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SHORT REPORT

Cerebellar heterokaryon formation increases with age and after irradiation

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Abstract Hematopoietic cells have been demonstrated to survive in many nonhematopoietic tissues after transplantation. Apparent "bone marrow-derived" cerebellar Purkinje cells in fact result from fusion events and it has been suggested that fusion may be a natural physiological phenomenon to rescue dysfunctioning cells. Here, we show that fusion of transplanted bone marrow cells with resident Purkinje cells is age-dependent and is strongly enhanced when Purkinje cells are damaged by high-dose irradiation. In addition, Purkinje heterokaryons occur in increased frequencies in the cerebellum of normal, unperturbed, aged mice compared to young animals. Our data suggest that age- and/or irradiation-induced dysfunctioning of Purkinje cells in the cerebellum is required for cell fusion.

Introduction

The developmental potential of stem cells in bone marrow has been the subject of heated discussions ever since it was demonstrated that after transplantation their progeny could be identified in many nonhematopoietic tissues (Krause et al., 2001). Although most of these transplanted cells were later shown to maintain their hematopoietic phenotypes, in several striking examples it was obvious that the transplanted bone marrow cells had adopted at least some characteristics of the tissue in which they newly lodged. Most notably, it was demonstrated that functional liver regeneration could be established after transplantation of purified hematopoietic stem cells (Lagasse et al., 2000). Similarly, after transplantation of GFP+ transgenic bone marrow cells green Purkinje cells could easily be identified in cerebella of recipient mice (Priller et al., 2001). However, as these donor-derived cells were invariably polyploid, it became evident that these phenomena resulted from fusion of bone marrow cells derived from the transplant with resident hepatocytes, cardiomyocytes, or Purkinje cells (Alvarez-Dolado et al., 2003; Wang et al., 2003).

The observed karyotypical abnormalities dampened the initial enthusiasm to use these cells for future therapeutic purposes. However, it is of interest to note that a significant fraction of hepatocytes, cardiomyocytes, and indeed also Purkinje cells are polynucleated under normal conditions. Therefore, it has been speculated that fusion of bone marrow-derived cells with, for example, Purkinje cells may
in fact be a natural phenomenon and may not be connected to the experimental transplantation procedure, which routinely involves high doses of total body irradiation (Kozorovitskiy and Gould, 2003). Potentially, fusion of circulating bone marrow-derived cells could be a normal process to maintain tissue homeostasis when cells become dysfunctional as a result of damage or during aging (Blau et al., 2001). In the present study we have addressed this issue by comparing fusion frequencies in normal nonirradiated young and old animals in the Purkinje cell layer, in bone marrow transplant recipients after a total body irradiation, and in animals in which the brains were protected from irradiation by shielding.

Results

Young adult C57BL/6 mice were irradiated with 9.5 Gy of X-rays, either completely (TBI) or while the entire brain was shielded with a 4-mm lead plate. After irradiation recipients received 10^7 unfractionated bone marrow cells isolated from enhanced green fluorescent protein (eGFP) transgenic donor mice. At various time points after transplantation chimerism levels in peripheral blood were monitored and reached values of 91 ± 4% in the TBI group and 88 ± 5% in the shielded group (not significantly different, Fig. 1A). Recipient mice were sacrificed at 8, 16, 20, and 32 weeks posttransplant, and the number of GFP^+ cells in the molecular layer, the Purkinje cell layer, and the granular layer was carefully quantified in the cerebellum of all animals. In mice that received TBI the number of GFP^+ cells in all three regions increased gradually with age (Fig. 1B). Strikingly, in animals in which the brain was shielded, the total number of donor-derived cells in the brain was substantially lower (Fig. 1C), although the chimerism levels in the peripheral blood were similar in both groups (Fig. 1A). Double stainings using antibodies that detect macrophages revealed that most if not all GFP^+ cells in the molecular and granular layer were of hematopoietic origin (Fig. 2). However, in the Purkinje cell layer GFP^+ cells that displayed the characteristic Purkinje cell phenotype and that did not coexpress hematopoietic markers were detected for the first time after 16 weeks, in numbers comparable to what has been described previously (Weimann et al., 2003). The number of green Purkinje cells significantly increased with age (Fig. 1B). The total number of green Purkinje cells per entire cerebellum was highly reproducible when seven irradiated animals were compared (Fig. 1D). However, shielding of the brain resulted in the complete absence of Purkinje cells in three of four mice and in the identification of a single green Purkinje cell in one recipient (Fig. 1D). All green Purkinje cells that we detected had a similar and typical morphology, all stained positive for calbindin (Figs. 3A and B), and in most cells two nuclei could be detected (Fig. 3C). The latter feature confirms the fusion-dependent origin of GFP^+ Purkinje cells.

