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Animal models

George E Georges, Brenda M Sandmaier and Rainer Storb

INTRODUCTION

Significant progress has been made in the past few years in the development of blood stem cell transplantation as a method for treatment of patients with hematologic and non-hematologic malignancies. Work using animal models has helped set the stage for these developments. This chapter will highlight the background history and recent progress of that work.

The primary animal models include inbred mice and rats and random-bred dogs, rabbits and non-human primates. The need for animal models is clear: there are unacceptable risks to patients when clinically untested modifications are made to transplant protocols. Animal models serve as the means with which to reliably assess the biology and the likely outcome of new approaches to stem cell transplantation. In general, the inbred mouse has been a very useful model for studying the basic biology of hematopoiesis, as well as for providing rapid readouts for studying basic principles of transplantation. The major caveat with the murine

Table 1.1 Red blood cell demands of different species[a]

Species	RBC per ml ($\times 10^9$)	Weight (kg)	Blood volume (ml)	RBC life span (days)	RBC per day	Life expectancy (years)	RBC per life of animal
Mouse	9.0	0.025	1.8	50	3.2×10^8	2	2.4×10^{11}
Human	6.0	70	4900	120	2.5×10^{11}	80	7.3×10^{15}
Cat	7.5	4	280	70	3.0×10^{10}	15	1.6×10^{14}
Dog	7.2	18	1550	115	9.7×10^{10}	13	4.6×10^{14}

[a]Adapted from Abkowitz et al.[57]

model is that the demands on stem cells are radically different and extrapolation of transplantation results from mice to humans for clinical use is questionable. These differences in demand are best illustrated by the fact that the mouse makes as many red blood cells in a lifetime as a human makes in one day or as a dog makes in 2.5 days (Table 1.1).

The outbred dog is a useful large animal model with consistently relevant outcomes that are similar to the clinical experience in human marrow transplantation. In addition, the large litters provide a model for sibling transplants, which are not easily done in non-human primates. The non-human primate model bears the closest resemblance to clinical transplantation, although monkeys and apes are more difficult to maintain as study subjects, and therefore discourage the development of truly novel transplantation approaches.

STEM CELLS CIRCULATE IN PERIPHERAL BLOOD

That blood stem cells circulate at a low frequency in the peripheral blood has been known for over 30 years. In 1962, Goodman et al[1] demonstrated that pooled blood isolated from normal F_1 hybrid donor mice could successfully restore hematopoiesis in supralethally irradiated and myeloablated homozygous parent mice. They specifically postulated the existence of a blood stem cell population with marrow repopulation potential that was present in the donor mouse's peripheral blood. Similar findings were reported by other investigators in guinea pigs[2] and dogs.[3] To determine the cell dose necessary for allogeneic engraftment in large animals, Storb et al[4] infused large doses of leukapheresed donor mononuclear cells (a total of 20×10^9 mononuclear cells collected daily over 7 days from peripheral blood) into lethally irradiated unrelated dogs, which resulted in restoration of marrow function (engraftment). The incidence of graft-versus-host disease (GVHD) was high. Sufficient hematopoietic progenitor cells were not obtained from the thoracic ducts of donors to enable autologous engraftment.[5] This suggested that stem cells did not circulate in the lymphatics. Cross-circulation experiments in baboons indicated that hematopoietic progenitor cells were present in the peripheral blood of non-human primates as well.[6,7]

While these studies proved that peripheral blood stem cells (PBSC) existed and were a potential source of cells for transplantation, marrow cells provided a more clinically reliable and easily accessible source of hematopoietic repopulation cells. In steady state, the best estimate of the ratio of hematopoietic progenitor cells in the blood to those in the marrow is approximately 1:100.[8] Due to the low frequency of PBSC, attempts to generalize the use of PBSC in lieu of marrow was not pursued. If, however, marrow harvest resulted in an insufficient number of cells needed for transplant, blood-derived stem cells could be used as a supplemental source to ensure engraftment. For example, in patients with aplastic anemia, where there is a higher risk of graft failure associated with low numbers of transplanted marrow cells, supplemental buffy coat infusion from donors have been successfully used to increase the number of stem cells infused.[9]

Although there had been some concern that PBSC had limited proliferation capacity and did not include the requisite pluripotent stem cell population necessary for long-term hematopoiesis,[10] canine models have convincingly demonstrated long-term repopulation by allogeneic cells.[11,12] As early as 1981, both Goldman et al[13] and Korbling et al[14] demonstrated that leukapheresed autologous cryopreserved PBSC could restore hematopoiesis after myeloablative chemotherapy in patients with chronic myelogenous leukemia. While this did not prove long-term repopulation, it supported the principle, since patients transplanted in blast crisis were converted into chronic phase after infusion of PBSC that had been collected in chronic phase.

In comparison with marrow, the unmanipulated leukapheresis product containing PBSC has an order of magnitude greater number of mature T cells. In the allogeneic mouse and dog transplant models, higher T-cell doses at time of

transplant have been correlated with increased severity of acute GVHD.[4,15,16] Since GVHD remains one of the major causes of morbidity and mortality, there has, up until the recent advent of mobilization strategies, been a significant disincentive for the use of PBSC as an alternative source for allogeneic grafts. High numbers of T cells in the PBSC leukapheresis product have not been relevant to the autologous transplant setting.

STEM CELL CHARACTERIZATION

Historically, a major obstacle to studying circulating hematopoietic progenitor cells was that the phenotype of the cells responsible for pluripotent reconstitution of hematopoiesis was unknown. Over the past decade there have been advances in the phenotypic characterization of hematopoietic progenitor cells, including identification of specific cell-surface markers (CD34 or c-kit) and cell metabolism-specific dyes. Despite this progress, stem cells remain best defined functionally as having the capacity to restore complete long-term hematopoietic cell differentiation and production in the lethally irradiated host.

