyte/macrophage colony-stimulating factor, and granulocyte colony-stimulating factor) for 4 months and were then maintained for further 8 months under cytokine-free conditions. In the peripheral blood, spleen, and implanted bone fragments in the SCID-hu mice that had received both a bone fragment and BM cells, human CD59+ cells were detected 1 year after transplantation; however, they were not detectable in SCID-hu mice that had received either a bone fragment or BM cells only. Thus, implantation of both a bone fragment and BM cells appears to provide a model of long-term adult human hematopoiesis in SCID mice.

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delivering a cytokine mixture of rhSCF (1 μg/mouse/d), rhIL-3 (1 μg/mouse/d), rhGM-CSF (1 μg/mouse/d), and rhG-CSF (1 μg/mouse/d) in saline plus 1% SCID serum was implanted beside the bone fragment at the same time. Twenty days after bone implantation, BM cells (1 to 5 × 10^7 cells/mouse) from the same donor were infused into the SCID mice through the tail vein. Further treatment with the cytokine mixture was performed by subcutaneous injection every 3 days for 4 weeks and then every 2 days for a further 12 weeks, followed by 8 months of maintenance under cytokine-free conditions. We designated the SCID-hu mice cotransplanted with a human bone fragment and BM cells “BT/BMT-SCID.”

As controls, SCID mice transplanted with only a bone fragment (BT-SCID) and SCID mice transplanted with only BM cells (BMT-SCID), which were administered the same cytokine treatments, were prepared.

**Immunofluorescence of PB cells of SCID-hu mice.** Mononuclear cell concentration in the PB of SCID-hu mice was evaluated by separation in Lymphoprep (Nycomed AS, Oslo, Norway) by centrifugation at 1,800 rpm for 20 minutes. After 2 washings with phosphate-buffered saline (PBS), the cell pellets were resuspended in 50 μL of PBS containing fluorescein isothiocyanate (FITC)-conjugated anti-CD59 rat IgG2b (Serotec, Oxford, England) at a dilution of 1:50 for 30 minutes at 4°C. The cells were washed twice with PBS and were resuspended in PBS. In each experiment, FITC-labeled isotype-matched antibodies were included as negative controls. Flow cytometry was performed using a FACScan system (Becton Dickinson & Co, Mountain View, CA).

The percentage of CD59⁻ cells was calculated as follows: % of CD59⁻ cells = (% of FL-1 bright cells with anti-CD59 monoclonal antibody [MoAb]) – (% of FL-1 bright cells with control MoAb).

**Detection of the human CD59 gene in spleen cells.** Detection of the CD59 gene in the spleen cells of SCID-hu mice 1 year after transplantation was performed by the polymerase chain reaction (PCR) protocol. Genomic DNA of SCID-hu spleen cells was collected by the standard protocol described previously.²⁴ Oligonucleotide primers were synthesized by the phosphoramidite method using an automated DNA synthesizer (Model 391 PCR-mate EP; Applied Biosystems, Foster City, CA) and purified on oligonucleotide purification cartridge (OPC) columns (Applied Biosystems). For amplification of human CD59 DNA, a CD-59/1 sense primer, ⁵’-TGG-GTAAAGTAGGGTTGGAG-3’, and a CD59/2 antisense primer, ⁵’-AGCTTATGTTGACAGGAGTG-3’, were used. The predicted amplification product was the 984-bp CD59, exon 2 DNA sequence. For amplification of mouse H-2K² DNA, a H-2K²/1 sense primer, ⁵’-ATCCAGTCTCTCCTGATCCCT-3’, and a H-2K²/2 antisense primer, ⁵’-ACCACCACAGATGCCCACCT-3’, were used. The predicted amplification product was the 1003-bp H-2K² sequence. One hundred micrograms of genomic DNA was added to PCR solution buffer (10 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl, and 1.5 mmol/L MgCl₂, containing each dNTP at 125 mmol/L and each primer at 0.5 mmol/L, in a final volume of 20 μL). After adding 2.5 U of Taq DNA polymerase (Perkin Elmer-Cetus, Branchburg, NJ), the samples were overlaid with 100 μL of mineral oil, denatured for 1 minute at 94 °C, and subjected to 28 cycles of PCR (at 92 °C for 40 seconds, at 60 °C for 40 seconds, and at 75 °C for 90 seconds) in

![Fig 1](http://www.bloodjournal.org) Flow cytometry analysis of PB cells from SCID-hu mice. (A) The data for forward scatter (FSC) and side scatter (SSC) of PB cells from SCID-hu mice are presented. PB cells of human (A-1) and SCID mice (A-2) were used as controls (B).
Fig 2. The expression of human CD59 antigens on PB cells of SCID-hu mice. The cells in gate 1 were CD59 antigen-positive like human cells.

