Dynamic tracking of human hematopoietic stem cell engraftment using in vivo bioluminescence imaging

Xiuli Wang, Michael Rosol, Shundi Ge, Denise Peterson, George McNamara, Harvey Pollack, Donald B. Kohn, Marvin D. Nelson, and Gay M. Crooks

The standard approach to assess hematopoietic stem cell (HSC) engraftment in experimental bone marrow transplantation models relies on detection of donor hematopoietic cells in host bone marrow following death; this approach provides data from only a single time point after transplantation for each animal. In vivo bioluminescence imaging was therefore explored as a method to gain a dynamic, longitudinal profile of human HSC engraftment in a living xenogeneic model. Luciferase expression using a lentiviral vector allowed detection of distinctly different patterns of engraftment kinetics from human CD34+ and CD34+CD38− populations in the marrow NOD/SCID/μ2mnull mice. Imaging showed an early peak (day 13) of engraftment from CD34+ cells followed by a rapid decline in signal. Engraftment from the more primitive CD34+CD38− population was relatively delayed but by day 36 increased to significantly higher levels than those from CD34+ cells (P < .05). Signal intensity from CD34+CD38−-engrafted mice continued to increase during more than 100 days of analysis. Flow cytometry analysis of bone marrow from mice after death demonstrated that levels of 1% donor cell engraftment could be readily detected by bioluminescence imaging; higher engraftment levels corresponded to higher image signal intensity. In vivo bioluminescence imaging provides a novel method to track the dynamics of engraftment of human HSC and progenitors in vivo.

Introduction

Key aspects of the biology of hematopoietic stem cells (HSCs) have been studied for many years using elegant murine models of bone marrow transplantation. More recently, immunodeficient murine models have been used successfully to study human HSCs. In all of these models, HSCs are infused into recipient animals, and donor engraftment is determined by analysis (usually flow cytometry) of bone marrow harvested after recipient animals are killed. This approach, although able to accurately measure the levels of engraftment and the contribution of donor HSCs to different hematopoietic lineages, has 2 main disadvantages. First, sampling limitations may occur by harvesting only certain parts of the marrow space. Thus, the anatomic distribution of donor engraftment is assumed to be equivalent within and between experimental animals. Second, engraftment is measured at a single time point (after death) and so the kinetics of engraftment within an individual animal are unknown.

Bioluminescence imaging is a technique that detects visible light released when the enzyme luciferase reacts with its substrate, luciferin. The development of a method to detect luciferase expression in vivo has facilitated the study of infectious disease, and tumor growth and response to therapy in small animal models. Lymphocyte trafficking into joints and the central nervous system (CNS) has also been demonstrated in vivo. We now show that by accomplishing high efficiency, stable transduction, and expression of luciferase in HSCs, bioluminescence imaging can reveal novel, dynamic information on the pattern of human HSC and progenitor engraftment and proliferation within the marrow space of living hosts.

Materials and methods

Isolation of human HSCs

Umbilical cord blood (CB) was obtained from normal deliveries under protocols approved by the Committee on Clinic Investigations of Childrens Hospital Los Angeles institutional review board. CD34+ cells were isolated from mononuclear cells to 80% to 90% purity with a magnetic-activated cell sorting (MACS) CD34 enrichment column (Miltenyi Biotec, Oberlin, CA). CD34+CD38− cells were isolated using a fluorescence-activated cell sorter (FACS Vantage; Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) from CD34+ enriched populations following fluorescence-labeled antibody staining with CD34-fluorescein isothiocyanate (FITC, BDIS) and CD38-phycocerythrin (PE; leu-17; BDIS), as previously described.

Lentiviral vector expressing the luciferase reporter gene

SMPU-R-MNCU3-LUC is a lentiviral vector based on HIV-1 that transduces the firefly luciferase gene. The backbone vector SMPU-R has
deletions of the enhancers and promoters of the HIV-1 long terminal repeat (LTR; SIN), has minimal HIV-1 gag sequences, contains the ePT/CFTS sequence from HIV-1, has 3 copies of the UES polyadenylation enhancement element from SV40, and a minimal HIV-1 BRE (gift from Paula Cannon, Childrens Hospital Los Angeles). The vector has the U3 region from the MND retroviral vector as an internal promoter driving expression of the firefly luciferase gene from SP-LUC+ (Promega, Madison, WI).

