ARTICLE IN PRESS
Biol Blood Marrow Transplant ■■ (2018) ■■–■■

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

ASBMT American Society for Blood and Marrow Transplantation

Preclinical Canine Model of Graft-versus-Host Disease after In Utero Hematopoietic Cell Transplantation

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Article history:
Received 23 March 2018
Accepted 15 May 2018

Key Words:
In utero hematopoietic cell transplantation
Graft-versus-host disease

A B S T R A C T

In utero hematopoietic cell transplantation (IUHCT) offers the potential to achieve allogeneic engraftment and associated donor-specific tolerance without the need for toxic conditioning, as we have previously demonstrated in the murine and canine models. This strategy holds great promise in the treatment of many hematopoietic disorders, including the hemoglobinopathies. Graft-versus-host disease (GVHD) represents the greatest theoretical risk of IUHCT and has never been characterized in the context of IUHCT. We recently described a preclinical canine model of IUHCT, allowing further study of the technique and its complications. We aimed to establish a threshold T cell dose for IUHCT-induced GVHD in the haploidentical canine model and to define the GVHD phenotype. Using a range of T cell concentrations within the donor inoculum, we were able to characterize the phenotype of IUHCT-induced GVHD and establish a clear threshold for its induction between 3% and 5% graft $CD3^+$ cell content. Given the complete absence of GVHD at CD3 doses of 1% to 3% and the excellent engraftment with the lowest dose, there is a safe therapeutic index for a clinical trial of IUHCT.

INTRODUCTION

In utero hematopoietic cell transplantation (IUHCT) is a nonmyeloablative approach capable of achieving allogeneic mixed hematopoietic chimerism and associated donor-specific tolerance (DST) without conditioning under specific experimental circumstances [1]. We previously demonstrated that an optimized approach in the preclinical canine model yields stable and consistent engraftment associated with levels of hematopoietic chimerism sufficient for the uniform induction of DST and that are potentially therapeutic for many hematopoietic disorders [2]. That study confirmed in a preclinical model the potential for IUHCT in the treatment of many hematopoietic disorders, including the hemoglobinopathies [3-6]. However, its risks need to be well understood before clinical translation. Although not observed in our canine model, graft-versus-host disease (GVHD) represents the greatest theoretical risk of this procedure, and this risk has not previously been well characterized in the context of IUHCT.

Some murine studies have suggested that GVHD may occur after IUHCT based on observations of decreased survival and/or upregulation of T cell regulatory populations [7], but efforts to define the relationship between IUHCT and GVHD in murine models have been hampered by a bone marrow (BM) T cell profile requiring artificial supplementation with splenocytes for sufficient T cell–effector function [8]. The dog model of GVHD has been well established in postnatal BM transplantation (BMT) as an excellent predictor of clinical results [9,10], and in this study we sought to (1) determine whether GVHD could be induced in recipients of IUHCT, (2) characterize the GVHD response in this context, and (3) define a safe T cell threshold for clinical application of IUHCT. We now report a canine model of IUHCT-induced GVHD dependent on T cell concentration within the graft, with clinical features similar to postnatal GVHD that supports a safe therapeutic index for clinical trials of IUHCT.

METHODS

Animals

Time-dated pregnant female beagles were purchased from Covance Research Products, Inc. (Cumberland, VA), which maintains a closed colony with an inbreeding coefficient of <.3%. Pregnancy was confirmed by transabdominal ultrasound (Acuson Sequoia; Siemens, Malvern, PA) upon arrival. Animals were housed in the Laboratory Animal Facility of the Abramson Research Center at The Children's Hospital of Philadelphia, which is approved

Financial disclosure: See Acknowledgments on page 6.
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https://doi.org/10.1016/j.bbmt.2018.05.020
1083-8791/

by the Association for Assessment and Accreditation of Laboratory Animal Care. All experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia and followed guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

BM Harvest and Processing

BM was harvested under sterile conditions from the pregnant mother under general anesthesia (see Supplemental Methods). Mononuclear cells were isolated using a Nycoprep 1.077A (Accurate Chemical, Westbury, NY) density gradient. CD3 depletion used monoclonal anti-canine CD3 CA17.2A12 (AbD Serotec, Raleigh, NC). Cells were then incubated with rat anti-mouse IgG1 microbeads and sorted via AutoMACS (Miltenyi, Auburn, CA). CD26 inhibition was performed as previously described [11] (see Supplemental Methods).

In Utero HCT

CD3 selected and depleted fractions after AutoMACS processing were analyzed for CD3 and CD34 content by flow cytometry, using anti-canine CD34PE (1H6) (BD Pharmingen, San Diego, CA) or anti-canine CD34FITC (ABD Serotec) and anti-canine CD3FITC (ABD Serotec). A portion of the $CD3^+$ population was added back to achieve final $CD3^+$ concentrations of 3%, 5%, or 7.5% of the total administered donor cells for each group. Additionally, 1 group underwent injection of whole maternal BM with a $CD3^+$ concentration of 16%. For injection, cells were resuspended in normal saline with 1% heat-inactivated donor serum, .03% DNAse, and .1% heparin. Injections were performed via laparotomy under ultrasound guidance, and fetal viability was confirmed.

