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Research article

Submyeloablative conditioning with busulfan permits bone marrow-derived cell accumulation in a murine model of Alzheimer's disease

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HIGHLIGHTS

- Busulphan can be used to produce bone marrow (BM) chimerism.
- Chimerism is needed to study BM-derived cells (BMDCs) in Alzheimer's disease (AD).
- In mixed strain recipients, BM chimerism requires natural killer (NK) cell antibodies.
- BMDCs accumulate in CNS of 3 × Tg mice.
- BMDCs are not associated with intracellular or extracellular β-amyloid.
- BMDC have a perivascular distribution in cortex and hippocampus.
- Intraparenchymal BMDCs are present in hypothalamus.

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ABSTRACT

Previous work has suggested that bone marrow (BM)-derived cells (BMDCs) accumulate within the CNS and could potentially associate with β-amyloid plaques in Alzheimer's disease (AD). To explore the accumulation of BMDCs in murine AD, we transplanted green fluorescent protein (GFP)-labeled BM cells into triple transgenic (3 × Tg) and wild-type (wt) mice using non-irradiative myelosuppresive conditioning with busulfan (BU). We find that BU (80 mg/kg) is sufficient to obtain adequate chimerism (>85%) in wt mice. In order to obtain appreciable non-irradiative chimerism in the 3 × Tg mice (>80%), anti-asialo ganglio-*N*-tetraosylceramide (α-ASGM-1) antibody was also used to reduce natural killer cell function and thereby abrogate the hybrid resistance of the 3 × Tg mouse strain. Using BU conditioning and α-ASGM-1 together, we observed sustained BM chimerism and BMDC accumulation within the CNS of the 3 × Tg and wt mice. In cortex and hippocampus, BMDC accumulation was perivascular in distribution and similar between 3 × Tg and wt mice, with no clear association between BMDCs and AD plaques. We conclude that non-irradiative BM chimerism can be achieved with BU in 3 × Tg mice, but requires α-ASGM-1 (or similar appropriate NK-cell depletion). Use of this chimerism protocol permits BMDCs accumulation in the CNS of mixed strain recipient mice although BMDCs appear to be largely perivascular within cortex and hippocampus.

1. Introduction

Alzheimer's disease (AD) is characterized pathologically by the presence of amyloid plaques and neurofibrillary tangles in cerebral cortex, hippocampus and other regions [1]. Also associated with amyloid plaques are microglia, the resident immune cells of the central nervous system [2]. Recent studies have suggested that microglial function is abnormal in AD and that modulation or replacement of brain-resident microglia might represent a therapeutic approach to treat AD [3,4]. The homing and expansion of bone marrow (BM)-derived cells (BMDCs) into the brains of murine models of AD has been hypothesized to lead to changes in the

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progression of AD, through the elimination of amyloid deposits. It has also been theorized that BMDCs may be more effective at the phagocytosis of β-amyloid than endogenous microglia [3]. However, a limitation to this approach is the requirement for lethal total-body irradiation (TBI) to achieve adequate BM chimerism, a protocol which can affect blood-brain barrier (BBB) permeability [5], as well as the function of endogenous microglia [6,7]. Several recent studies have demonstrated the effectiveness of the milder myelosuppressive conditioning agent busulfan (BU; busulfex) for achieving BM chimerism [8–12], compared to BM conditioning by TBI. We evaluated BU in a murine model of AD to determine if BU was capable of producing BM chimerism, as well as BMDC accumulation in the CNS. Previously, we used a non-myeloablative BU regimen to establish sustained chimerism in both wildtype (wt) mice and an ALS model overexpressing mutant human superoxide dismutase 1 (mSOD). We observed engraftment of BMDCs in the spinal cords of both wt and mSOD mice following treatment with 60–100 mg/kg BU and transplantation of GFP$^+$ BM cells [11].

2. Material and methods

2.1. Animals

Homozygous 3 × Tg AD mice (B6;129-Psen1tm1Mpm Tg(APPSwe tauP301L)) on a hybrid 129/C57BL6 background were obtained from Jackson Labs [13] and bred at Simon Fraser University (SFU). Control, wt animals comprised C57BL/6 mice. Donor animals comprised mice ubiquitously expressing green fluorescence protein (GFP) under a β-actin promoter (C57BL/6; GFP/CD45.2). 3 × Tg and wt mice were used as recipients for BM transplantation at 20 weeks of age and were sacrificed at approximately 52 weeks of age. Protocols governing the use of animals were approved by the Animal Care Review Committee of SFU and were in compliance with guidelines published by the Canadian Council of Animal Care (CCAC) and NIH.

