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Engraftment Efficiency after Intra–Bone Marrow versus Intravenous Transplantation of Bone Marrow Cells in a Canine Nonmyeloablative Dog Leukocyte Antigen-Identical Transplantation Model

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A B S T R A C T

An intra–bone marrow (IBM) hematopoietic stem cell transplantation (HSCT) is assumed to optimize the homing process and therefore to improve engraftment as well as hematopoietic recovery compared with conventional i.v. HSCT. This study investigated the feasibility and efficacy of IBM HSCT after nonmyeloablative conditioning in an allogeneic canine HSCT model. Two study cohorts received IBM HSCT of either density gradient (IBM-I, n = 7) or buffy coat (IBM-II, n = 6) enriched bone marrow cells. An historical i.v. HSCT cohort served as control. Before allogeneic HSCT experiments were performed, we investigated the feasibility of IBM HSCT by using technetium-99m marked autologous grafts. Scintigraphic analyses confirmed that most IBM-injected autologous cells remained at the injection sites, independent of the applied volume. In addition, cell migration to other bones occurred. The enrichment process led to different allogeneic graft volumes (IBM-I, 2×5 mL; IBM-II, 2×25 mL) and significantly lower counts of total nucleated cells in IBM-I grafts compared with IBM-II grafts (1.6×10^8/kg versus 3.8×10^8/kg). After allogeneic HSCT, dogs of the IBM-I group showed a delayed engraftment with lower levels of donor chimerism when compared with IBM-II or to i.v. HSCT. Dogs of the IBM-II group tended to reveal slightly faster early leukocyte engraftment kinetics than intravenously transplanted animals. However, thrombocytopenia was significantly prolonged in both IBM groups when compared with i.v. HSCT. In conclusion, IBM HSCT is feasible in a nonmyeloablative HSCT setting but failed to significantly improve engraftment kinetics and hematopoietic recovery in comparison with conventional i.v. HSCT.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is an established treatment for a variety of malignant and nonmalignant hematologic diseases. Successful engraftment after HSCT mainly depends on pre- and post-transplantation immunosuppression, type and composition of the graft, and the number of infused HSCs. Although immunosuppression can be influenced and the graft composition can be modified as well (eg, by the depletion of T cells [1] or cell subpopulations [2]), low numbers of available HSCs (weak grafts) remain a clinical challenge. Transplantation of weak grafts with limited HSC numbers into a recipient is usually accompanied by a delayed hematopoietic engraftment, an increased risk of graft failure [3,4], and a delayed immune reconstitution [5]. Accordingly, novel concepts aiming at an improvement of HSCT results are strongly desirable.

After conventional i.v. administration, most HSCs get trapped in peripheral organs, mainly in the lung, liver, and spleen [6-8]. Only a small fraction of the infused HSCs can reach the bone marrow cavity [9,10]. One approach to optimize engraftment, particularly of weak grafts, might be the targeted infusion of HSCs directly into the bone marrow. In rodent models intra–bone marrow (IBM) HSCT has been shown to improve the homing efficiency and resulted in a

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faster hematopoietic recovery and engraftment of HSCs compared to i.v. HSCT [11-13]. Prolongation of the resting time of HSCs in the injected bone marrow by fixation of the HSCs in a collagen gel or a magnetic forces approach using magnetic beads could even further improve the engraftment compared with unmodified IBM controls [14,15]. In addition, the first studies in humans indicated that IBM HSCT decreases the incidence of graft-versus-host disease as well [16].