Based on animal models and clinical data thus far, successful allogeneic PBSC engraftment requires the equivalent intensity of conditioning that is necessary for marrow transplantation. Similarly, a minimum threshold cell dose of PBSC is necessary to ensure successful allogeneic transplants. In the allogeneic dog model, a minimum of 0.6×10^8 mononuclear marrow cells per kilogram is necessary for successful engraftment after myeloablative conditioning (9.2 Gy total-body irradiation). In contrast, at least one order of magnitude higher number of non-cytokine-mobilized peripheral blood cells (2×10^9 mononuclear cells/kg) are necessary for allogeneic engraftment.[5,17] This is due to the lower frequency of stem cells in the blood compared with marrow, and does not appear to be due to functional differences in stem cells derived from peripheral blood versus marrow.

Several investigators have developed assays to quantify the number of stem cells necessary for successful transplantation. The colony-forming unit spleen (CFU-S), colony-forming unit granulocyte–macrophage (CFU-GM) and other derivative assays, initially developed in the mouse model, are indirect measures of primitive precursor cells present in the marrow or blood. More recently, these assays have been used to determine optimal stem cell mobilization regimens, which will be discussed in subsequent sections of this chapter.

IN VITRO ASSAYS FOR STEM CELLS

Till and McCulloch described the CFU-S in 1961.[18] In this assay, normal mouse marrow cells are injected into lethally irradiated syngeneic mice. Macroscopically visible multilineage (myeloid and lymphoid) colonies of cells in the spleens of the irradiated recipients were identified at either 8 or 14 days after injection. Neither the 8- nor the 14-day CFU-S cells were true stem cells, however, since they were incapable of long-term hematopoiesis.[19,20] A subpopulation of these CFU-S were capable of generating multilineage colonies in spleens of secondary irradiated recipient mice.[21]

This assay has been subsequently refined into the pre-CFU-S assay, which is a double transplantation technique.[20] It defines a population of primitive hematopoietic progenitor cells as being capable of reconstituting hematopoiesis (14 days after primary transplantation) and then differentiating into CFU-S in secondary irradiated recipients. While the pre-CFU-S assay does not definitively identify true clonal hematopoietic stem cells, it provides a quantitative means of comparing primitive hematopoietic stem cell activity in various cell populations.

Another approach to identifying cells with long-term repopulating capacity in the mouse is the competitive repopulating unit (CRU) assay (also referred to as competitive repopulating index (CRI)).[22,23] This is an in vivo limiting dilution assay, which quantifies genetically marked cells that are individually capable of regenerating at least 5% of the entire hematopoietic

system, including both myeloid and lymphoid cells and maintaining production of these cells for at least 6 months. These relatively long-term pluripotent cells are infused simultaneously with short-term myeloprotective cells (or unmanipulated, distinctly marked marrow cells) into irradiated recipient mice. Thus, CRU cells must compete with committed progenitors for long-term repopulation.

A similar but more quantitative in vitro approximation of stem cell number is the long-term-culture initiating cells (LTC-IC) assay.[24] LTC-IC are immature, multipotent progenitor cells, which are quantitated by limited dilution analysis. Another commonly used in vitro assay to describe more committed progenitor cells measures CFU-GM. This quantifies the number of precursor cells that develop characteristics of granulocytes after a shorter duration of culture (typically 10–14 days).

These in vitro assays have been developed to assess the number of progenitor cells in peripheral blood and marrow. They serve only as surrogate markers for truly primitive pluripotent stem cells in a given animal model. It remains unproven whether or not increased frequencies of in vitro progenitor cells correlate with increased numbers of long-term marrow repopulating stem cells.

STEM CELL PHENOTYPE

Much work has been devoted to identifying the specific cellular phenotype of stem cells. A more detailed discussion of the characteristics of stem cells can be found in later chapters. While a unique cell surface antigen has not been identified in stem cells, multistep selective depletion of differentiated hematopoietic cells results in a subpopulation of 'lineage-negative' cells that include stem cells. CD34, a stem cell marker, has been identified and characterized in humans, dogs and mice. In non-human primates, monoclonal antibodies to human CD34 are cross-reactive, and identify a population of stem cells.[25] Additionally, c-kit and Ly-6A/E (or Sca-1) have been identified as stem cell markers in mice.[26] These cell surface proteins distinguish more primitive hematopoietic cells from lineage-specific cells.[27] Not all CD34$^+$ or c-kit$^+$ cells have self-renewal potential; most are multipotent for lymphoid and myeloid lineages.[28]

During steady-state conditions, the subset of primitive cells that have evidence of self-renewal potential are resistant to 5-fluorouracil (5-FU) or other active cell-cycle toxic agents.[29] Because stem cells replicate infrequently and are quiescent, they would be expected to have low numbers of active mitochondria. Rhodamine-123 (Rh-123) is a dye that binds to cells high in mitochondrial content. Several investigators have shown that primitive hematopoietic cells (lineage-negative, c-kit$^+$) with low Rh-123 staining maintain long-term repopulating capacity.[30,31] A second metabolic agent, Hoechst 33342 (Ho) (a DNA-affiliating dye), can identify cells not in cell cycle. Thus, a combination of the two metabolic dyes and monoclonal antibodies have been used to identify a primitive, pluripotent progenitor cell that is Ho-low, Rh-123-low, lineage-negative, c-kit$^+$ and capable of long-term hematopoiesis.[32] Wolf[33] has shown that injection of single Ho-low, Rh-123-low, cells into lethally irradiated mice results in donor hematopoietic reconstitution in 15% of cases.

Thus, significant progress in the isolation and characterization of stem cells has been made over the past few years. Despite the in vitro assays to characterize stem cells, the ability of progenitor cells to repopulate ablated marrow for long-term hematopoiesis remains the key functional definition of stem cells.

