Transpl Int (2000) 13 [Suppl 1]: S541–S546

S. Janczewska
A. Ziolkowska
B. Interewicz
T. Majewski
W. L. Olszewski
B. Lukomska

Vascularized bone marrow transplanted in orthotopic hind-limb stimulates hematopoietic recovery in total-body-irradiated rats

S. Janczewska · A. Ziolkowska · B. Interewicz · T. Majewski · W. L. Olszewski · B. Lukomska (✉)
Department for Surgical Research and Transplantology, Medical Research Center Institute, Polish Academy of Sciences, 5, Pawinskiego Street, 02 106 Warsaw, Poland

W. L. Olszewski
Norwegian Radium Hospital, Montebello, 03–10 Oslo, Norway

Abstract Hematopoietic recovery after bone marrow transplantation (BMT) is reported to be slow with long-lasting immune deficiency. This may be attributable to lack of a proper microenvironment for hematopoietic cell proliferation and differentiation. We have designed a model in which complete hematopoietic reconstitution of lethally irradiated rats can be achieved by vascularized bone marrow transplantation (VBMT) in an orthotopic hind-limb graft. The aim of the study was to investigate the process of repopulation of bone marrow cavities and peripheral blood of irradiated rats after VBMT and, in particular, to follow the contribution of grafted BM cells and residual recipient BM cells in hematopoietic regeneration. Lewis hind-limbs were transplanted orthotopically to totally irradiated (8 Gy) syngeneic sex-mismatched recipients (VBMT). In the control group 8×10^7 BM cells in suspension were injected intravenously (BMCT). After 10 days BM and peripheral blood (PB) cells were collected from the recipient. For cell subset analysis cytomorphological evaluation of BM smears and flow cytometry of PB cells were performed. Additionally, PCR was performed using specific primers for rat Y chromosome (sex-determining region Y-*Sry*) to detect male (donor or recipient) cells in sex-mismatched BM graft recipients and the products were analyzed by electrophoresis. VBMT brought about much faster replenishment of nucleated cells in BM and PB than did BMCT. Cytometry analysis of PB cells revealed more lymphocytes in VBMT than in BMCT recipients. The amount of donor DNA of bands corresponding to Y-*Sry* was also higher in PB cells of VBMT than of BMCT recipients. The presence of host DNA was observed in PB cells of VBMT rats but was not detected in PB population of BMCT recipients. VBMT is highly effective in hematopoietic reconstitution of irradiated recipients. The fast cell maturation and repopulation may be due to the presence of stromal cells transplanted in a normal spatial relationship with donor hematopoietic cells in hind-limb graft. Self renewal of radioresistant host cells was seen after VBMT but not after BMCT.

Key words Bone marrow transpantation · Stromal cells · Hematopoietic repopulation

Introduction

Bone marrow transplantation (BMT) is routinely used for treatment of hematologic diseases. However, graft failure is a major complication in bone marrow (BM) recipients. Reconstitution of hematopoiesis is important to reduce morbidity and mortality in patients subjected to radiotherapy and chemotherapy before BMT. The mechanisms that regulate hematopoiesis remain incompletely understood but involve interactions of progenitors with stromal cells and extracellular matrix components in the respective microenvironment [2, 22, 26]. Routinely bone marrow is transplanted as a suspension of cells infused intravenously. This population contains hematopoietic but not stromal cells. Delayed engraftment of transplanted progenitor cells may be attributable to lack of a proper hematopoietic microenvironment in patients after myeloablative therapy. Recently it has been demonstrated that the recruitment of donor-derived stromal cells by bone grafts effectively prevents graft failure [10, 11]. Transplantation of bone along with BM cells has been shown to facilitate reconstitution of the host with hematopoietic cells of donor origin [8, 20]. The question arises whether this improved hematopoietic recovery is due only to the expansion only of transplanted progenitors or also of host radioresistant residual cells. Since interaction of progenitor cells with the BM stromal microenvironment is required for development, maturation, and differentiation of hematopoietic cells, we used a model of vascularized bone marrow transplantation (VBMT) in hind-limb graft where lymphohematopoietic progenitors remain in contact with stromal cells.