Transduction of HSCs and progenitors with luciferase vector

CD34+ and CD34+/CD38− cells were transduced in fibronectin-coated plates with virus supernatant containing SMPU-R-MNCU3-LUC vector in the presence of interleukin 3 (IL-3), IL-6, and stem cell factor (SCF), as described. A second cycle of transduction was performed 8 hours later by removing old medium and adding new virus supernatant and medium. Twenty-four hours after the initial transduction, cells were thoroughly washed 3 times with phosphate-buffered saline (PBS) before transplantation or in vitro analysis.

Detection of luciferase expression in CD34+ cells by immunohistochemistry

An aliquot of transduced CD34+ cells was cultured for 3 days in IL-3, IL-6, and SCF, after which cells were harvested and prepared on cytopsin slides. Slides were stained with monoclonal antiluciferase antibody (Novus, Littleton, CO) 1:100 for 1 hour, followed by donkey polyclonal antibody to mouse IgG-FITC (Novus) 1:100 for 30 minutes. The slides were mounted with Vectashield medium with DAPI (4',6-diamidino-2-phenylindole; Vector Laboratory, Burlingame, CA). Cultured nontransduced CD34+ cells were used as negative controls.

HSC and progenitor transplantation and bioluminescent imaging

Eight- to 10-week old NOD/SCID/β2mnull mice, obtained from Jackson Laboratories (Bar Harbor, ME), were sublethally irradiated with 300 cGy (Promega) at a dose of 125 mg/kg, as described. General anesthesia was then induced with 5% isoflurane and the mouse was placed in the light-tight heated chamber; anesthesia was continued during the procedure with 2% isoflurane introduced via nose cone. The imaging system consists of a cooled, back-thinned charge-coupled device (CCD) camera to capture both a visible light photograph of the animal taken with light-emitting diodes and the luminescent image, and a rotating mirror and translatable animal stage that allow for images to be acquired over 360° if desired.

Flow cytometry detection of engraftment

At the end of each experiment, mice were killed by inhalation of a mixture of 75% CO2/25% O2 and marrow was harvested from different parts of the skeleton. Direct immunofluorescence staining was performed with antibody against human leukocyte common antigen CD45 (BDIS). Cells were analyzed by FACS Calibur (BDIS).

Statistical analysis

Data were presented as mean ± SD. The unpaired t test was used for comparisons between groups at each time point. P < .05 was considered significant.

Results

Expression of luciferase transgene in HSCs and progenitor cells

Luciferase expression in HSCs and progenitor cells was accomplished using a lentiviral (HIV-1)-based vector. The proportion of CD34+ cells expressing the luciferase transgene was assessed in vitro by analyzing an aliquot of transduced cells after 3 days of culture. Using immunohistochemical detection of luciferase protein, high-efficiency gene expression was noted (Figure 1; Table 1).

Time course of signal increase and decay after luciferin administration

The time course of peak and decay of the bioluminescent signal after each administration of the luciferin substrate is an important consideration in quantitative and comparative studies. We therefore studied the signal kinetics using luciferase-expressing human CD34+ cells in our immunodeficient mouse model. Mice that received transplants of luciferase-expressing CD34+ cells 2 weeks earlier were given intravenous or intraperitoneal injections of luciferin. Images of the animals were acquired continuously beginning immediately after injection, each with a 3-minute exposure. Peak signals were detected within 5 minutes after intravenous injection and then decreased rapidly over the next few minutes (data not shown). These kinetics are unlike many previous animal models using luciferase in which time to both peak signal and decay are more delayed. The route of injection of luciferin

<table>
<thead>
<tr>
<th>No. of luciferase-positive cells</th>
<th>Total no. of cells</th>
<th>Luciferase-positive cells, %</th>
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<tbody>
<tr>
<td>Nontransduced cells</td>
<td>6</td>
<td>75</td>
</tr>
<tr>
<td>Transduced CD34+ cells</td>
<td>109</td>
<td>123</td>
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(intravenous or intraperitoneal) produced no significant differences in signal kinetics. Therefore, to standardize the measurement of signal in this model, all images in subsequent experiments were acquired 2 minutes after intravenous injection of luciferin.