In larger animals it has not been possible to isolate in vitro a homogeneous population of pluripotent primitive stem cells. However, immunoaffinity selection of a stem-cell-containing population using anti-CD34 monoclonal antibodies has been possible in baboons.[25] While the CD34 antigen does not define stem cells, it permits 100- to 1000-fold enrichment of the population of stem cells isolated from blood or marrow. With the cloning of the canine *CD34* gene[34] and the development of monoclonal antibodies to the recombinant CD34 antigen, more sophisticated in vitro blood stem cell manipu-

lation will be possible in that model (McSweeney et al, personal communication). It will be possible to investigate the long-term engraftment potential of allogeneic purified $CD34^+$ cells and the specific role of T cells or natural killer cells in the dog. Since cellular manipulation of PBSC (such as subtraction or addition of T cells) for non-malignant or currently treatable malignant diseases in humans can be ethically questionable, especially in the allogeneic transplant setting, animal models are necessary for these studies.

MOBILIZATION OF STEM CELLS

Along with the development of improved assays to identify progenitor cells from marrow or blood, one of the critical advances in blood stem cell transplantation has been the observation that stem cells can be mobilized from marrow into the peripheral blood. In 1977, Cline and Golde[35] and Richman et al[36] reported that the number of CFU-GM were markedly increased in humans given endotoxin, dextran sulfate or chemotherapy.

Animal models have been useful in studying new strategies for stem cell mobilization, since they have responses similar to stem cell mobilization in humans. Although the use of chemotherapy-mobilized stem cells is now clinically widespread in the autologous clinical transplant setting, animal models were necessary to define the kinetics and show clinical feasibility.

In the autologous dog model, Abrams et al[37] demonstrated that infusion of cryopreserved PBSC mobilized after chemotherapy was able to restore hematopoiesis after myeloablative total-body irradiation (TBI). Peripheral blood mononuclear cells (PBMC) were collected 14–16 days after myelosuppressive cyclophosphamide (CY) treatment – during the rapid leukocyte recovery phase following the CY-induced leukocyte nadir. Following collection and cryopreservation, as few as 0.5×10^8 PBMC/kg could protect dogs from otherwise lethal TBI (9.0 Gy). In comparison with non-mobilized peripheral blood, chemotherapy-mobilized leukapheresed cells provided 11 times more CFU-GM and were 12.5 times more potent in reconstituting hematopoiesis. Potency was defined in terms of the total number of nucleated cells needed for successful engraftment. Thus, the collection and infusion of PBSC had become significantly more efficient.

In a similar manner, Appelbaum et al[38] demonstrated successful autologous transplantation of dogs with lymphoma using leukapheresed PBSC obtained after combination chemotherapy. The leukapheresis of chemotherapy-mobilized stem cells has rapidly developed into a clinically widespread technique for autologous transplantation of patients with a variety of malignancies, including lymphoma, acute myeloid leukemia, breast and ovarian cancer.

One of the most compelling reasons for the rapid widespread clinical application of autologous PBSC transplantation has been the more rapid engraftment seen with mobilized PBSC compared with marrow. This results in a reduction in the amount of supportive care and inpatient hospital days. It has contributed to the improved clinical safety of autologous transplants.[39] The accelerated recovery of hematopoiesis is due to the infusion of large numbers of mature, proliferating committed progenitor cells in addition to sufficient long-term marrow-repopulating stem cells with the leukapheresis cell product. This is reflected in the marked increase in the number of committed precursor cells in assays of CFU-S (mice) and CFU-GM (canine and primate) in animal models of chemotherapy-mobilized stem cells.

In autologous clinical transplants, there has been concern that the risk of relapse of the underlying malignancy is significantly increased when there is infusion of contaminating malignant cells with cryopreserved stem cells following high-dose chemotherapy.[40] Clinical studies are currently underway involving purging of PBSC via $CD34^+$ immunoaffinity selection, thereby reducing the relapse rate following autotransplants.[41] There have been no published reports of blood stem cell tumor purging in animal models.

CYTOKINE-MOBILIZED STEM CELLS

The discovery and cloning of the many cytokines involved in hematopoiesis have been key factors in the tremendous advances in the understanding of cell growth regulation. Many of the cytokines have the additional property of stimulating marked increases in hematopoietic progenitor cells circulating in the peripheral blood. Cytokines are thought to act on primitive or precursor cells at different stages of differentiation. Thus, recent efforts have been focused on combining different cytokines to maximize the number of stem cells in circulation. If the efforts were successful, the number of apheresis procedures necessary to achieve a sufficient collection of PBSC could be reduced to one brief session. (Refer to Table 1.2 for a summary of cytokines studied for mobilization.)

The first cytokines shown to mobilize stem cells into the peripheral blood were granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF). During Phase I clinical trials, increases of up to 100-fold in the number of CFU-GM were observed with both cytokines.[42,43] These observations led to speculation that cytokines could be used to mobilize transplantable stem cells into the peripheral blood.

The following section summarizes results from murine, canine and non-human primate models of stem cell mobilization and transplantation. Many of the cytokines have cross-species reactivity, which has permitted rapid assessment of biological effects in several animal models. Other cytokines appear to have suboptimal biologic activity in unrelated species and have required the cloning of that species' homologous cytokine.

MURINE STEM CELL MOBILIZATION

Molineux et al[44] published the first study demonstrating the transplantation potential of G-CSF-mobilized PBSC in the mouse. Donor mice were subcutaneously administered G-CSF (250 μg/kg/ day × 4 days) until maximal progenitor cell circulation was achieved. As little as 10 μl of blood (2.5×10^5 cells) from male $B6D2F_1$ mice restored complete hematopoiesis in 15.2 Gy (^{60}Co γ over 16 hours) lethally irradiated female syngeneic recipients. Donor chimerism of 100% was shown for up to 10 months after transplant, and there were no discernible differences in hematopoiesis between the G-CSF-mobilized PBSC and marrow control chimeras.