The aim of the study was to investigate the process of repopulation of bone marrow cavities and peripheral blood of irradiated rats after VBMT and to evaluate the contribution of grafted donor BM cells and residual BM cells of the recipient to the hematopoietic regeneration. Syngeneic sex-mismatched BMT that does not evoke graft-versus-host disease or need immunosuppressive drugs was chosen since it gave more direct insight into the mechanisms involved in hematopoietic recovery after BMT.

Materials and methods

Animals

Three-month-old male and female Lewis rats (RT1 l), bred and maintained in our own facilities, were used throughout this study. BM graft recipients were exposed to total body γ-irradiation (8 Gy) from a ^{60}Co source (Theratron) at the dose rate of 150 cGy/min. This dose was selected as it caused BM death in 100% of irradiated rats without BMT after 11–16 days.

Vascularized bone marrow in hind-limb transplantation

Donor preparation

The donor hind-limb was amputated at groin level with the femoral and iliac arteries and veins dissected above this level to obtain a long vascular stump for anastomoses.

Recipient operation

A hind-limb of the recipient was amputated at the mid-thigh level. The femur was fixed with an intramedullary metallic stent. End-to-end anastomoses of the arteries and veins were made with the recipient vessels using 10.0 monofilament sutures. The stumps of the sciatic nerve were stitched. Muscles were joined and the skin was closed.

Experimental design

Studies were carried out in four experimental groups. BM was transplanted in syngeneic sex-mismatched recipients in order to determine whether repopulating hematopoietic cells were of donor or recipient origin. In group I, total body irradiation was followed by VBMT in hind-limb from male rats into female recipients. In group II, total-body-irradiated (TBI) female rats received intravenous infusion of male BM cell suspension (8×10^7 cells). In group III, TBI male rats received VBMT from female donors. In group IV, female BM cells were transplanted intravenously into TBI male rats.

Cell collection

Ten days after irradiation and BMT, BM from the recipient tibia and peripheral blood (PB) were harvested.

Cytomorphologic evaluation

Differential morphologic evaluation of BM cells was performed on Giemsa-stained cytospins under light microscopy. At least 1000 cells were examined for each spin.

Flow cytometry analysis

Cell subsets from PB were analyzed using mouse anti-rat monoclonal antibodies CD43 (W3/13), CD15 (HIS 48), CD14 (ED1), CD19 (OX12), CD5 (OX1), CD4 (W3/25), CD8 (OX8), CD56 (3.2.3) (Serotec, Oxford). For cell labeling, each of the various antibodies was used at an optimal dilution based on preliminary dose-response titration. The second-step antibody was $F(ab')_2$ rabbit anti-mouse IgG conjugated with FITC. One-color analysis was performed on FACStar (Becton Dickinson, San Jose, California). Data were analyzed using Lysys II software from Becton Dickinson.

PCR reaction

Genomic DNA was isolated from BM and PB cells of BM recipients using DNAzol Reagents (Gibco BRL). Quantification of DNA was performed spectrophotometrically on GeneQuant.

PCR reaction mixture contained 5 μg DNA, 1.5 U Taq DNA polymerase (PCR Beads, Pharmacia, Biotech) and 25 pmol rat Y-*Sry*-specific oligonucleotide primers (sense, 5' GAGAGAGGCACAAGTTGGC-3'; antisense, 5' AATACCAGTGGATGTGATGCGG-3'). PCR amplification was carried out in a Thermal Cycler (MJ Research) by 5 min denaturation at 94 °C, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (54 °C, 30 s), extension (72 °C, 30 s), and a final extension step (72 °C, 5 min). The products were analyzed by electrophoresis in 12.5% PAGE (Phast System, Pharmacia Biotech), followed by silver staining (Silver Staining Kit, Pharmacia, Biotech). The gels were scanned and analyzed by One-Dscan software (Scanalytics, Inc.).

Statistics

Results were presented as mean percentage ± SD. For statistical analysis Student's *t*-test was used. Differences in the data were considered significant when $P < 0.05$.