Histologic examination of transplanted bone fragments. Conventional histologic sections (5-μm thick) were cut from formalin-fixed, paraffin-embedded materials. The sections were mounted on poly-L-lysine-coated glass slides and were air-dried overnight at room temperature. Histologic examination was performed by standard light microscopic evaluation of sections stained with hematoxylin and eosin.

Detection of human CD59 gene in bone xenografts. DNA of the cells in bone xenografts 1 year after bone implantation was eluted from paraffin-embedded samples (see Fig 4C) as described previously.35-36 For amplification of human CD59 DNA, a CD-59/1 sense primer 5’-CTTTCCTTCTTACGCT-3’, and a CD59/2 antisense primer, 5’-AATGGGACACTGGCTTT-3’, were used. The predicted amplification product was the 121 bp of human CD59, exon 2 DNA sequence. The samples were overlaid with 100 μL of mineral oil, denatured for 1 minute at 95°C, and subjected to cycles of PCR (at 95°C for 1 minute, at 55°C for 1 minute, and at 72°C for 2 minutes) in a Thermal Cycler. A last single cycle of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 7 minutes was performed. The amplification products were analyzed by gel electrophoresis on 10% polyacrylamide gel (Daiichi-kagaku, Tokyo, Japan) and stained with ethidium bromide.

RESULTS

The intensities of forward scatter and side scatter of PB cells from SCID-hu mice. Figure 1 showed the intensities of forward scatter versus side scatter of the PB cells from SCID-hu mice. Human PB cells had a typical population, indicated by the ellipse in gate 1 (Fig 1A), but SCID mice did not have such a cell population (Fig 1B). PB samples from all SCID-hu mice, BT-SCID, BMT-SCID, and BT/BMT SCID, 4 months after transplantation showed a cell population resembling that of human cells. However, this population disappeared with time after termination of the

Table 1. Percentages of CD59+ Cells in SCID-hu Mice

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<th>4 mo</th>
<th>8 mo</th>
<th>12 mo</th>
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<tr>
<td>BT-SCID</td>
<td>13.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BMT-SCID</td>
<td>5.7</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>BT/BMT SCID</td>
<td>15.2</td>
<td>11.8</td>
<td>6.1</td>
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We calculated the percentage of CD59+ cells by subtracting the percentage of FL-1 bright positive cells shown by FITC-labeled anti-CD59 MoAb from that shown with the control MoAb.
HUMAN HEMATOPOIESIS IN SCID-HU MICE

Fig 3. Detection of CD59 gene-positive cells in SCID-hu mice 12 months after transplantation using the PCR. (A), Human control; (B), SCID-mouse control; (C), BT-SCID; (D), BMT-SCID; and (E), BT/BMT-SCID. DNA from human PB cells as a positive control and DNA from the spleen of BT/BMT SCID showed the CD59 exon 2 gene.

cytokine injections and was undetectable 8 months after BT in BT-SCID and 12 months after BMT in BMT-SCID (Fig 1C). Only BT/BMT SCID contained the human cell population 1 year after transplantation.

Detection of human CD59+ cells in PB cells and human CD59 gene of spleen cells from SCID-hu mice. CD59+ cells in PB cells from SCID-hu mice were detected by flow cytometry analysis. Clearly, the particular cell population indicated in gate 1 in Fig 1A was brightly positive for CD59 expression, as shown in Fig 2. Table 1 shows the percentages of CD59+ cells calculated using the formula described in Materials and Methods. The proportion of CD59+ cells in BT-SCID was 13.7% at 4 months and 20 days after bone transplantation, but none could be found at 8 months after transplantation. In BMT-SCID, although CD59+ cells constituted 5.0% of the population 8 months after BM transplantation, none were found at 12 months after transplantation. In BT/BMT SCID, CD59+ cells were present at 15.2%, 11.8%, and 6.1% 4, 8, and 12 months after bone transplantation, respectively. The percentage of the particular cell population indicated in gate 1 paralleled the percentage of CD59+ cells (Fig 1 and Table 1). PCR examination showed that human CD59 exon 2 DNA was positive in the spleen cells of BT/BMT SCID 12 months after transplantation but was negative in those of BT-SCID and BMT-SCID (Fig 3).

Histologic findings of transplanted bone fragments and detection of human CD59 gene. Figure 4 shows histologic sections of bone grafts in BT/BMT SCID. Hematopoietic cells were found in the BM of the grafts 4 months after bone transplantation (Fig 4A). However, at 1 year after bone transplantation, necrotic and/or fibrous changes were found in the BM of the xenograft, where no clear foci of hematopoietic cells were evident (Fig 4B). Hematopoietic foci were observed only in the area facing the osmotic pump (Fig 4C). PCR examination also confirmed the presence of human CD59 gene-positive cells in this area of the bone xenograft (Fig 5).

DISCUSSION

In this study, we aimed at establishing SCID-hu murine model of long-term human hematopoiesis by coimplantation of a bone fragment and BM cells from an adult human.