However, several questions remain unanswered. Which is the best graft composition? Is there an impact of graft volume on engraftment due to the limited available space in the bone marrow cavity? Furthermore, nearly all previous studies used myeloablative conditioning approaches; therefore, it remains unknown if IBM HSCT is also feasible after nonmyeloablative HSCT, a setting in which the stress on blood cell homeostasis is significantly less compared with myeloablative HSCT. To address these issues, we performed an IBM HSCT study in a canine nonmyeloablative HSCT model. Herein, we aimed to evaluate 2 different methods of graft processing for reduction of the graft volume for IBM injection. In the run-up to allogeneic HSCT the migratory capacity of the modified grafts was analyzed in vivo using technetium-99m (^{99m}Tc) labeled grafts in an autologous setting. This trafficking study was performed separately from all other investigations because of the known toxic potential of ^{99m}Tc on labeled cells. The analyses regarding engraftment and hematologic recovery were subsequently realized in the allogeneic HSCT model.

METHODS

Laboratory Animals

Litters of random-bred beagles were purchased from a laboratory animal breeder (Harlan-Winkelmann, Borchen, Germany) possessing a license for animal breeding and husbandry according to section 11 of the German animal protection law. Thirteen dog leukocyte antigen–identical donor–recipient sibling pairs were selected on the basis of matching for highly polymorphic MHC class I and class II microsatellite markers [17,18]. Three recipient dogs and 11 donors were of male gender. At the beginning of the study, recipient dogs were a median age of 1.5 years old (range, 1.0 to 3.0) with a median weight of 14.6 kg (range, 9.0 to 17.0). All dogs were dewormed and vaccinated against rabies, distemper, parvovirus, leptospirosis, hepatitis, and parainfluenza virus. The animals were housed in an accredited facility in standard indoor and outdoor runs and were provided commercial dog chow and tap water ad libitum. Kennels were equipped with dog houses and mats. Dogs had daily contact to keepers and investigators in a minimum 2-week period of familiarization before starting experiments and were trained to get used to handling and staff. Furthermore, they were kept in packs except for the early pretransplant phase. All experiments were approved by the review board of the state Mecklenburg-West Pomerania, Germany (State Institute for Agriculture, Food Safety and Fishery Mecklenburg-West Pomerania, Germany; Permit Number: LALLF M–V/TSD/7221.3-1.1-073/08).

Analyses of Autologous Cell Migration after IBM HSCT

Scintigraphic imaging was used to analyze the migratory capacity of IBM-transplanted cells. Therefore, bone marrow cells were collected under general intramuscular anesthesia with a mixture of 10 mg/kg ketamine and 2 mg/kg xylazine. A median volume of 74 ml (range, 70 to 80) bone marrow blood was harvested from the right humerus and femur of the dog. Metamizol was applied as standard prophylaxis (1250 mg, i.m.) and therapeutically if required (500 mg, oral or i.m.) to avoid postoperative pain.

HSCs were enriched using either buffy coat followed by Ficoll density gradient centrifugation (IBM_{auto}-I: total volume, 5 mL; $CD34^+$ cell count, 1.0 and 1.2×10^6/kg; n = 2) or buffy coat centrifugation only (IBM_{auto}-II: total volume, 25 mL; median $CD34^+$ cell count, 1.0×10^6/kg; range, .3 to 2.0×10^6/kg; n = 4). Cells were tagged with ^{99m}Tc-hexamethylpropyleneamine oxime (^{99m}Tc-HMPAO; Ceretec, GE Healthcare, Hamburg, Germany) containing an activity of 550 and 583 MBq, respectively (IBM-I) or a median of 854 MBq (range, 669 to 1350; IBM-II). The autologous cells were injected into a single site of the left humerus over an administration period of 5 minutes under general anesthesia. Scintigraphic measurements were performed 10 minutes, 50 minutes, 6 hours, and 24 hours after IBM injection using a PRISM 2000 XP gamma camera (Philips, Hamburg, Germany). Distribution of HSCs was assessed visually through the whole body. For control, ^{99m}Tc-HMPAO diluted in sodium chloride was used.

