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Perivascular, but not parenchymal, cerebral engraftment of donor cells after non-myeloablative bone marrow transplantation

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ABSTRACT

Myeloablative (MyA) bone marrow transplantation (BMT) results in robust engraftment of BMT-derived cells in the central nervous system (CNS) and is neuroprotective in diverse experimental models of neurodegenerative diseases of the brain and retina. However, MyA irradiation is associated with significant morbidity and mortality and does not represent a viable therapeutic option for the elderly. Non-myeloablative (NMyA) BMT is less toxic, but it is not known if the therapeutic efficacy observed with MyA BMT is preserved. As a first step to address this important gap in knowledge, we evaluated and compared engraftment characteristics of BMT-derived monocytes/microglia using several clinically relevant NMyA pretransplant conditioning regimens in C57BL/6 mice. These included chemotherapy (fludarabine and cyclophosphamide) with or without 2 Gy irradiation, and 5.5 Gy irradiation alone. Each regimen was followed by transplantation of whole bone marrow from green fluorescent protein-expressing wild type (wt) mice. While stable hematopoietic engraftment occurred, to varying degrees, in all NMyA regimens, only 5.5 Gy irradiation resulted in significant engraftment of BMT-derived cells in the brain, where these cells were exclusively localized to perivascular, leptomeningeal, and related anatomic regions. Engraftment in retina under 5.5 Gy NMyA conditions was significantly reduced compared to MyA, but robust engraftment was identified in the optic nerve. Advancing the therapeutic applications of BMT to neurodegenerative diseases will require identification of the barrier mechanisms that MyA, but not NMyA, BMT is able to overcome.

Introduction

Myeloablative (MyA) pretransplant conditioning followed by bone marrow transplantation (BMT) is neuroprotective in a variety of animal models of neurodegenerative disease, including Alzheimer's disease (Keene et al., 2010; Malm et al., 2008; Naert and Rivest, 2012; Simard et al., 2006), amyotrophic lateral sclerosis (Corti et al., 2004; Ohnishi et al., 2009), Huntington's disease (Kwan et al., 2012), and glaucoma (Anderson et al., 2005). The anatomic distribution, phenotype, and turnover of monocytes/microglia within the central nervous system (CNS) appear to be crucial for the modulation of neurological disease (Djukic et al., 2006; Malm et al., 2005; Mildner et al., 2007; Priller et al., 2006). Successful MyA BMT achieves engraftment of circulating donor monocytes within the CNS as perivascular and parenchymal monocytes/microglia (Priller et al., 2001; Simard and Rivest, 2004), resulting in a chimeric CNS monocyte–microglia population that can modulate disease-related innate immune response to mediate a reduction in neurotoxicity (Cobbold et al., 1986; Hanisch and Kettenmann, 2007; Pollack et al., 2009; Prinz et al., 2011; Ransohoff and Cardona, 2010; Rivest, 2009; Sharabi and Sachs, 1989; Shie et al., 2009).

Clinically, however, MyA BMT is associated with significant morbidity and mortality and is used almost exclusively to treat life-threatening malignant cancers of the blood, including leukemias and lymphomas. MyA BMT is poorly tolerated in elderly patients and is therefore not likely to be used to treat age-related neurodegenerative diseases even if BMT-mediated neuroprotection in rodents could be recapitulated in human disease. By contrast, non-myeloablative (NMyA) BMT regimens have been developed specifically to treat patients with hematologic malignancies, such as the elderly, who are too frail or sick to tolerate conventional MyA BMT. In addition, NMyA BMT applications are currently under intense clinical investigation for multiple sclerosis (Burt et al., 2009), lupus (Burt et al., 2006), diabetes (Voltarelli et al., 2007), and other non-malignant conditions (Annaloro et al., 2009; Tyndall and Saccardi, 2005). Thus, NMyA preconditioning could provide a more appropriate risk/benefit ratio to elderly patients in the early stages of

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; CNS, central nervous system; MyA, myeloablative; NMyA, non-myeloablative; BMT, bone marrow transplant; GCL, ganglion cell layer; GFP, green fluorescent protein; Gy, Gray; HBSS, Hank's Balanced Salt Solution; IPL, inner plexiform layer; OPL, outer plexiform layer; PBS, phosphate buffered saline; wt, wild type.

