Cytokine-Induced Osteopoietic Differentiation of Transplanted Marrow Cells

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Abstract

Transplantation of whole bone marrow (BMT) leads to engraftment of osteoprogenitors as well as hematopoietic cells; however, the robust osteopoietic chimerism seen early after BMT decreases with time. Using our established murine model, we demonstrate that a post-BMT regimen of either granulocyte-colony stimulating factor, growth hormone, parathyroid hormone or stem cell factor each stimulates greater donor osteoblast chimerism at 4 months post-transplantation than saline-treated controls and approximates the robust osteopoietic chimerism seen early after BMT. However, only growth hormone led to significantly more donor-derived osteocytes compared to controls. Importantly, there were no adverse hematologic consequences of the different treatments. Our data demonstrate that these cytokines can stimulate the differentiation of transplanted donor marrow cells into the osteopoietic lineage after BMT. Post-BMT cytokine therapy may generate durable osteopoietic engraftment which should lead to sustained clinical benefit and render BMT more applicable to bone disorders.
Introduction

Both hematopoietic and osteopoietic progenitors reside within bone marrow and engraft in recipients after bone marrow transplantation (BMT).\textsuperscript{1-4} In principle, BMT should be effective therapy for disorders of osteoblasts as well as hematopoiesis. We tested this notion by undertaking the first clinical trial of BMT for a genetic disorder of bone formation, severe osteogenesis imperfecta (OI). We demonstrated donor-derived osteoblasts which was associated with marked clinical benefits,\textsuperscript{5,6} findings recently corroborated in an animal model of BMT for OI.\textsuperscript{3} Follow-up of our patients, however, revealed that the rate of improvement slowed after the first 6 to 12 months post BMT.\textsuperscript{6}

Studies in mice have shown that BMT leads to robust donor-derived osteopoiesis early after transplantation but the osteopoietic chimerism declines to negligible levels between 6 and 12 months after BMT,\textsuperscript{7} a time course that closely correlates with the observed slowing of clinical improvement. These data suggest that the underlying explanation for the impermanent rate of improvement in OI patients is transient donor osteopoietic engraftment. A durable osteopoietic graft, then, should lead to substantially improved long-term clinical outcomes. Here we report the use of cytokines/hormones, selected for their capacity to stimulate hematopoietic and/or mesenchymal cells, to recruit transplanted donor marrow cells into the osteopoietic differentiation pathway after BMT.
Methods

Bone marrow transplantation and cytokine treatment. Bone marrow (3 x 10^6 cells/mouse) obtained from GFP-transgenic donors (FVB/N background) was transplanted into lethally irradiated (1125 cGy) FVB/N recipients by tail vein injections as previously described. At 8 weeks post-transplantation, mediators were administered by daily intraperitoneal injection for 4 consecutive weeks as follows: Recombinant human G-CSF (Amgen, Thousand Oaks, CA), 250 μg/kg/day, 4 days/week; rat PTH, (Bachem Americas Inc., Torrance, CA), 80 μg/kg/day, 5 days/week; porcine GH (National Hormone and Peptide Program, Torrance, CA), 4 mg/kg/day, 6 days/week; recombinant mouse SCF (Prospec Bio, Rehovot, Israel), 100 μg/kg/day, 4 days/week; sterile saline (control). Mice were sacrificed for analysis at 1 or 4 weeks after the completion of the treatment regimen. All animal protocols were approved by the Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia.

Complete blood counts. Peripheral blood was collected by retro-orbital bleed and counts were obtained using a Hemavet hematology analyzer (Model HV950FS; Drew Scientific, Waterbury, CT).

Immunohistochemistry. Sections were immunostained for GFP expression as previously described. GFP+ osteoblasts and osteocytes were enumerated by visual
examination of stained slides by 2 investigators, who were blinded to the experimental conditions. (Additional details provided in Supplemental Material).

**Flow Cytometry.** Peripheral blood and bone marrow were analyzed for GFP expression and lineage specific markers using commercially available antibodies as previously described. (Additional details provided in Supplemental Material).

**Histomorphometry.** Bone histomorphometry was performed at the Bone Histomorphometry Core Laboratory at MD Anderson Cancer Center using standard protocols.

**Statistical methods.** Data are presented as mean ± SE. Differences were considered statistically significant by one-way or two-way ANOVA followed by Bonferroni’s multiple comparison test if they attained $p < 0.05$ (Prism, v4, GraphPad Software, Inc., San Diego, CA).