Allogeneic HSCT

The day (ie, 16 hours) before allogeneic HSCT, recipients were treated with 2 Gy total body irradiation (.25 Gy/min, n = 9; .1 Gy/min, n = 4). Grafts with a volume of 10 mL/kg body weight of the recipient were collected from the iliac crest, humerus, and femur of the respective donor under general anesthesia as mentioned above. Bone marrow aspirates were processed as described above to transplant 7 buffy coat/Ficoll density gradient enriched grafts (IBM-I group, total volume 2×5 mL) and 6 buffy coat enriched grafts (IBM-II group, total volume 2×25 mL). IBM HSCT on day 0 was performed under general anesthesia by concomitant intraosseous graft application through a single site into each the left humerus and femur over a period of 5 minutes.

Chimerism and cellularity analyses were conducted on bone marrow samples from the injection site and the contralateral side. Investigation of cellular graft composition regarding quantity of total nucleated cells (TNCs), $CD34^+$, $CD14^+$, and $CD3^+$ cells were performed by flow cytometric analysis. Pre- and postgrafting immunosuppression consisted of cyclosporine A (starting dose 15 mg/kg; days –1 to +35; twice daily, orally) in combination with mycophenolate mofetil (20 mg/kg; days 0 to +27; twice daily, orally). Clinical status of recipients was assessed daily and included the examination of body weight, ingestion, defecation, oral mucosa, eyes, and activity. Hematotoxicity was determined daily by leukocyte and platelet blood counts during early post-transplantation period (days 0 to +28) and weekly thereafter.

Assessment of Hematopoietic Chimerism

Genomic DNA of peripheral blood mononuclear cells (PBMCs), granulocytes, and bone marrow was isolated weekly up to day +70 and biweekly later on. Analyses of chimerism were performed via PCR amplification of polymorphic tetranucleotide repeats followed by a capillary gel electrophoresis [19]. Engraftment was defined as detection > 5% of donor-derived DNA in all analyzed cell populations. Graft rejection was defined as missing donor-derived DNA in 2 subsequent chimerism analyses.

Analyses of Cellularity after Allogeneic IBM HSCT

On days +7, +14, +28, +56, +91, and +182 after allogeneic HSCT, bone marrow biopsy samples were collected from the recipient's injected and contralateral bones. After a 4-hour incubation in Stieve's fixative, the samples were placed in 1-propanol followed by EDTA (Chelaplex II, 3 to 7 days, room temperature) and paraffin embedded. Slices with a layer thickness of 4 μm were stained with hematoxylin and eosin. Microscopic analyses with a magnification of 100× or 200× were performed.

Statistical Analysis

Distribution of data was described using medians and ranges. IBM HSCT data were compared with our own historical i.v. HSCT data previously generated in the same canine nonmyeloablative HSCT model using unstimulated bone marrow grafts. Comparisons between treatment groups were performed by using the Mann-Whitney U-test followed by Bonferroni correction for multiple comparisons. Differences were considered statistically significant if $P < .0167$. The Wilcoxon matched-pairs signed-ranks test was used for comparison between the paired injected and noninjected bone marrow data within a group. Uni- and multivariate Cox regression analyses were performed to assess the influence of the cell dose of the graft on allograft rejection. The potential influencing variables TNC counts, $CD34^+$ cell counts, donor gender, recipient gender, age, and weight of the recipient were considered in these analyses. Statistical significance in Wilcoxon and Cox regression analyses was declared at $P < .05$.

RESULTS

Cell Migration after Autologous IBM HSCT

To check for the feasibility of IBM HSCT, a series of autologous HSCT was performed. Infusion of grafts directly into the bone was feasible without cellular backflow at the injection sites after up to 5 minutes of compression (no leakage outside the body, no localized swelling) and without any other side effects independent of graft composition and volume. The ^{99m}Tc-HMPAO-control setting allowed the identification of structures as the bladder, kidneys, and brain (Figure 1A). Additionally, the signal in the injected bone could be detected.

The double-enriched ^{99m}Tc-HMPAO-tagged bone marrow cells (5 mL; IBM_{auto}-I) accumulated in organs like the lungs,