Figures 1 and 2 showed the existence of the typical cell population resembling that of human cells in PB from BT/BMT SCID 1 year after BMT. In previous studies, human hematopoietic cells in animal models were distinguished from those of the recipient animals by immunocytochemical analysis using MoAbs against surface markers or by fluorescence in situ hybridization. In our experiment, we used a FITC-conjugated anti-CD59 MoAb to distinguish human-derived cells form mouse-derived cells. CD59 antigen was expressed on many hematopoietic and nonhematopoietic cells from humans. The particular cell population indicated in gate 1 in Fig 1A was clearly bright positive for CD59 expression (Fig 2). Human CD59+ cells in BT/BMT SCID 1 year after bone transplantation were present at 6.1%. PCR examination showed that human CD59 exon 2 DNA was positive in the spleen cells of BT/BMT SCID 1 year after transplantation (Fig 3). These data suggested that normal human hematopoietic cells were present in the PB and in the spleens from BT/BMT-SCID. Some previous reports have indicated that cytomegalovirus-transformed human cells can survive in SCID-hu mice for a long time. The spleen cells of all CD59+ human cells used in our experiment were negative for cytomegalovirus infection in PCR examinations (data not shown).

It was expected that mature human-derived blood cells might indicate a xenograft reaction in SCID mice. A graft-versus-host reaction in SCID-hu mice has been observed by some but not all groups. In our models, no evidence of graft-versus-host reaction was observed.

The BT-SCID and BT/BMT-SCID showed loss of hair around the bone xenograft, whereas the hair of BMT-SCID was not affected. The loss of hair in BT-SCID mice recovered after termination of the cytokine treatment, but that in BT/BMT-SCID continued for over 1 year. The bone xenograft in BT-SCID was forced out from the skin, but that of BT/BMT-SCID was not. The reason for this phenomenon is now being investigated.

Angiogenesis into the implanted bone fragment was found in BT/BMT-SCID but not in BT-SCID. The angiogenesis was observed in the area that faced the cytokine-delivering osmotic pump. We examined this area in the samples histologically after staining them with hematoxylin-eosin.
topoietic cells were found in the area facing the osmotic pump 1 year after bone transplantation (Fig 4C). The cells in this field responded to optical rhGM-CSF stimulation to form GM-CSF colonies (3 colonies were detected; data not shown). Because rhGM-CSF showed no cross-reactivity with mouse GM-CSF, the GM colony formation response to human GM-CSF indicated that human hematopoietic cells were present in the bone xenograft. PCR examination also confirmed the presence of human CD59 gene-positive cells in this area of the bone xenograft (Fig 5). Because mouse-derived cells were human CD59−, these results indicated the existence of human cells in the implanted bone fragment.

These findings indicated that transplantation of both a human bone fragment and BM cells with long-term cytokine treatment could provide a stromal microenvironment suitable for human hematopoietic progenitor cells and maintain it for a long time. In our study, because we did not have a sufficiently large sample for determining the maturation of human cells in the bone xenograft, it was not clear whether pluripotent stem cells were present in the implanted bone fragment. The differentiation of human-derived PB cell into lymphoid, erythroid, myelomonocytic, and megakaryocytic cells was not clear, because antibodies against human CD4, CD8, and CD3 were cross-reactive with mouse spleen cells in our study.

SCID-hu mice have already been used as models of leukemia, lymphoma, acquired immunodeficiency syndrome, and some autoimmune diseases.1-15 Our aim is to study the further application of SCID-hu models for in vivo evaluation of immunotherapy, especially immunogene therapy. Some reports have suggested the usefulness of huPBL-SCID mice as the models of immunotherapy.16-24 However, because the percentage of human cells in SCID-hu mice was very low, we could not use this model as a tool for investigating immunotherapy. Total body irradiation or treatment with antiasialo GM1 antibody could augment the transplantability of human cells in SCID-hu mice. Therefore, we are planning to transplant a human bone fragment and BM cells into SCID mice treated with antiasialo GM1 antibody to increase the transplantability of human cells.

Another problem is that other human lymphoid organs such as lymph nodes can not be implanted in our SCID-hu model mice. For a normal immune response against cancer, implantation of these organs might be necessary. We have already confirmed that a high degree of transplantability of lung cancer cells into SCID mice can be obtained. At the present stage, although there are still some problems to be solved, long-term adult human hematopoiesis in SCID-hu mice achieved by cotransplantation of both a bone fragment and BM cells in conjugation with long-term cytokine treatment is thought to be an important first step for producing a useful model of immunotherapy.

**Fig 5. Detection of CD59 gene in bone grafts from BT/BMT SCID-hu mice 4 months and 1 year after bone transplantation using the PCR.** DNA of the cells in the bone xenograft in the BT/BMT SCID was purified from the paraffin-embedded samples in Figs 4A and C by the methods reported previously. (A), SCID mouse control; (B), BT/BMT SCID (4 months after BT); (C), BT/BMT SCID (1 year after BT); and (D), human control.
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