2.2. Pre-treatment and BM transplantation

BU (Busulfex, Otsuka Pharmaceuticals, Japan; 3 mg/ml, IP) at 20 mg/kg BU was given for 4 consecutive days. 24 h after the last BU injection, 1.5×10^7 BM cells harvested from GFP$^+$ donors were injected into recipient mice by lateral tail vein. For BU + cyclophosphamide (CY; Procytox, Baxter Oncology GmbH, Germany; 10 mg/ml) treatments, mice were treated for 4 days with BU as above, followed by 2 days of 100 mg/kg CY. For NK cell function depletion, 30 μL of anti-asialo ganglio-*N*-tetraosylceramide (α-ASGM-1; Wako Chemicals, USA) antibody in 300 μL PBS was injected IP 2 h before BM transplant and again at 14 days post-transplant. To monitor blood chimerism, blood was drawn weekly via the lateral saphenous vein and analyzed on a Guava EasyCyte flow cytometer (EMD Millipore, Billerica, MA).

2.3. Tissue processing

Mice were sacrificed with CO_2, transcardially perfused with 30 mL PBS followed by 30 mL of 4% paraformaldehyde (w/v; PFA). The skull was removed and fixed overnight in 4% PFA at 4 °C. The brain was dissected out, and left in a 20% sucrose solution overnight at 4 °C. Tissue was then embedded in Tissue Tek O.C.T. (Sakura Finetek, USA) and stored at −80 °C, before being sliced at a thickness of 30 μm. Coronal brain sections were made using the Zivic rodent brain slicer matrix (Zivic Instruments, Pittsburgh, PA) at a distance of 9.0 mm posterior to the anterior aspect of the olfactory lobe (or 3.0 mm posterior to the bregma) for the counting of cortical and hippocampal GFP$^+$ cells. Hippocampal and cortical orientations were then used to ensure consistency between sections. For the counting of hypothalamic GFP$^+$ cells, sections were taken at 1.0 mm posterior to the bregma and the lateral ventricles were used as a landmark. Free-floating sections were immunohistochemically labeled as previously described [11] (Iba-1 1:1000; CD31 1:800; 6E10 1:1000). Congo Red staining was performed by first rinsing free floating brain sections in PBS, followed by 20 min incubation in alkali NaCl, and 25 min in alkali Congo Red solution (Sigma–Aldrich, USA).

2.4. Analysis

GFP$^+$ cells in cerebral cortex, hippocampus and hypothalamus were manually counted, based on morphology. Brain sections were analyzed using a Leica epifluorescence microscope with a Leica digital camera (Leica Microsystems, Germany). GFP$^+$ cells in the cortex, hippocampus and hypothalamus of 3 brain sections per mouse were manually counted as previously described [14]. The area of tissue evaluated was outlined using microscope analysis software and the cell density determined using the area measurement tool on the Leica microscope software (Leica Application Suite; Leica Microsystems, Switzerland) to determine the mean number of GFP$^+$ cells/mm^3 tissue. Briefly, cells were classified as round (<9 μm diameter), rod (~20 μm in length with rounded ends), amoeboid (>9 μm diameter), elongated (>20 μm in length) or stellate (ramified processes; [15]). Statistical analysis of cell counts was done using a one-way randomized groups ANOVA on SPSS software and significance taken as $p < 0.05$. Additional methods are described in Supplementary material.

3. Results

3.1. Establishment of chimerism in 3 × Tg and wt mice

In wt mice, high-level chimerism was achieved following BU (80 mg/kg) and BM transplantation by 3 weeks post-transplant, similar to what we have found previously [11]. However when we attempted to use the same transplantation protocol in the 3 × Tg mouse model of AD, peripheral blood chimerism of 35% ± 8% ($n = 13$) was observed at 2 weeks post-transplant using 80 mg/kg BU. Additionally, blood chimerism was not sustained, dropping to 13% ± 4% by 3 weeks and 5% ± 1% by 4 weeks post-transplant (Fig. 1A 'no α-ASGM-1; $n = 13$'). No 3 × Tg animals demonstrated sustained chimerism of >10% past 5 weeks. This was in contrast to C57BL/6 wt mice which were treated in an identical manner and developed high-level peripheral blood chimerism of 67% ± 8% at 2 weeks, and 88% ± 4% at 3 weeks (data not shown). This high-level of chimerism in wt mice was sustained for 32 weeks ($n = 5$). The fall in BM chimerism in the 3 × Tg animals was suggestive of C57BL/6 donor cell rejection by the hybrid strain 129/C57BL6 recipients. This phenomenon, known as hybrid resistance, occurs when a mixed-strain recipient, such as the 129/C57BL6, rejects donor tissue of exclusively one parental origin (in this case C57BL/6 or 129), and is attributed to the action of natural killer (NK) cells directed toward the composite strain [16,17]. To circumvent this problem we tried a variation of the BU protocol where NK cell activity was temporarily eliminated with an anti-asialo-GM-1 antibody (α-ASGM-1) which acts on the cell surface glycolipid asialo-GM-1, damaging the cell membrane and abolishing NK cell function [18]. α-ASGM-1 was administered on the day of transplant and 2 weeks post-transplant to allow time for donor BM to engraft, since it has been reported that 50% of NK cell activity returns 14 days post-injection of α-ASGM-1 [19]. While the average chimerism for the non-depleted group rapidly dropped by 4 weeks post-transplant (to <10%; $n = 13$), the average chimerism of the α-ASGM-1 treated group remained significantly higher up