Table 1 Cellular analysis of BM isolated from tibia 10 days after total body irradiation and BMT. Results presented as mean percentage ± SD ($n = 7$)

	Normal rats	VBMT recipients	BMCT recipients
Myeloid cells			
Myeloblasts	2.2 ± 0.9	4.2 ± 2.9	3.4 ± 1.7
Promyelocytes	6.7 ± 0.8	6.7 ± 2.4	7.6 ± 3.4
Myelocytes	8.0 ± 3.6	7.4 ± 1.0	7.7 ± 2.9
Metamyelocytes	10.8 ± 1.3	6.5 ± 2.0	7.2 ± 1.9
Juvenile neutrophils	12.2 ± 1.5	8.1 ± 2.3	6.2 ± 1.7
Segmented neutrophils	9.3 ± 3.9	6.1 ± 2.8	6.7 ± 3.0
Monocytes	1.4 ± 1.5	0.7 ± 1.2	0.9 ± 0.8
Lymphoid cells			
Lymphocytes	9.2 ± 4.3	6.6 ± 3.3	3.5 ± 1.4
Plasma cells	2.1 ± 0.1	0.8 ± 0.1	0.8 ± 0.5

Results

Bone marrow cells

The BM cell yield of the TBI recipients of VBMT was $10.8 \pm 5.4 \times 10^7$/g bone, compared with $33.0 \pm 5.5 \times 10^7$/g in the normal, nonirradiated rats. In groups who received intravenous BMCT cell yield was 3.4×10^7/g bone. There were no differences in the proportions of myeloid cell subsets between VBMT and BMCT recipients and normal rats. In granulocytic cell lines the percentage of immature forms (i.e. myeloblasts, promyelocytes, myelocytes, metamyelocytes, juvenile neutrophils and segmented neutrophils) in BM of VBMT and BMCT recipients resembled those observed in the control rats. However, the proportions of T lymphocytes present in BM of VBMT recipients was higher than in BMCT recipients and normal rats (6.6 ± 3.3 vs 3.5 ± 1.4 vs 9.2 ± 4.3%, $P = 0.02$ respectively) (Table 1).

Leukocyte composition of PB

PB leukocyte yield of TBI recipients of VBMT was $23.8 \pm 3.4 \times 10^2$/μl and that of TBI BM cell-infused rats was $4.4 \pm 9.4 \times 10^2$/μl, in comparison to $47.0 \pm 12.4 \times 10^2$/μl in normal, nonirradiated rats. Significant differences were found in the percentages of various cell subsets between normal, VBMT, and BMCT rats. Ten days after TBI and BM transplantation, PB of VBMT and BMCT recipients revealed more granulocytes than that of the normal rats. However, the percentage of $CD15^+$ cells was much lower in VBMT rats than in BMCT recipients (42.5 ± 10.8% vs 61.0 ± 8.6%, $P = 0.01$). After both types of BM transplantation the relative number of T cells was significantly lower than in normal controls. In our studies T lymphocyte recovery was more evident in VBMT than in BMCT recipients. There were more T cells and different T cell subsets in PB cells after VBMT than after BMCT (40.6 ± 2.1% vs 23.0 ± 1.6% $CD5^+$, 25.4 ± 3.3% vs 16.8 ± 0.8% $CD4^+$, 18.7 ± 3.5% vs 8.2 ± 4.3% $CD8^+$ cells, $P = 0.01$ respectively). Fewer B cells, NK cells, and monocytes were observed in the PB population of BM-transplanted rats than in normal rats. No difference was found in the percentage of these cells between VBMT and BMCT recipients (1.7 ± 0.7% vs 1.3 ± 0.8% $CD19^+$, 1.1 ± 0.5% vs 0.6 ± 0.9% $CD56^+$ and 2.4 ± 0.4% vs 1.4 ± 0.6% $CD14^+$ cells, respectively) (Figs. 1, 2).