Neben et al[45] compared the marrow and peripheral blood compartments for the presence of stem cells after mobilization. C57B1/6J (B6) mice were treated with CY 200 mg/kg, followed by or in lieu of rhG-CSF 250 mg/kg/day × 4 days. At day +6 after onset of mobilization, limited numbers of Ficoll-Hypaque gradient separation mononuclear cells – obtained from either marrow, spleen or peripheral blood – were transplanted into syngeneic lethally irradiated recipients (12.5 Gy, split course 3 hours apart at 1.11 Gy/min).

Compared with non-mobilized controls, the committed progenitor CFU-S content in peripheral blood increased 11-fold following G-CSF mobilization, 15-fold following CY and 36-fold following CY plus G-CSF. The primitive stem cell content of mobilized peripheral blood approached that of normal marrow in the donors treated with CY, with G-CSF and with CY plus G-CSF (competitive repopulating index, CRI, of normal marrow and normal peripheral blood was 0.43 and 0.006 respectively, while CRI of blood mobilized with CY, with G-CSF and with CY plus G-CSF was 0.21, 0.41 and 0.28 respectively). In addition, the stem cell content reversibly decreased in the marrow of mice treated with a combination of CY plus G-CSF, but remained unchanged following G-CSF alone.

These studies demonstrated that the hematopoietic stem cells can be temporarily and reversibly mobilized into the peripheral blood and nearly approach marrow levels. The differences in the repopulating ability following specific techniques used for mobilization suggested that different subpopulations of stem cells may have been mobilized. This has led to attempts to identify other cytokines that may

Table 1.2 Summary of cytokines used to mobilize PBSC

Stimulus	Timing of mobilization	Mouse	Dog	Primate	Rescue of lethally irradiated recipient
G-CSF	4–7 days	5–20-fold ↑ CFU-GM[46] 11–18-fold ↑ CFU-S[45,55]	3–7 fold ↑[62] CFU-GM	10–50 fold ↑ CFU-GM[64,65]	Mouse, allogeneic dog, baboon, allogeneic rabbit
GM-CSF	5–14 days			12-fold ↑ CFU-GM[70]	
SCF	7–10 days	1.5–3.7-fold ↑ CFU-GM[46] 14-fold ↑ CFU-S[53]	1.4–12-fold ↑[62] CFU-GM	0–10-fold ↑ CFU-GM[64]	Mouse, autologous dog
SCF + G-CSF	5–10 days	13–20-fold ↑ CFU-GM[46] 7–10-fold ↑ CFU-S[48]	12–45-fold ↑[62] CFU-GM	50–300-fold ↑ CFU-GM[64,65]	Mouse, allogeneic dog, baboon
IL-1β	Single dose	10-fold ↑ CFU-S[77] 30-fold ↑ CFU-GM[77]		100-fold ↑ CFU-GM[68]	Mouse, autologous monkey
IL-3	11–14 days			14-fold ↑ CFU-GM[70]	
IL-3 + GM-CSF	11–14 days IL-3 followed by 5 days GM-CSF			65-fold ↑ CFU-GM[70]	
IL-6	14 days	8.7-fold ↑ CFU-S[50]			Mouse
IL-6 + G-CSF	14 days	132-fold ↑ CFU-S[50]			
IL-7	7 days	6-fold ↑ CFU-S[51]			Mouse
IL-8	Single dose	20-fold ↑ CFU-GM[52]		10–100-fold ↑ CFU-GEMM[66]	Mouse
IL-11	7 days	1.9-fold ↑ CFU-S[53]		60–140-fold ↑ CFU-GM[67]	
IL-11 + SCF	7 days	10.6-fold ↑ CFU-S[53]			Mouse
IL-11 + G-CSF	4 days IL-11 followed by 4 days G-CSF			90–350-fold ↑ CFU-GM[67]	
FLT-3 + G-CSF	5 days	384-fold ↑ CFU-S[55]			
MIP-1α	Single dose	2-fold ↑ CFU-S[56]			
MIP-1α + G-CSF	2 days G-CSF followed by single-dose MIP-1α	25-fold ↑ CFU-S[56]			
Anti-VLA-4	4 days			10–100-fold ↑ CFU-GM[71]	
Anti-VLA-4 + G-CSF	5 days followed by 2 days anti-VLA-4			200–1300-fold ↑ CFU-GM[71]	

even further enhance the ability to mobilize PBSC efficiently.

Accordingly, Briddell et al[46] administered low-dose recombinant rat stem cell factor (rrSCF) and recombinant human (rh)G-CSF in combination, which resulted in even higher concentrations of both primitive and mature progenitor cells in the peripheral blood than if either cytokine was used singly. Splenectomized male (C57BL/6J × DBA/2J)F_1 mice were mobilized with rrSCF (pegylated form, 25 μg/kg/day), rhG-CSF (200 μg/kg/ day) for 7 days alone or in combination. Mobilized PBSC obtained as fractionated low-density peripheral blood cells were infused in a cell dose escalation study into syngeneic lethally irradiated (11.5 Gy, equal split dose, 4 hours apart) female mice. PBSC mobilized by SCF plus G-CSF resulted in greater survival at 90 days compared with mice receiving an equal number of mononuclear cells mobilized with G-CSF. The synergistic effect of the two cytokines may be explained by the SCF expanding both primitive and mature hematopoietic progenitor cells and the G-CSF mobilizing progenitor cells into the periphery.

Yan et al[47] have shown that PBSC mobilized with a combination of SCF and G-CSF could be sequentially transplanted to tertiary recipients and reconstitute hematopoiesis for more than 26 months. At 12–24 months after transplant, the female recipients had donor engraftment greater than 90%, as documented by Y-chromosome specific polymerase chain reaction (PCR) probe in spleen, thymus and lymph node cells. Marrow cells from these recipients were further transplanted to syngeneic secondary female recipients (myeloablated with 12.0 Gy, split dose, 4 hours apart). At 6 months, 98% of the secondary recipients had male-derived hematopoiesis by whole-blood PCR analysis, with single-colony marrow culture PCR analysis confirming greater than 90% male-derived hematopoiesis. Marrow cells from the secondary recipients were further passaged to tertiary recipients, with 90% of mice exhibiting male-derived hematopoiesis 6 months after transplant. In contrast, fewer than 50% of secondary recipients of PBSC mobilized with G-CSF alone showed sustained male-derived hematopoiesis by single-colony marrow culture PCR analysis. This indicated that PBSC mobilized with SCF plus G-CSF provided increased numbers of long-term reconstituting stem cells compared with PBSC mobilized with G-CSF alone.