**Results and Discussion**

Eight weeks after BMT, prior to cytokine treatment, all four experimental groups (G-CSF, PTH, GH, and SCF) showed similar complete blood counts, complete (>90%) donor hematopoietic chimerism, and donor contribution to the leukocyte subsets (data not shown). One week after the completion of the mediators (13 weeks after BMT), donor contribution to all peripheral blood lineages and leukocyte subsets were not statistically different compared to controls. G-CSF and SCF stimulated an increase in
total leukocytes, neutrophils, and monocytes, but none of the cell counts were substantially greater than physiologic cell counts for healthy mice (Figure 1A). Bone marrow cellularity and GFP expression among marrow cells was >90% in all groups and controls (Figure 1A). Four weeks after the completion of mediator treatment (16 weeks after BMT), the donor contribution to all peripheral blood lineages, leukocyte subsets, and marrow cells remained similar to controls, and the G-CSF stimulated increase of total leukocytes persisted (Figure 1B). As expected, these data show that hematopoietic reconstitution is robust and the mediators are not detrimental to marrow function supporting their clinical use after BMT.

Collectively, cytokine treatment led to significantly increased osteoblast chimerism in epiphysis and metaphysis at 4 weeks ($p < 0.05$) and chimerism was significantly greater at 4 weeks compared to 1 week after the regimens ($p < 0.001$, Figure 2A.). Specifically, the G-CSF- and GH-treated mice showed significantly greater chimerism than controls at 4 weeks after the treatment. The osteoblast chimerism in the controls at 1 and 4 weeks after the treatment regimens was low and stable, consistent with prior time course studies ($p < 0.05$) (Figure 2A). The PTH-treated mice also showed a stable donor osteoblast chimerism at 1 and 4 weeks (Figure 2A). In contrast, G-CSF-treated mice showed significantly increased donor osteoblast chimerism at 4 weeks compared to 1 week, and the GH group showed a trend, suggesting a surge of osteopoietic differentiation followed the cessation of the G-CSF and GH treatments (Figure 2A). Christopher and Link reported that G-CSF suppressed osteoblast maturation and increased the osteoprogenitor pool leading to a rebound in osteoblast number after G-CSF treatment resulting from recruitment of new osteoblasts.$^{11}$ Our data, which are
consistent with those findings, suggest that the new osteoblasts are, in part, recruited from transplanted, donor-derived marrow cells. The overall range of donor chimerism (12-18%) among the four groups paralleled the chimerism observed 3 weeks after BMT in mice. The clinical importance of this observation is that the level of donor osteopoietic chimerism at the later time points approximates that found early after BMT, which has proved beneficial in children with OI treated with BMT. We anticipate that the cytokine-induced heightened osteopoietic chimerism will decrease over time so that repeated cycles of cytokine treatment will likely be required to maintain high-level osteopoietic chimerism in patients long-term and sustain the associated clinical benefits.

Histomorphometry of the bone revealed that the G-CSF-treated mice had a significantly greater number of osteoblasts per mm of bone surface, percent of bone covered with osteoid, and percent of bone surface lined with active osteoblasts compared to controls at both 1 and 4 weeks (Figure 2B). Using the absolute number of osteoblasts per mm of bone surface in the metaphysis and the osteoblast donor chimerism, the calculated absolute numbers of donor cells per mm of bone surface for the G-CSF, GH and saline mice were 1.26, 0.88, and 0.32, respectively. Thus, G-CSF appears to stimulate a 4-fold increase and GH a 3-fold increase in the absolute number of donor-derived osteoblasts. While long-term continuous treatment with G-CSF results in osteopenia in children, the intermittent dosing schedule of G-CSF used in our studies may be anabolic for bone, analogous to use of PTH.

Osteocyte chimerism was most improved in the GH group, with these animals showing the greatest increase between 1 and 4 weeks in both the epiphysis and metaphysis (Figure 2C). Moreover, GH-treated mice were the only group to show
significantly more donor-derived osteocytes than saline controls (Figure 2C). Despite the high donor osteoblast chimerism in the G-CSF group, the osteocyte chimerism was not increased, indicating that these cells do not give rise to the osteocytes. Both G-CSF and GH increase the osteoprogenitor pool, but G-CSF is thought to inhibit osteoblast differentiation,\textsuperscript{11,14} while GH promotes osteoblast differentiation.\textsuperscript{15} Since only about 1 of 5 osteoblasts is thought to give rise to an osteocyte,\textsuperscript{16} one possible explanation for our data would be that the G-CSF leads to the recruitment of osteoblasts that comprise the hematopoietic niche, while GH recruits osteoblasts that give rise to osteocytes. If proven true, these cytokines, G-CSF and GH, may find specific clinical applications in therapy directed to the endosteal hematopoietic niche or the structural bone, respectively.