Determination of donor and recipient cells by PCR analysis

Cellular DNA extracted from BM and PB of VBMT and BMCT female recipients of male BM graft demonstrated the donor-derived Y chromosome fragment. The absorbance of amplified DNA bands corresponding to the sex-determining region Y-*Sry* in BM and PB cells of VBMT female rats was higher than in BMCT female recipients ($A = 0.67$ vs 0.27 and 0.41 vs 0.22, respectively). Moreover, in the female to male BMT a high amount of recipient DNA was found in BM and PB cells of VBMT but not BMCT recipients. Amplification with the Y-*Sry*-specific primers demonstrated PCR products of $A = 0.32$ in BM cells and 0.41 in PB cells of VBMT rats.

Discussion

The results of our studies indicate that VBMT in syngeneic hind-limb is highly effective in cellular reconstitution of BM and PB. The fast repopulation of BM cavi-

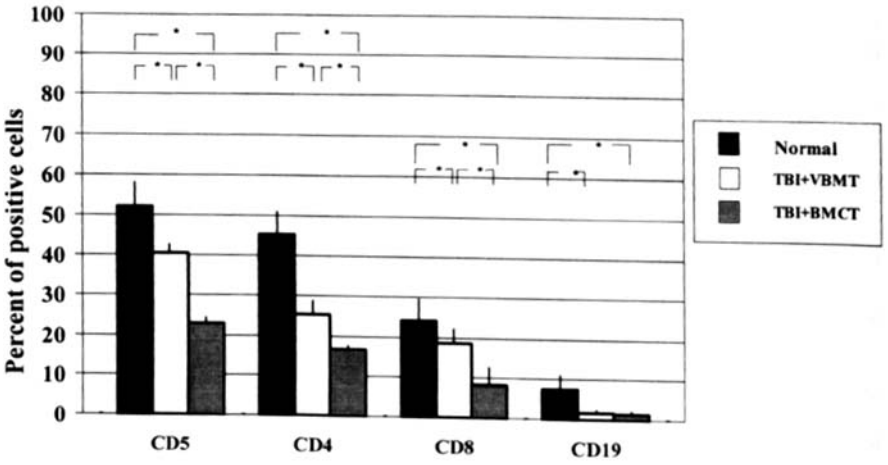

Fig. 1 Cell subset analysis in peripheral blood of total-body-irradiated rats transplanted with bone marrow. Results are presented as the mean percentage of CD43-, CD15-, CD14-, and CD56-positive cells ± SD ($P < 0.02$)

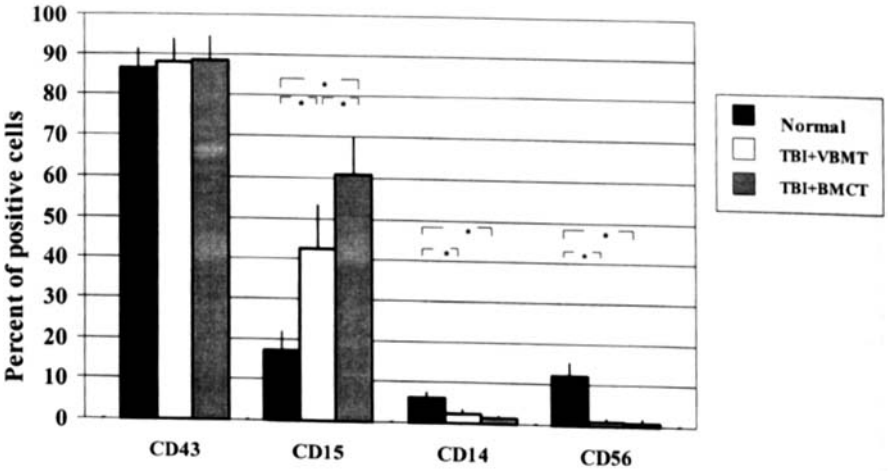

Fig. 2 Phenotype analysis of peripheral blood leukocytes of total-body-irradiated rats transplanted with bone marrow. Results are presented as the mean percentage of CD5-, CD4-, CD8-, and CD19-positive cells ± SD ($P < 0.02$)

ties from hind-limb graft, containing BM in a most natural form, remains in sharp contrast with the results obtained in rats inoculated intravenously with BM cell suspension. It seems that transplantation of BM cells together with stromal cells in their natural spatial relationship allows immediate resumption of hematopoietic activity of the graft.