These studies have been further supported by the findings of Bodine et al,[48] who used a quantitative competitive repopulation assay to assess the ability of combined SCF and G-CSF to mobilize repopulating stem cells. Comparing results in C57BL/6J splenectomized mice receiving SCF plus G-CSF for 3–7 days with those given an equal number of marrow cells from unstimulated HW80 mice (genetically identical to C57BL/6J except at the β-globin locus), there was a dramatic shift in hematopoiesis in favor of the mice treated with SCF plus G-CSF. Specifically, 80–90% of the hemoglobin was derived from C57BL/6J (donor) mice 4 months post-transplant in the histocompatible recipient WBB6F_1 mice irradiated with 9.0 Gy from a ^{137}Cs source. In addition, the efficiency of recombinant retroviral human multidrug-resistance gene (*MDR-1*) transfer into PBSC mobilized with SCF plus G-CSF was 2–5-fold greater than into PBSC mobilized with standard (5-FU) chemotherapy.

Based on quantitative models of hematopoiesis that were originally described by Chervenick,[49] these studies indicate that splenectomized mice treated with SCF plus G-CSF undergo a threefold expansion in total absolute number of stem cells, from a baseline of 3000 to an increase of 9000 per mouse. In addition, the cytokine combination mobilized 70% of stem cells from the marrow compartment to the peripheral blood compared with <0.1% of stem cells in the blood of non-mobilized controls.

Several of the interleukin (IL) cytokines have been studied to determine their effect on mobilization of stem cells in mice. There have been recent reports on the use of these cytokines as single agents or in combination with G-CSF to rapidly mobilize PBSC and reconstitute hematopoiesis in myeloablated recipients. IL-6 is a cytokine previously reported to stimulate primitive hematopoietic stem cells. IL-6 has

been used to support and expand progenitor cells in vitro. Suzuki et al[50] have shown that rhIL-6 (10 μg/day × 14 days) combined with rhG-CSF (0.35 μg/day × 14 days) resulted in a 132-fold increase in CFU-S compared with control mice. IL-6 or G-CSF alone resulted in 8-fold and 11-fold increases respectively in CFU-S. Survival of lethally irradiated (10 Gy) syngeneic C57BL/6 mice at day 100 after infusion of 100 μl of blood from animals mobilized with combined IL-6 and G-CSF was 92%. Mice rescued with IL-6 or G-CSF single-cytokine-mobilized blood had survival rates of 31% and 46% respectively. This compared with 0% survival of mice rescued with non-mobilized blood. The relatively poor survival of mice rescued with G-CSF-mobilized PBSC in that study is most likely related to differences in G-CSF administration compared with other investigators. Specifically, G-CSF dose (0.35 μg vs 4 μg per day) and method of administration (continuous infusion for 14 days vs subcutaneous daily injection for 5–7 days) were different. Possible synergy between the three cytokines SCF, IL-6 and G-CSF awaits further investigation.

Grzegorzewski et al[51] have reported that rhIL-7 at 5 μg twice daily for 7 days in mice stimulated a sixfold increase in CFU-S in peripheral blood. As few as 10^6 peripheral blood leukocytes (PBL) from IL-7-treated C57BL/6 (Ly 5.1) donors rescued 90% of lethally irradiated (11 Gy, equal split dose 3 hours apart) recipient C57BL/6 (Ly 5.2) mice compared with no survivors from the same number of PBL from control donors. In recipients transplanted with IL-7-mobilized PBSC, >90% donor hematopoiesis (Ly 5.1 antigen) was documented at 6 months post-transplant in marrow, spleen and thymus. These studies suggest that IL-7 can efficiently mobilize PBSC; whether or not the IL-7-mobilized stem cells are capable of the long-term hematopoiesis necessary in larger animals is unknown. Potential synergies between IL-7 and other cytokines to mobilize stem cells remain to be examined.

IL-8 is a cytokine that is involved in chemotaxis and activation of neutrophils. In large animals, injection of IL-8 results in immediate neutropenia, followed by granulocytosis, neutrophil margination and infiltration, plasma exudation, and angiogenesis. Laterveer et al[52] have reported that mice given 30 μg of rhIL-8 as a single intraperitoneal dose showed a 20-fold increase in CFU-GM in peripheral blood within 15 minutes of injection. As few as 1.5×10^6 PBMC isolated from IL-8-treated Balb/c male mice reconstituted hematopoiesis in 100% of myeloablated (8.5 Gy) female recipients (studied ≤60 days post-transplant). This compared with no survivors among controls. The rapid mobilization followed by rapid reversal of PBSC following IL-8 may provide synergy with G-CSF or SCF for more efficient stem cell mobilization.

Mauch et al[53] showed that either twice daily subcutaneous or continuous infusion of 250 μg/day rhIL-11 for 7 days in mice resulted in an increase in the progenitor cell (CFU-S) content but not the primitive cell content (measured by CRI) of blood and spleen. Six-month repopulating ability of PBSC, spleen or marrow cells from B6-Hbb^s-treated mice mixed 1:1 with B6-Hbb^d marrow cells was assessed in lethally irradiated (12.5 Gy, split dose) B6-Hbb^s. The combination of IL-11 and SCF synergistically increased the capacity of PBSC and spleen cells to provide 6-month hematopoiesis. However, as single agents in splenectomized donor mice, SCF increased the long-term competitive repopulating ability of blood (CRI = 1.63) while IL-11 did not (CRI = 0). IL-11 enhanced SCF mobilization of stem cells from marrow to blood, since CRI of marrow from splenectomized donor mice after combination IL-11 and SCF decreased to 0.3 while CRI of marrow after SCF treatment was 0.8 (control = 1.0). Although IL-11 may not be useful as a single-agent cytokine for PBSC mobilization, its synergistic effect with SCF was clearly demonstrated.