Finally, we evaluated the occurrence of heterokaryons in normal, unperturbed (nonirradiated and nontransplanted) young and aged mice. Whereas in a total of three young animals we did not find a single heterokaryon among 742 analyzed cells, in 12-month-old mice we detected 10 binucleated cells of 693 analyzed Purkinje cells (i.e., 1.44%) (Fig. 4). Although these numbers are still rather

Figure 1  The presence of bone marrow-derived cells in the cerebellum increases after irradiation and with age. (A) The levels of donor-derived leukocytes in the peripheral blood of totally irradiated or shielded recipients at 8 and 20 weeks posttransplant. (B) The mean number of GFP^+ cells per cerebellar section in the molecular layer (ML), Purkinje cell layer (PCL), and granular layer (GL) of mice at 8, 16, 20, and 32 weeks after total body irradiation and bone marrow transplantation. In (C) the number of GFP^+ cells in these three cerebellar regions is depicted for mice that received a total body irradiation (closed bars) or in which the brain was selectively shielded (open bars), 20 weeks after bone marrow transplantation. (D) The effect of shielding the brain from irradiation on the number of GFP^+ Purkinje cells at 8 and 20 weeks posttransplant. Each data point refers to an individual mouse.
modest, the difference in fusion frequency between Purkinje cells in old and young brains is significant.

Discussion

Our data show that fusion of bone marrow-derived cells with Purkinje cells is significantly increased after irradiation of the brain. These observations are in line with an earlier report that documented the absence of bone marrow–Purkinje cell heterokaryons in parabiotic animals, in which the peripheral circulation of two mice was joined without radiation (Wagers et al., 2002). We also show that the total number of GFP+ cells that can be detected in the molecular layer, Purkinje cell layer, and granular layer in the cerebellum increases after irradiation and in irradiated animals steadily increases with age. Most of these cells continued to express hematopoietic markers, and they are likely cells of monocytic origin that invade in increasing numbers the damaged/aged cerebellum. Why some of these bone marrow-derived cells fuse with Purkinje cells remains unknown. The simplest explanation would be that the large size of the Purkinje cells is instrumental, as it increases the odds of encountering invading donor cells. However, if that were the case one would expect to observe at least occasionally other GFP+ neuronal cell types, which we and others (Massengale et al., 2005; Wagers et al., 2002) have not. As it is experimentally impossible to selectively protect Purkinje cells from an otherwise irradiated cerebellum, it is formally possible that the increased fusion frequency of bone marrow cells with Purkinje cells is a mere reflection of the number of GFP+ donor cells present after radiation. A more likely hypothesis suggests that Purkinje cells, which are known to contain little if any regenerative capacity, are most vulnerable to loss of function during normal aging. This latter process is strongly enhanced by irradiation. Very recent studies have documented that damaging Purkinje cells by chemical toxins, in addition to normal aging, indeed substantially increases fusion rates (Magrassi et al., 2007). Our data suggest that age- or radiation-induced dysfunctioning of Purkinje cells in the cerebellum contributes to the process of cell fusion.