**Bioluminescence imaging of engraftment patterns of human progenitor and HSC populations in immunodeficient mice**

The pattern of short- and long-term human HSC and progenitor engraftment was assessed with longitudinal imaging studies in immunodeficient (NOD/SCID/β2mnull) mice. Mice were sublethally irradiated 2 hours before intravenous infusion with transduced human CD34+ cells or the more primitive CD34+CD38− subpopulation believed to contain HSCs (Figure 2).

Serial images were acquired over the course of 7 to 15 weeks after transplantation at intervals of 2 to 7 days, beginning 1 to 8 days after cell infusion. Luciferin was injected intravenously no more than 2 minutes before each imaging run. Mice that had not received human cells were used as negative controls to assess background luminescence; no detectable signal was apparent either before or after luciferin injection in the negative controls.

In mice that received CD34+ transplants, human cells could be detected by bioluminescence imaging as early as 24 hours after transplantation. Whole body signal measurements over the course of the experiment showed a sudden increase in signal 2 to 3 weeks after injection of CD34+ cells, after which time the signal rapidly decreased. Low signal levels were detected beyond 100 days after transplantation (Figure 3A).

A different pattern of engraftment kinetics was seen consistently when CD34+CD38− cells were transplanted (Figure 3B-C). Signals were still detectable early, but were initially at lower levels than in the CD34+ transplants. Luminescence signals began to rise during the second week after transplantation and continued to increase in mice that received CD34+CD38− transplants throughout the period of study (108 days). Quantification of signal intensity from mice that received transplants of CD34+ cells (n = 5) and mice that received CD34+CD38− cells (n = 3) showed a highly reproducible pattern of engraftment kinetics for each population (Figure 4). From day 36, the signal in mice that received CD34+CD38− cells was significantly higher than that in mice that received CD34+ cells (P < .05).

**Anatomic distribution of bioluminescent signals in the murine skeleton**

Throughout the course of imaging, the strongest signals appeared in the vertebral column, pelvis, and femur; lower signals were detected in the skull and humeroscapula (Figure 5A).

At the end of the experiments, mice were killed and the percentages and total numbers of human cells in each part of the skeleton were quantified by harvesting all the bone marrow from each area separately and measuring the presence of cells that express the pan-leukocyte human-specific marker CD45 (Figure 5B-C). Consistent with the pattern of distribution of the luminescence signal, the highest percentage of human CD45+ cells was found in marrow harvested from the spine, femur, and pelvis. Engraftment levels as low as 1.3% as detected by flow cytometry corresponded to readily detectable signals by bioluminescence imaging. The higher bioluminescent signal in the mice that received CD34+CD38− transplants corresponded to higher levels of engraftment based on FACS analysis. These results demonstrate the ability to detect low levels of engraftment of human HSCs and progenitors and to quantitate different levels of engraftment using bioluminescence imaging.
transplants of luciferase-transduced human CD34

fraction of photon

animals that received CD34

Summary of data from 5 animals that received transplants of CD34

substances, are not as sensitive as bioluminescence imaging, and

3-dimensional information and can also be applied both in small

and magnetic resonance imaging (MRI), bioluminescence imaging

photon (PET), single photon emission computed tomography (SPECT),

ence imaging to detect marrow engraftment of HSCs. Compared

the light emitted by the firefly luciferase-luciferin reaction has a

peak wavelength at about 560 nm, because a significant portion of

its light emission occurs above 600 nm, a wavelength where light

more readily passes through tissue. Light-emitting reporters that

emit at longer wavelengths (in the red and near-infrared range) may

provide advantages in the future in terms of tissue penetration of

their signal, but they have not yet been well studied in vivo.