The recently cloned rhflt-3 ligand[54] had a synergistic effect in combination with rhG-CSF in increasing peripheral blood mobilization of CFU-S.[55] Mice injected with both flt-3 ligand 20 μg/kg and G-CSF 250 μg/kg for 5 days yielded a 384-fold increase in CFU-S compared with an 18-fold increase in CFU-S with G-CSF alone. It is not yet known if these findings translate into the ability of flt-3 ligand to

mobilize stem cells that are capable of long-term hematopoiesis in lethally irradiated recipients.

Lord et al[56] reported that BB-10010, a variant of human macrophage inflammatory protein-1 (MIP-1α), mobilized mouse PBSC in synergy with G-CSF. Thirty minutes after a single subcutaneous dose of MIP-1α (2.5 μg/kg of BB-10010), there was a twofold increase in circulating CFU-S and progenitors with marrow repopulating ability (MRA, similar in design to the pre-CFU-S assay). Following 2 days of G-CSF treatment (twice daily injections of 100 μg/kg), circulating CFU-S and MRA increased by 25- and 27-fold respectively. A single administration of MIP-1α after 2 days of G-CSF treatment increased circulating CFU-S and MRA even further to 38- and 100-fold. Splenectomized mice had even greater increases in CFU-S and MRA, with similar pattern of mobilization. The long-term engraftment potential of rapidly mobilized primitive progenitor cells using the combination of cytokines awaits future study.

The important conclusions from these studies are that PBSC transplants in the mouse are feasible, and that the technology for assessing long-term hematopoiesis makes the mouse a useful tool for quickly comparing mobilization strategies. It is important to stress that 6-month hematopoiesis in the mouse does not translate into long-term hematopoiesis in humans, however. The various cytokines assayed indicate that there are multiple agents capable of mobilizing stem cells. The synergistic effect of combined or sequential cytokine administration may promote higher yields of mobilized stem cells. Further progress in this area awaits the elucidation of the specific molecular and cellular mechanisms by which cytokines influence stem cell mobilization. That so many cytokines have been shown to mobilize stem cells suggests that complex signalling within the hematopoietic cell differentiation can be strongly influenced at several points to enhance the peripheralization of progenitor cells. Elucidation of the cellular mechanisms of mobilization will lead to better understanding of the control of hematopoiesis.

LARGE ANIMALS

While mouse studies have shown the enormous potential for using cytokine-mobilized blood stem cells as a source of stem cells for hematopoietic transplantation, large-animal studies are necessary to determine the PBSC's efficacy to maintain long-term hematopoiesis after transplant. Compared with large animals, mice have much more limited hematopoietic demands over the lifespan of the animal. For example, Abkowitz et al[57] have estimated that the cat has at least 3750 times more red blood cells produced per lifetime compared with the mouse. Humans have over 30 000 times the number of blood cells produced per lifespan compared to the mouse (Table 1.1). Thus, questions of stem cell self-renewal potential and long-term hematopoiesis can really be convincingly studied only in larger animals in order to draw meaningful clinical comparisons.

In addition to the questions of long-term hematopoiesis, the large-animal models are more accurate at predicting the incidence and severity of GVHD in humans. For example, with most mouse strain combinations, GVHD is not observed after crossing non-major histocompatibility complex (MHC) H-2 class barriers.[15,16,58] In many cases, even MHC H-2 disparity may not result in fatal GVHD. In contrast, acute GVHD is uniformly seen in MHC-incompatible canine, simian or human marrow stem cell graft recipients.[59,60] Canine studies were the first to show fatal GVHD in recipients of marrow from DLA-identical littermates when transplants were done without postgrafting immunosuppression, a finding that was subsequently confirmed in humans.[61] Thus, the large-animal models have been more accurate and more relevant to predicting clinical outcome in humans. For this reason, large-animal models will continue to be necessary to assess new treatment/mobilization regimens and hematopoietic stem cell manipulation procedures prior to use in humans.

Canine stem cell mobilization

Studies in outbred dogs showed recombinant canine (rc)G-CSF and rcSCF to be synergistic with regard to mobilizing transplantable progenitor cells into the peripheral blood.[62] rcG-CSF (10 μg/kg/day) for 7 days led to a 5.4-fold increase in CFU-GM/ml of blood, compared with 7 days of high-dose rcSCF (200 μg/kg/day), which gave an 8.2-fold increase. Low-dose SCF (25 μg/kg/day) administered for 7 days had no effect on CFU-GM. However, rcG-CSF plus low-dose SCF resulted in a 21.6-fold increase in CFU-GM, a significant difference compared with low-dose SCF alone ($p = 0.03$). The 25 μg/kg/day dose level for low-dose SCF was based on the observation that this may approximate the maximum tolerated dose in humans.[63]

To assess the ability of G-CSF and SCF to increase the circulation of cells capable of rescuing dogs after lethal TBI, 1×10^8 mononuclear cells/kg were collected and cryopreserved from dogs after 7 days of treatment with G-CSF, SCF or a combination of the two. One month later, dogs were exposed to 9.2 Gy TBI and transplanted with the previously collected cells. All three control animals transplanted with 1×10^8 non-mobilized PBMC/kg died with marrow aplasia 11–29 days after TBI. Similarly, dogs give PBSC mobilized with low-dose SCF only failed to recover. In contrast, all 15 dogs given PBSC collected after G-CSF, high-dose SCF, or low-dose SCF plus G-CSF recovered granulocyte function. The mean period to obtain 500 PMN/μl was 17 days (G-CSF), 18.8 days (SCF) and 13.6 days (low-dose SCF plus G-CSF). The mean period to reach 20 000 platelets/μl was 42 days (G-CSF), 46 days (SCF) and 37 days (low-dose SCF plus G-CSF). In the combination group, all five dogs survived with stable trilineage engraftment to >180 days. This study was not designed to study long-term repopulation, since PBSC may have provided only short-term repopulation until reconstitution of autologous hematopoiesis from radiation-resistant stem cells occurred. The study showed that both SCF and G-CSF dramatically increased the level of PBSC and that these growth factors act synergistically to mobilize stem cells from the marrow into the blood.