Collectively, our data suggest a quiescent stem/progenitor cell resides in the transplanted marrow compartment that can be stimulated to enter the osteopoietic differentiation pathway. Future efforts will be directed toward elucidating the mechanism of the cytokine-induced osteopoietic differentiation of transplanted marrow cells and developing approaches to harness these pathways to advance bone marrow cell transplantation for disorders of bone.
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AUTHOR CONTRIBUTIONS

SO designed, performed, and analyzed research and assisted with manuscript preparation, VR performed research, RB performed research, TJH performed research, MD designed and analyzed research, EMH oversaw the entire project, designed and analyzed research, and prepared the manuscript.

The authors declare no competing financial interests.
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FIGURE LEGENDS

Figure 1. Hematopoiesis and hematopoietic chimerism. (A) Hematopoietic chimerism, determined by flow cytometric analysis for GFP expression, of peripheral blood (top, left; n=10 for each cytokine group) and leukocyte subsets (bottom, left; n=10) at 1 week after cytokine treatment (13 weeks after BMT). Absolute cell counts of leukocytes (top, middle; n=10), neutrophils (top, right; n=10) and monocytes (bottom, middle; n=10) and donor chimerism of total bone marrow cells (bottom, right; n=5 for each cytokine group) all at 1 week after cytokine treatment. (B) The same analyses as in panel (A) with data obtained at 4 weeks after cytokine treatment (16 weeks after BMT; n=5 for each cytokine group). All data are presented as mean ± SE. Statistically significant relationships are indicated. RBC, red blood cells; PLT, platelets; WBC, white blood cells; BMC, bone marrow cells. CD11b, Gr-1, CD3, and B220 are lineage markers for monocytes, neutrophils, T-lymphocytes, and B-lymphocytes, respectively.

Figure 2. Osteopoietic chimerism and histomorphometry. (A) Osteoblast (OB) chimerism, determined by immunohistochemical staining for GFP expression, in the epiphysis (left) and metaphysis (right) at 1 and 4 weeks after cytokine treatment. (n=5 for each cytokine group at each time point, 8 sections per mouse were analyzed). (B) Histomorphometric analyses of bone from G-CSF-, GH-, and saline-treated mice obtained at 1 (left) and 4 (right) weeks after completion of the cytokine regimen. (n=5 for each cytokine group,). (C) Osteocyte (OC) chimerism, determined by immunohistochemical staining for GFP expression, in the epiphysis (left) and metaphysis (right) at 1 and 4 weeks after cytokine treatment. (n=5 for each cytokine
group at each time point, 8 sections per mouse). All data are presented as mean ± SE. Statistically significant relationships are indicated. ***$p < 0.001$, **$p < 0.01$, * $p < 0.05$ compared to the saline group at the same time point.
Figure 1

A

1 w after last injection

- G-CSF
- PTH
- GH
- SCF
- saline

GFP (+) cells (%)

RBC, PLT, WBC

WBC-1w

- G-CSF
- PTH
- GH
- SCF
- saline

Number of WBC (x 10^9/L)

- G-CSF
- PTH
- GH
- SCF
- saline

Number of Neutrophils (x 10^9/L)

- G-CSF
- PTH
- GH
- SCF
- saline

1 w after last injection

CD11b, Gr-1, CD3, B220

Monocytes-1w

- G-CSF
- PTH
- GH
- SCF
- saline

Number of Monocytes (x 10^6/L)

BMC-1w

- G-CSF
- PTH
- GH
- SCF
- saline

Number of GFP (+) cells (%)

4w after last injection

- G-CSF
- PTH
- GH
- SCF
- saline

GFP (+) cells (%)

RBC, PLT, WBC

WDC-4w

- G-CSF
- PTH
- GH
- SCF
- saline

Number of WBC (x 10^9/L)

- G-CSF
- PTH
- GH
- SCF
- saline

Number of Neutrophils (x 10^9/L)

- G-CSF
- PTH
- GH
- SCF
- saline

4w after last injection

CD11b, Gr-1, CD3, B220

Monocytes-4w

- G-CSF
- PTH
- GH
- SCF
- saline

Number of Monocytes (x 10^6/L)

BMC-4w

- G-CSF
- PTH
- GH
- SCF
- saline

Number of GFP (+) cells (%)

Leukocyte Subsets
Figure 2

A

OB in epiphysis

\( \rho < 0.01 \)  

1w  

4w

OB in metaphysis

\( \rho < 0.01 \)  

1w  

4w

B

Number of osteoblasts per mm of bone surface

1w  

4w

Percent of bone surface lined with active osteoblasts

1w  

4w

Percent of bone surface covered with osteoid

1w  

4w

C

OC in epiphysis

\( \rho < 0.05 \)  

1w  

4w

OC in metaphysis

\( \rho < 0.01 \)  

1w  

4w
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