Chimerism Analysis

Chimerism was assessed using the variable number of tandem repeats assay, which identifies microsatellite repeats with a sensitivity of approximately 2%, as previously described [2,11]. Because of overlap between donor peak and stutter patterns, no more than 2 primers were informative for any pairing; in most cases only 1 could be used. This assay was validated against both quantitative Taqman PCR for the *SRY* gene and CD18 FACS, using long-term chimeras from our previous study [11]. As reported previously, variable number of tandem repeats results correlated well with both techniques [2].

Clinical Observation

Animals were examined at least twice daily after birth to evaluate for signs of GVHD, including skin lesions (flaking, erythema), weight loss, poor feeding, and oral/mucosal lesions. For all groups tube feedings were initiated upon evidence of weight loss persisting on 3 subsequent measurements. All animals showing consistent signs of distress (lethargy, pain, inability to tolerate oral intake) were killed in accordance with our protocol. Oral steroids and rapamycin were administered as deemed clinically appropriate by the supervising veterinarian. Surviving pups were observed for a period of 15 to 22 months.

Immunohistochemistry

Tissue specimens were fixed in 10% buffered formalin and embedded in paraffin using a Sakura Tissue-Tek embedder (Sakura Finetek USA, Torrance, CA). Using a paraffin microtome (Leica RM2035; Instrument GmbH, Nussloch, Germany), 4-μm sections were obtained. Paraffin sections were incubated overnight at 55°C and deparaffinized in serial xylene washes, followed by rehydration through a graded alcohol series to deionized water. Slides were blocked for specific protein for 30 minutes at room temperature. For CD3 immunoperoxidase staining, mouse anti-canine CD3 (ABD Serotec) was applied as primary antibody, and slides were incubated overnight at 4°C. The slides were washed with PBS at room temperature for 10 minutes, and species-specific secondary antibody was applied (anti-mouse IgG; Vector Lab, Burlingame, CA). After room temperature incubation for 30 minutes, slides were rinsed in PBS for 10 minutes, and avidin-biotin complex (1:200 dilution; Vector Lab) was added for 30 minutes. After a PBS rinse the slides were developed with peroxidase substrate kit SK-4100 (Vector Lab), lightly stained with Harris hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted using Acrymount (Statlab Medical Products, Lewisville, TX). Slides were imaged using a DMR microscope (Leica Microsystems, Wentzler, Germany).

Statistics

A chi-square test was applied to compare survival with birth among groups.

RESULTS

GVHD after IUHCT in the Optimized Canine Model

As per our previous study [2], we elected to target gestational age (GA) 40 to 42 days using intracardiac administration of maternal BM. Our published cohort consisting of 29 fetuses of 6 pregnant dams served as a control group at a $CD3^+$ concentration of 1%, with absolute $CD3^+$ counts ranging from 3.7×10^8 to 2.7×10^9/kg. In the 3% group 15 fetuses of 2 pregnant dams were injected at GA 37 to 43 days, with absolute $CD3^+$ counts ranging from 4.5×10^8 to 1.2×10^9/kg. Seven fetuses of a single pregnant dam were injected at GA 43 days using a graft containing 5% $CD3^+$ cells with an absolute $CD3^+$ count of 8.75×10^8. Five fetuses of a single pregnant dam were injected at GA 41 days using a $CD3^+$ concentration of 7.5%, with an absolute $CD3^+$ count of 2.25×10^9/kg, and 9 fetuses of a single pregnant dam were injected at GA 43 days with whole maternal BM at a $CD3^+$ concentration of 16%, resulting in an absolute $CD3^+$ count of 2.0×10^9/kg (Table 1). Absolute $CD3^+$ counts were determined based on estimation of fetal weight using ultrasound measurement of crown–rump length at the time of injection [12], resulting in some overlap in absolute counts between groups.

Differences in survival to birth between groups did not reach statistical significance (chi-square statistic, 4.141; $P = .39$), and no mucosal or skin lesions or other evidence of GVHD was clinically evident at birth in any animal. Our 1% cohort had 86% survival to birth (25/29), as previously reported [2], which compares favorably with our historical intraperitoneally injected animals [11] and survival rates reported previously by Blakemore et al. [13]. Fetuses neither live nor stillborn were likely resorbed after the injection procedure, which has been reported to occur at a rate of 11% to 25% during normal canine gestation [14,15], and thus no pathologic evaluation could be conducted. In the 3% group 9 of 14 pups were live born (65%), and an additional pup was stillborn because of birth complications but without evidence of GVHD on pathologic evaluation. The 5% group yielded 5 of 8 live-born pups (63%), whereas 3 of 5 pups in the 7.5% group were live born after a difficult labor lasting > 21 hours and assisted by oxytocin for failure to progress. One additional pup in this litter was stillborn because of the prolonged labor but without clinical or pathologic abnormality. In the

Table 1
Injection Summary and Graft Composition in All Groups

Dose	No. of Live-Born Injected Pups	GA at IUHCT (days)	CD3 cells/kg	CD34 cells/kg	GVHD
1%	20	39-41	$3.71\text{-}27.2 \times 10^8$	$5.8\text{-}58.2 \times 10^8$	N
3%	8	38-43.5	$4.5\text{-}12.6 \times 10^8$	$5.0\text{-}9.8 \times 10^8$	N
5%	5	43	8.75×10^8	5.39×10^8	Y
7.5%	3	41.5	2.26×10^9	7.0×10^8	Y
16% (Whole BM)	7	43	2.0×10^9	5.1×10^8	Y

Significant overlap in CD3 cells/kg suggests that the concentration, rather than the absolute CD3 content of the donor graft, is a significant contributing factor to the development of GVHD.