Non-human primate stem cell mobilization

Andrews et al[64,65] have shown that rhSCF in combination with rhG-CSF mobilized greater numbers of progenitor cells that could be collected by a single 2-hour leukapheresis than did rhG-CSF alone. One group of baboons was administered SCF (25 μg/kg/day) plus G-CSF (100 μg/kg/day), while a second group received G-CSF alone (100 μg/kg/day). Each animal underwent a single 2-hour leukapheresis on the day that the number of progenitor cells per volume of blood was maximal (day 10 for SCF plus G-CSF and day 5 for G-CSF-treated animals). For baboons administered SCF plus G-CSF, the leukapheresis products contained eightfold more progenitor cells (CFU-GM) compared with animals treated with G-CSF alone.

In baboons that were exposed to a lethal dose of 10.7 Gy TBI followed by administration of cryopreserved leukapheresed cells, more rapid autologous trilineage engraftment was observed using PBSC mobilized by SCF plus G-CSF compared with G-CSF alone. In animals transplanted with PBSC mobilized by SCF plus G-CSF, the times to engraftment of neutrophils (ANC > 500) and platelets (>20 000) were 12 and 8 days respectively, compared with 24 and 42 days using PBSC mobilized by G-CSF alone. These results demonstrate enhanced mobilization of progenitor cells with long-term engraftment potential using SCF plus G-CSF in non-human primates, which is similar to findings in dogs and splenectomized mice and in preliminary results from phase I/II studies in humans.

In findings similar to those described in mice, Laterveer et al[66] reported that IL-8 led to a dose-dependent 10–100-fold increase in CFU-GEMM in peripheral blood of rhesus monkeys, within 30 minutes of injection. By 4 hours after injection, CFU-GEMM had returned to pretreatment levels. Hastings et al[67] reported that concomitant administration of rhIL-11 (100 μg/kg/day) and rhG-CSF (10 μg/kg/day) subcutaneously for 7 days in *Cynomolgus* monkeys resulted in a 61–141-fold increase in CFU-GM above baseline. This was not significantly different than G-CSF administration alone. However, monkeys receiving IL-11 × 4 days followed by G

CSF × 4 days showed a 90–350-fold increase in CFU-GM above baseline. This suggested that IL-11 followed by G-CSF enhances stem cell mobilization, and that IL-11 acted via a distinct hematopoietic cell differentiation mechanism.

Gasparetto et al[68] showed in *Cynomolgus* monkeys that rhIL-1β (1 μg/kg, single intravenous dose) resulted in a 100-fold increase in CFU-GM 4 days after injection and a 10–15-fold increase in secondary CFU-GM (similar to LTC-IC assays) for 2–7 days after injection. Autologous PBSC (1×10^7 mononuclear cells) collected either 24 or 72 hours after IL-1β was able to rescue all four monkeys following 10 Gy TBI, but only two of the four survived with normal neutrophil counts beyond 60 days. One of two non-mobilized control monkeys survived long-term following PBSC autotransplant. In addition, Gasparetto et al reported that neither a single injection of IL-3 (20 μg/kg), G-CSF (50 μg/kg) nor GM-CSF (50 μg/kg) resulted in significant CFU-GM or secondary CFU-GM increases. It is not clear if humans could tolerate the dose of IL-1β needed to mobilize PBSC in this study; dose-limiting toxicity including fever and hypotension was reported in patients receiving 0.1 μg/kg IL-1β following 5-FU administration.[69]

In rhesus monkeys, Geissler et al[70] compared IL-3 (33 μg/kg/day subcutaneously for 11–14 days) followed by GM-CSF (5.5 μg/kg/day for 5–14 days) with either GM-CSF or IL-3 alone. There was a 65-fold increase in peripheral blood CFU-GM in the sequential protocol compared with 12- and 14-fold respectively in the others. This study contrasts with the findings of Gasparetto et al, emphasizing the point that optimal PBSC mobilization depends upon dose, duration and method of administration of specific cytokines.

It is unknown what specific molecular signals cause mobilization of hematopoietic progenitor cells from marrow to peripheral blood. However, it appears that cellular interactions between hematopoietic cells and their microenvironment (such as marrow stromal cells) are important for development and function, and several adhesion molecules may be critical for these interactions.

Investigating the role of the cytoadhesion molecule VLA-4 on hematopoietic cells, Papayannopoulou and Nakamoto[71] have shown that systemic treatment of primate with antibody to the VLA-4 integrin molecule (HP1/2 administered intravenously for 4 days at a dose of 1 mg/kg) resulted in a 10–100-fold increase in circulating CFU-GM. There was a synergistic effect observed with G-CSF (30 mg/kg/day for 5 days) followed by anti-VLA-4 antibody administration (HP1/2, 1 mg/kg/day for 2 days), with a significant increase in CFU noted compared with G-CSF alone. The total CFU/ml increased from 2500 (G-CSF) to 13 000 (G-CSF plus anti-VLA-4 antibody). This suggests independent mechanisms by which G-CSF and anti-VLA-4 antibody can mobilize stem cells. Based on studies in mice and humans, VLA-4 is an adhesion molecule on hematopoietic cells. The anti-VLA-4 antibody appears to block the interaction between hematopoietic stem cells and marrow stroma, resulting in increased stem cell mobilization. The anti-VLA-4 antibody may be clinically useful to increase the yield of PBSC for transplantation. The identification of additional cell-surface/adhesion molecules that are involved in stem cell mobilization may reveal additional mechanisms with which to improve blood stem cell yield for clinical use.

