Inconsistent donor-specific efforts at IUHCT resulted in minimal or no engraftment and cells. However, until recently most clinical and experimental development to achieve donor-specific transplantation (IUHCT) is the exploitation of normal immunologic ontogeny to allow allogeneic hematopoietic engraftment without myeloablative conditioning. Host hematopoietic competition is among the primary barriers to engraftment in IUHCT. In the murine model this can be partially overcome by delivery of larger donor cell doses, but volume is limiting. Enrichment of donor hematopoietic stem cells (HSCs) would seem to offer a more efficient approach, but such enriched populations have engrafted poorly in existing models of IUHCT. To increase HSC dose while maintaining the presence of accessory cells, we used a less stringent enrichment protocol of single-step lineage depleted cells alone (lin-) or in combination with whole donor bone marrow mononuclear cells. Our results confirm that increasing doses of HSCs in combination with bone marrow accessory cells can dramatically improve engraftment after IUHCT. This represents a practical and clinically applicable strategy to maximize the engraftment potential of the donor graft without risk of treatment-associated toxicity.

INTRODUCTION

The primary rationale for in utero hematopoietic cell transplantation (IUHCT) rests on exploitation of normal events during hematopoietic and immunologic ontogeny to allow allogeneic hematopoietic engraftment without myeloablative conditioning. Host hematopoietic competition is among the primary barriers to engraftment in IUHCT. In the murine model this can be partially overcome by delivery of larger donor cell doses, but volume is limiting. Enrichment of donor hematopoietic stem cells (HSCs) would seem to offer a more efficient approach, but such enriched populations have engrafted poorly in existing models of IUHCT. To increase HSC dose while maintaining the presence of accessory cells, we used a less stringent enrichment protocol of single-step lineage depleted cells alone (lin-) or in combination with whole donor bone marrow mononuclear cells. Our results confirm that increasing doses of HSCs in combination with bone marrow accessory cells can dramatically improve engraftment after IUHCT. This represents a practical and clinically applicable strategy to maximize the engraftment potential of the donor graft without risk of treatment-associated toxicity.

METHODS

Mice

Mice were derived from breeding colonies maintained by our laboratory. BALB/c (H2Kd+) mice were used for time-dated mating, and pregnant females underwent IUHCT at E14. All donor BM was harvested from 6- to 12-week-old MHC-H2KdGFP+C57BL/6TgN(Act-EGFP)OsbY01 mice (B6GFP) (originally provided by M. Okabe, Genome Information Research Center, Osaka University, Osaka, Japan). All experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children’s Hospital of Philadelphia.
University and followed guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In Utero Transplantation
Donor low-density mononuclear cells (MNCs) were separated by Ficoll gradient centrifugation and resuspended in PBS. For enriched transplants, lineage depleted (lin-) cells were isolated using a MACS lineage depletion kit (Miltenyi Biotec, Auburn, CA). For cKit+ Sca-1+ lin- (KSL) transplantation the lineage depletion was further enriched using CD117(c-kit) APC (eBioscience, San Diego, CA), Sca-1 PE-Cy7 (BD Pharmingen, San Jose, CA), and biotinylated lineage antibody cocktail (Miltenyi Biotec), followed by streptavidin APC-Cy7 (eBioscience) as a secondary stain. KSL cells were isolated via flow cytometric sorting using a FACSaria (BD, Franklin Lakes, NJ). At E14 each fetus was injected with either 10^5 BM-MNCs (WBM), 5 x 10^5 lin- cells, 1 x 10^5 KSL cells, or 10 x 10^5 BM-MNCs (WBM) + 5 x 10^5 lin- cells in 20 µL PBS as previously described [11]. Surviving pups were placed with a foster dam within 24 hours of birth.

Chimerism

At 1, 2, 3, 4, and 8 months of age peripheral blood from E14-injected mice was obtained via retro-orbital puncture. After mice were killed, liver, spleen, and BM were assessed after mechanical homogenization and passage through a 70-µM filter and RBC lysis. Donor cell chimerism was assessed as the percentage of CD45<sup>+</sup> cells that were GFP<sup>+</sup> by flow cytometry. Similarly, multilineage chimerism was determined based on the percentage of each cell type found to be GFP<sup>+</sup>, as described above.

Flow Cytometry

Donor cells were analyzed for KSL content using CD117(c-kit) APC (eBioscience), Sca-1 PE-Cy7 (BD Pharmingen), and biotinylated lineage antibody cocktail (Miltenyi Biotec), followed by streptavidin APC-Cy7 (eBioscience) as a secondary stain. KSL cells were stained with CD3 PE (BD Pharmingen), B220 PE-Cy5 (BD Pharmingen), CD11b PE-Cy7 (BD Pharmingen), and CD11c PE (BD Pharmingen). Flow cytometry was performed on a FACS Calibur (BD).

Statistics

The significance of differences among groups was determined using the Student’s t-test for 2 samples assuming unequal variances. A 2-tailed P ≤.05 was considered significant.

RESULTS

HSC Content within Donor Cells

To compare donor cell populations for HSC content, we determined the (KSL frequency within each population to estimate total donor HSCs administered per fetus. Our goal was to administer HSC doses in the experimental limbs that were each significantly increased relative to our control BM dose of 10 x 10^6 low-density MNCs. In our control BM-MNCs the KSL fraction was determined to be 0.2%, giving a calculated total donor HSC content of 2 x 10^5 HSCs per fetus. Within the lin- population, KSL frequency increased to 3.6%, representing an 18-fold enrichment (Figure 1). Thus, fetuses in the KSL group received 1 x 10^5 HSCs, fetuses receiving 5 x 10^6 lin- cells (enriched group) received 1.8 x 10^5 HSCs, and fetuses receiving 10 x 10^6 BM-MNCs + 5 x 10^6 lin- cells (combined group) received 2 x 10^5 HSCs. Lin- cells represented 1% to 3% of whole bone marrow, and MACS lineage depletion conferred >90% purity.