The limitations of light propagation through tissue currently

restrict the application of bioluminescence imaging to small

animals where the signal can easily penetrate at all depths.

High-resolution MRI appears to be another useful way to track

cells in vivo, especially in humans, and can provide the added

component of 3-dimensional information. However, MRI requires

high loading of cells with magnetic label and comparatively long

imaging times, and is not as sensitive or quantifiable as biolumines-

cence imaging. Although PET and SPECT imaging provide 3-di-

mensional information and can also be applied both in small

animals as well as in humans, they require the use of radioactive

substances, are not as sensitive as bioluminescence imaging, and

generally demand longer imaging times. In the case of PET,

expensive cyclotron facilities are also required. The development

of multiple reporter systems that combine optical and radionuclide

probes will enable multimodality imaging and provide the dual

benefits of high sensitivity and precise anatomic localization.

In the studies reported here, the bioluminescence model pos-
sessed the sensitivity to detect and quantify different levels of

chimerism of human donor cells within the marrow space, provid-
ing a dynamic profile of engraftment and proliferation in live

recipient animals. The technique was able to demonstrate the

biologic differences between the cell populations transplanted. The

increase in luminescence signal at weeks 2 to 3 after transplantation

with CD34+ cells is consistent with engraftment and expansion

from short-term repopulating progenitors. The subsequent lower

but stable signal is consistent with stable engraftment of a small

fraction of long-term repopulating cells within the CD34+

population. These findings are compatible with studies by Glimm et al.,

who demonstrated that the NOD/SCID/β2mnull model supports 2

distinct populations of engrafting human cells. Within the CD34+

population, short-term repopulation (3 weeks) is derived mainly

from CD34+CD38− cells, whereas 90% of long-term repopulation

is from the more rare and primitive CD34+CD38− cells. Other

investigators have demonstrated that all long-term engraftment in a

similar immunodeficient mouse model, the NOD/SCID mouse, is

generated by CD34+CD38− cells.

Consistent with these studies, bioluminescence imaging showed

that late-term engraftment predominated when purified

CD34+CD38− cells were transplanted. These strikingly different

kinetics of engraftment between CD34+ and CD34+CD38− cells

were consistently seen in all animals studied and demonstrate the

ability of bioluminescence imaging to detect populations with

intrinsically different engraftment profiles in vivo.

Thus, by combining 2 novel tools, lentiviral vectors to effi-
ciently and stably express luciferase in human HSCs and in vivo

bioluminescence imaging, a dynamic profile of progenitor and

HSC engraftment is now possible. All areas of the marrow can be

simultaneously assessed and changes in donor engraftment and

proliferation over time in individual animals can be appreciated.

This approach will have great utility in studies of homing and

migration of different stem and progenitor cells and will be

particularly useful when studying cell populations for which the

timing and distribution of engraftment are unknown.

Discussion

These studies are the first to describe the use of in vivo biolumi-

nescence imaging to detect marrow engraftment of HSCs. Compared

with other imaging modalities such as positron emission tomo-

graphy (PET), single photon emission computed tomography (SPECT),

and magnetic resonance imaging (MRI), bioluminescence imaging

offers higher sensitivity, ease of use, and high throughput for

imaging of small animals. This high sensitivity holds even though

the light emitted by the firefly luciferase-luciferin reaction has a

peak wavelength at about 560 nm, because a significant portion of

its light emission occurs above 600 nm, a wavelength where light

more readily passes through tissue. Light-emitting reporters that

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Stanford University, for initial helpful discussions.

Figure 5. Anatomic distribution of human cell engraftment. Mice receiving transplants of CD34+ and CD34+CD38− cells were scanned 9 weeks after transplantation (A) and then killed. Bone marrow cells were harvested from each region of the skeleton and analyzed by FACS for percentage (B) and total number (C) of donor human CD45+ cells. Scap indicates scapula; CD34+; and CD34+CD38−.


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