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neurodegenerative diseases. While several studies have established that recruitment of donor cells to the CNS parenchyma after BMT requires some level of preconditioning irradiation (Grathwohl et al., 2009; Malm et al., 2005; Mildner et al., 2007; Simard et al., 2006; Stalder et al., 2005), this level is not yet known.

NMyA preconditioning regimens consist of low dose irradiation (Shelburne and Bevans, 2009) and/or low dose chemotherapy (Cartier et al., 2009) delivered prior to the BMT. The sublethal irradiation dose used in NMyA preconditioning has been proposed to enhance long-term donor marrow chimerism by inducing proliferative signals after the initial phase of homing (Andrade et al., 2011). However, in order to be a useful therapy for neurodegenerative disease, NMyA preconditioning would probably also require CNS engraftment of BMT-derived cells. We sought to address this critical gap in knowledge by characterizing CNS engraftment of BMT-derived cells under clinically relevant NMyA preconditioning regimens that result in stable hematopoietic engraftment in the host.

Materials and methods

Mice

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). BMT was performed in 2-month-old female recipient mice using 6-week-old male mice homozygous for green fluorescent protein (GFP) as donors. GFP expression is under control of the β-actin promoter and cytomegalovirus enhancer. The mice were kept under standard conditions with food and water ad libitum. All protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Pre-transplant conditioning regimens

We chose three BMT pretransplant conditioning regimens that result in stable hematopoietic engraftment in rodents. Regimen I is a chemotherapeutic approach used in a mouse model of diabetes mellitus to induce robust tolerance to allogeneic islet cell transplants (Guo et al., 2003). We administered fludarabine (400 mg/kg) and cyclophosphamide (200 mg/kg) intraperitoneally (IP) in volumes of 10 μl/10 g body weight on day −1 before BMT (n = 6). Regimen II combines sublethal dose irradiation and chemotherapy to achieve stable hematopoietic engraftment (Luznik et al., 2001). We administered fludarabine (200 mg/kg) and cyclophosphamide (100 mg/kg) intraperitoneally on day −1 followed by 2 Gy irradiation on day 0 before BMT the following day (n = 7). Regimen III uses irradiation only and is based on experiments by Down et al. (1991) that demonstrated 50% hematopoietic engraftment between 4 and 6 Gy whole body irradiation. We exposed mice to 5.5 Gy whole body irradiation on day 0 followed by BMT the next day (n = 8). Regimen IV is the positive control group who received MyA BMT consisting of 10.5 Gy lethal dose irradiation on day 0 followed by BMT one day later (n = 9). In all cases, irradiation was performed with single dose irradiation to the whole body at 2 Gy per minute from a Cesium-137 source (JL Shepherd, Model 81–14, San Fernando, CA). Mice were monitored for 4 months and CNS engraftment was then determined. Hematopoietic engraftment was determined 1, 2 and 4 months after BMT. Additional groups received MyA BMT one (n = 5) and two (n = 5) months post-transplantation.

Generation of chimeric mice

BMT was performed as previously described (Keene et al., 2010). Whole bone marrow was isolated from 6-week-old male C57BL/6;GFP transgenic mice by flushing the femurs and tibias with RPMI media with 10% fetal bovine serum. The samples were combined, passed through a 25-gauge needle filtered through a 70 μm nylon mesh, and centrifuged. Erythrocytes were lysed in ammonium chloride potassium (ACK) buffer (Invitrogen, Carlsbad, CA) and the remaining leukocytes were resuspended in sterile PBS at a concentration of ~2.5×10^4 viable nucleated cells per μl. All the mice received ~5×10^6 cells via retroorbital venous plexus injections one day after preconditioning with different regimens. Mice were sacrificed for analysis of engraftment 1, 2 or 4 months after BMT.