Methods

Wild-type C57BL/6 mice, 6 to 8 weeks of age, were obtained from Harlan (Horst, the Netherlands) and used as recipients. Enhanced GFP C57BL/6-TgN (ActbeGFP) mice were bred in the animal facility of the University Medical Center Groningen and used as donor mice for GFP-positive whole bone marrow transplantations. All experiments were approved by the ethical committee on animal testing of the University of Groningen.

Wild-type mice were treated with either 9.5 Gy total body irradiation of X-rays (Philips CMG 41 X, 200 kV, 10 mA, 5 Gy/min) or received 9.5 Gy total body irradiation except for the brain, which was shielded with a 4-mm lead plate. Recipients were transplanted with 10^7 unfractionated GFP+ bone marrow cells that were obtained by flushing the femoral content with α-MEM (Gibco BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 2% FBS. Nucleated cell numbers were measured prior to transplantation on a Coulter Counter Model Z2 (Coulter Electronics, Hialeah, FL, USA). Chimerism levels were measured in the peripheral blood from 8 weeks after transplantation on a FACSCalibur flow cytometer (Becton – Dickinson, Erembodegem, Germany). Animals were sacrificed at 8, 16, 20, and 32 weeks posttransplantation to investigate the migration of bone marrow GFP+ cells to the brain.

Figure 2  (A and B) The effects of irradiation on the influx of GFP+ cells in the midbrain. Most, if not all, GFP+ cells are also Iba-1+, revealing their macrophage origin. TBI increases the number of GFP+ cells significantly. (C and D) Similar characteristics are seen in the cerebellum.
Figure 3  Morphology of GFP⁺ Purkinje cells. (A) A representative example of a calbindin⁺, binucleated, GFP⁺ Purkinje cell in the cerebellum of a mouse 20 weeks after total body irradiation and bone marrow transplantation. Two additional examples are shown in (B). (C) A magnification of the soma of the same cell shown in (A), demonstrating the presence of two DAPI⁺ (blue) nuclei (arrowheads) in the same calbindin⁻ (red), GFP⁻ (green) Purkinje cell.
Cerebella of recipient animals were evaluated at 8, 16, 20, and 32 weeks posttransplantation. Animals were anesthetized with sodium pentobarbital (Nembutal) and perfused transcardially with 2% polyvinylpyrrolidone and 0.4% NaNO2 in 0.1 M phosphate buffer (PB), pH 7.4, followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB, pH 7.4. Brains were removed and stored in the same paraformaldehyde – PB solution at 4 °C.

Fixed cerebella were cut in sagittal sections (35 μm) on a Vibratome and stored at 4 °C in fixation solution, prior to labeling. Free-floating sections were washed in PBS, permeabilized with 0.2% Triton X-100, and incubated for 2 h in a preincubation medium containing 2% normal goat serum, 1% bovine serum albumin, 0.0025% cold-water fish skin in PBS, followed by overnight incubation with monoclonal antibody anti-calbindin-D28 k (Sigma, St. Louis, MO, USA) diluted 1:20,000. After being washed, sections were incubated with the secondary Cyt3 antibody (Jackson Laboratories, West Grove, PA, USA) for 1 h at room temperature, followed by a DAPI staining (2 mg/ml; Sigma) to visualize nuclei. Sections were evaluated with a Leica AF 6000 fluorescence or SP2 UV BME confocal microscope (Leica, Mannheim, Germany).

To assess the number of bone marrow-derived cells in the cerebellum, we counted the GFP+ cells in the different layers of the cerebellum. We determined the number of cells in the molecular layer, granular layer, and Purkinje cell layer in one-half of each cerebellum. To this end the cells were counted in each section made from half of the cerebellum, calculated from the inner side. Ten cerebellar sections per animal were counted using the confocal microscope at 20x magnification. Four animals were analyzed in each experimental group (shielded or TBI).

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References


Figure 4 Heterokaryon in cerebellum of a 12-month-old mouse. (A) A calbindin+ Purkinje cell in the cerebellum of a nonirradiated, nontransplanted mouse. In (B) Topro nuclear staining identifies the two nuclei in this same cell. (C) The overlay of these images.