Enrichment of the Donor Cell Graft Increases Allogeneic Engraftment

Although KSL cells alone failed to engraft, mean levels of peripheral blood chimerism at 1 month were significantly increased in the enriched group as compared with WBM controls (27.4% versus 11.8%, P < .001). Unlike the WBM controls, in which chimerism levels continued to decrease throughout the period of analysis, the enriched group stabilized by 3 months and showed no further decrease through 8 months (Figure 2A).

Addition of Whole Bone Marrow Cells Increases the Engraftment Potential of an Enriched Graft

Strikingly, the KSL cells entirely failed to engraft in the absence of nonenriched BM cells, despite a 5-fold increase in HSC content over the control group. Initial mean chimerism in the combined group was significantly higher than the lin- cells alone (50.3% versus 27.4%, P = .03). The combined group maintained stable engraftment averaging approximately 25% (Figure 2A), which was significantly higher than either of the other groups and suggests that a cell population within the BM may facilitate HSC engraftment. Other hematopoietic organs demonstrated similar relative improvements in engraftment.
No significant differences in survival were observed between groups.

**Multilineage Engraftment after Enriched IUHCT**

Multilineage engraftment was analyzed within the donor cell population in all mice when they were killed. The persistence of stable, multilineage chimerism at 8 months of age in all groups confirms HSC engraftment. The lineage analysis demonstrated balanced donor lineage populations without skewing of lineage representation (Figure 3), once again confirming the contribution of donor HSCs rather than any lineage-committed progenitor population.

**DISCUSSION**

IUHCT represents a promising strategy for the treatment of any disorder that can be diagnosed early in gestation and can be treated by postnatal HSCT. Potential target disorders include the hemoglobinopathies and immunodeficiency syndromes, among others.

There are 3 theoretical barriers to engraftment after IUHCT: an immune response to donor cells, “space” within the host hematopoietic niche, and competition from host hematopoiesis. Work in our laboratory has shown that the previously recognized adaptive alloresponse limiting engraftment in our model [11] was secondary to maternal immunization and transfer of maternal antibodies via breast milk [3]. This can be avoided completely by maternal fostering, which was performed in this study, excluding immune response as a confounding factor and leaving host hematopoiesis as the remaining barrier. The simplest approach to overcoming host hematopoietic competition is to increase the dose of donor HSCs transplanted. We have previously increased donor cell dose and improved engraftment by use of the intravascular technique, which allows injection of a much greater volume of cells than our previous intraperitoneal technique. However, even with maximization of the dose of nonenriched BM cells, mean levels of donor chimerism remain below the threshold likely to be required for cure of many hematopoietic diseases [12-15]. Enrichment of donor cell grafts represents an obvious and clinically applicable approach.
approach to increase donor HSC dose. However, all our previous attempts to engraft highly enriched HSCs by IUHCT have been disappointing with little or no engraftment achieved in allogeneic systems and very minimal engraftment observed in congenic systems. The reason for failure of engraftment of highly enriched allogeneic cells after IUHCT is speculative but includes a detrimental effect on homing or engraftment caused by the enrichment method or other cells in the BM that may be required to “facilitate” engraftment in the fetus.

These results confirm our prior findings and are supportive of either or both of these reasons. The use of a single lineage depletion step allowed a 9-fold increase in donor HSC dose (despite transplanting half the number of total cells) and resulted in a more than doubling of mean donor cell chimerism at 1 month of age and a sustained 5-fold increase at 8 months of age. This is particularly striking when compared with the absolute lack of engraftment with a similar number of highly enriched KSL cells. Thus, a much less stringent enrichment protocol appeared to markedly improve engraftment. The combination of whole BM-MNCs (containing a negligible additional number of HSCs but a much higher number of cells) resulted in an additional major increase in early and late chimerism, supporting a facilitating effect of some component of the BM-MNCs on HSC engraftment. Engraftment remained twice that of the enriched group long after the anticipated contribution of short-term repopulating activity, demonstrating that their increased chimerism is the result of an increase in the absolute number of engrafted HSCs despite an equivalent number within the donor inoculum. The mechanism of the facilitating effect requires further study. Results in large animal models show that highly enriched HSCs may have engraftment defects that are reversible on addition of even low doses of T cells [16-18]. Likewise, stromal cell progenitors [19,20] and dendritic cell precursors [21] may play some role. Alternatively, the co-transplantation with BM cells may provide growth factors or paracrine effects that enhance donor cell homing, engraftment, or subsequent competition. Although this would be a likely explanation for a short-term increase in donor chimerism, it is a less likely to explain an increase in long-term engraftment [22].

This study is best viewed as a proof in principle that enrichment strategies can potentially improve engraftment after IUHCT and that if delivered in very high doses, adult HSCs can effectively compete in the fetal hematopoietic environment. This is a particularly attractive strategy because it requires no pharmacologic manipulation of the mother, fetus, or donor cells. It also negates the need for use of more competitive but more controversial sources of HSCs such as fetal HSCs or embryonic stem cells. Further studies are needed to determine the optimal enrichment strategy for clinical application.

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REFERENCES