The optimal time to harvest PBSC mobilized by either chemotherapy, cytokines or antibodies to adhesion molecules has been identified as the day(s) of peak CFU after mobilization. For clinical purposes, this is a cumbersome assay since there is significant interlaboratory variation in the measurement of CFU, and since progenitor assays take 10–14 days to read. Thus, there has been interest in using CD34 antigen flow cytometric analysis as a rapid and reliable clinical marker for measuring peak PBSC. Based on recent evidence in human studies, it appears that the day(s) of peak $CD34^+$ cells in the peripheral blood after cytokine mobilization correlates with the optimal time for leukapheresis of transplantable PBSC.[72]

GRAFT-VERSUS-HOST DISEASE AND BLOOD STEM CELL TRANSPLANTATION

There have been no published reports describing the use of non-syngeneic donors in the

mouse model to evaluate graft rejection or GVHD. However, the potential clinical application of allogeneic PBSC makes it vital to study the parameters influencing graft rejection and GVHD in large animals. This is particularly important in view of the larger numbers of T cells in unfractionated leukapheresed PBSC.[73] Previous studies in both small and large animals have shown high T-cell (10^7 cells/kg) doses to result in increased acute GVHD.[4,15,16] In humans given buffy coat infusion post-transplant (which has a 1- to 2-log increase in T-cell content compared with marrow), there is an increased incidence of chronic GVHD.[74]

The dog has been a very useful model for studying clinically relevant problems of GVHD in allogeneic marrow transplantation. The question of whether or not PBSC are more likely to cause acute or chronic GVHD remains an important issue. While preliminary clinical evidence suggests that there is no increased incidence of acute GVHD in allogeneic human PBSC transplants, the incidence of chronic GVHD remains unknown. Thus, it was important to determine the feasibility of the dog model to assess GVHD in this setting.

Sandmaier et al[75] used mobilized PBSC obtained via leukapheresis for allogeneic transplantation in dogs. PBSC were obtained after either 7 days of low-dose rcSCF (25 μg/kg/day) plus rcG-CSF (10 μg/kg/day), or after G-CSF alone. Donors showed up to 25-fold increases in peripheral blood CFU-GM after growth factor treatment. Eighteen dogs were given a median of 17.1×10^8 mononuclear cells/kg (median number of CFU-GM infused was 27×10^4 kg) from littermate donors after 9.2 Gy of TBI. No post-grafting immunosuppression was given. In DLA-haploidentical littermate recipients given PBSC mobilized with SCF plus G-CSF or G-CSF, the rate of engraftment was higher (100%) than observed with marrow alone, where 75% of dogs failed to engraft. All haploidentical recipients developed fatal hyperacute GVHD. Recipients of DLA-identical transplants all developed GVHD, which was fatal in 30% and transient in 70%. The incidence and severity of acute GVHD was similar to that expected after marrow grafts. This model will allow for further treatment, including T-cell depletion to minimize GVHD without increasing graft rejection.

The only other reported animal model of allogeneic G-CSF-mobilized PBSC used rabbits.[76] Adult outbred red Burgundy rabbits served as donors of histoincompatible New Zealand White recipients of the opposite sex. Three different schedules of PBSC collection and infusion were tested, none of which showed significant differences in engraftment rate or incidence of GVHD. PBSC were mobilized with rhG-CSF (10 μg/kg/day administered subcutaneously for 14 or 18 days), collected during either three or six apheresis sessions, and infused at a single time or 4 or 5 days after recipients were given a single dose of 10 Gy TBI delivered at 0.2 Gy/min from a ^{60}Co source. This dose of TBI was not lethal, and resulted in 50% recovery of autologous hematopoiesis. Recipients were administered cyclosporin as GVHD prophylaxis. Repetitive collections and infusions of PBSC decreased the period of aplasia compared with historical controls receiving unmanipulated marrow. Two of the 15 animals died of TBI toxicity, while all others engrafted. Two animals became long-term complete chimeras, 8 died of acute GVHD and 3 died of infection. These results indicated that PBSC mobilized with G-CSF could engraft across MHC barriers with an incidence of GVHD that was no different from unmanipulated marrow.

These large-animal studies suggest that allogeneic mobilized PBSC have a higher rate of sustained engraftment than marrow and cause no higher incidence of GVHD despite the infusion of increased numbers of lymphocytes. The improved rate of engraftment in mismatched transplants could be due to increased numbers of transplanted hematopoietic progenitor cells or T cells in the graft. It remains to be determined if there is any immunologic difference between growth-factor-mobilized PBSC compared with marrow.

SUMMARY

Animal models have been developed that are very useful for the study of PBSC in both autologous and allogeneic transplantation. Although

the existence of stem cells in the peripheral blood has been known for over 30 years, clinical use of PBSC has become feasible only recently with the advent of mobilization of these cells from marrow to blood with the help of hematopoietic growth factors. This chapter has reviewed studies that have been critical in identifying cytokines capable of enhancing PBSC mobilization. Large-animal models such as dogs and non-human primates, which have been historically instrumental for the development of clinical marrow transplantation, represent ideal settings for evaluating clinically relevant issues in allogeneic PBSC transplantation.

Animals such as the mouse and rat with limited hematopoietic demands lend themselves to the study of stem cell mobilization and the basic molecular biology of hematopoiesis. However, large-animal models will continue to be crucial for studying long-term hematopoiesis with its increased stem cell demands. Attempts to improve the safety and effectiveness of PBSC for allogeneic transplantation with in vitro manipulation of hematopoietic cells using molecular biology and immunologic techniques will continue to require the use of large-animal models.

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