Tissue collection and processing

Tissue dissection

Mice were anesthetized with Avertin 4 months post-transplantation. Blood was drawn via cardiac puncture before the mice were transcardially perfused with ice-cold phosphate buffered saline (PBS). Brains were rapidly removed from the skulls and divided by mid-sagittal section. One hemibrain was dissected on ice and cortex, striatum and hippocampus were immediately placed in cold Hank's Balanced Salt Solution (HBSS) and processed for flow cytometric analysis of BMT-derived cell engraftment. The other hemibrain was postfixed for 2 days in 4% paraformaldehyde, pH 7.6 and then placed in PBS solution containing 30% (w/v) sucrose for 2 days at 4 °C. Eyes were rapidly oriented and then enucleated and the cornea and lens were removed under a dissecting microscope. Eye cups were then post-fixed in 4% PFA for 24 h at 4 °C and then cryoprotected in 5%, 10% and 20% sucrose for 30 min each. Eye cups were oriented, embedded in Optimal Cutting Temperature Compound (Tissue Tek, Torrance, CA), and stored at −80 °C.

Tissue sectioning

Frozen brains were mounted on a microtome and cut into 40 μm coronal sections on a Leica CM3050 cryostat (Leica Microsystems GmbH, Wetzlar, Germany). Sections were collected in cold cryoprotectant solution (0.05 mol/l sodium phosphate buffer, pH 7.3, 30%

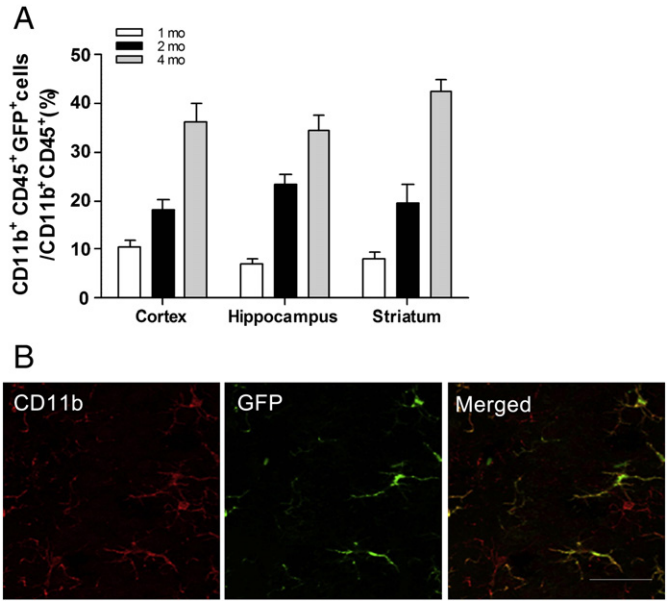

Fig. 1. Kinetics of BM-derived mononuclear cell engraftment in the brain with MyA BMT. A. Monocytic cells isolated from the cortex, hippocampus and striatum of chimeras preconditioned with 10.5 Gy were analyzed using flow cytometry. Monocytes/microglia were identified by $CD11b^+CD45^+$ staining. Quantitation of the percentage of GFP^+ cells in $CD11b^+CD45^+$ cells in the cortex, hippocampus and striatum of mice that received myeloablative BMT at different time points. Data are mean ± SEM, n = 6–9 (*P < 0.05, **P < 0.01, two-way ANOVA analysis using Bonferroni post-hoc test). B. Representative immunofluorescence photomicrographs of the cerebral cortex from MyA BMT recipient mouse at 4 months post-transplant. Microglia are identified with anti-CD11b stains (red) and BMT-derived cells are GFP^+ (green). Merged images demonstrate that all GFP^+ cells are also $CD11b^+$. Scale bar, 25 μm.