Successful engraftment with BM cells depends on several factors, among them a sufficient number of stem cells with expressed homing receptors for adhesion molecules in the BM compartment and stromal cells actively expressing surface ligands for stem cell receptors, releasing hematopoietic cytokines and extracellular matrix proteins sequestering growth factors from the microenvironment. The necessity of stem cell–stromal cell cooperation has been well documented [1, 14, 25]. The results of our studies strengthen this view, showing evident differences in tibial BM cell yield between the group with VBMT in hind-limb graft and the group with an equivalent number of BM cells transplanted in suspension (BMCT) 10 days after grafting. BM cell yield after BMCT was found to be three times lower than after VBMT. Although no differences in the percentage of various BM cell subsets of myeloid lineage were generally seen, the lymphocyte count was lower in the BMCT group.

Ten days after BMCT, PB of graft recipients contained predominantly granulocytes with a small number of lymphocytes. Reappearance of neutrophils and platelets is often considered the endpoint of hematopoietic recovery after chemotherapy and BM transplantation. This ignores the question of lymphoid reconstitution. A significant decrease of T cells has been reported after BM transplantation [9, 17–19, 24]. Generally, T cells can regenerate along two different pathways: one is thymus dependent and might be considered as a recapitulation of ontogeny, while in the other, T cells can be reconstituted through peripheral expansion of mature T cells cotransplanted with the BM graft. In most patients inadequate thymic function means that peripheral expansion of T cells is the primary pathway [4, 5, 17, 18]. We observed that, in contrast to BMCT, after VBMT the number of T lymphocytes was reconstituted fairly rapidly [6, 7, 13, 15, 16]. The faster recovery of T

cells observed after VBMT than after BMCT might be due to the presence of a greater number of T lymphocytes in hind-limb graft than in BM cell suspension infused intravenously. It could also depend on extrathymic maturation of donor stem cells. T cell differentiation occurs extrathymically in the lymph nodes, spleen, BM, liver, and intestine. T lymphocytes generated in the liver or the BM have the potential to disseminate and repopulate irradiated recipients [4, 21, 23]. In our studies, in the female recipients of male BM transplants a higher amount of donor DNA was observed in BM and PB cells of VBMT recipients. The absorbance of amplified DNA bands, corresponding to the sex-determining region Y-*Sry*, was higher after VBMT than after BMCT. It resulted from higher number of donor DNA copies subsequently to higher concentration of donor cells in VBMT recipient tissue in comparison with BMCT rats.

It has been reported previously that after BM transplantation hematopoietic cells may also reconstitute from residual recipient cells [9, 27]. These cells might be seeded in the BM or lymph organs and contribute to lymphohematopoietic regeneration after BM ablation. Ishida et al. reported on the presence of radioresistant (9.5 Gy) stem cells in mice [12]. There is also evidence that rare recipient T cells can survive conditioning regimens used for BMT [3]. Using PCR amplification of DNA regions Y-*Sry* we were able to determine the donor origin of cells isolated from TBI male recipients of female BM graft. Our data showed that BM and PB cells of VBMT rats originated from the host cells. These cells were not observed in BMCT recipients. It suggests that the mechanism of VBMT stimulation of hematopoietic cell engraftment is different from that of BMCT. In BMCT recipients hematopoietic cells were of donor origin, whereas after VBMT, BM and PB cells had a mixed origin with cells derived from both transferred donor and surviving host cells.

In conclusion, the results of our study show that VBMT is highly effective in hematolymphoid reconstitution of irradiated rats. The fast cell maturation and repopulation may due to the presence of stromal cells transplanted with donor hematopoietic cells in hind-limb graft. Interestingly, apart from efficient donor cell proliferation, self-renewal of radioresistant host cells occurred after VBMT. This was not observed after intravenous BMCT.

Acknowledgements This work was supported by grant no. 4PO5AO4915 from the Committee for Scientific Research.

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