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RADIATION DAMAGE INCREASES PURKINJE NEURON HETEROKARYONS IN NEONATAL CEREBELLUM

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INTRODUCTION

Recent reports have demonstrated that bone marrow-derived cells (BMDCs) can fuse with cells in brain, liver, muscle, heart, intestine and kidney.^{1–9} The factors that trigger in vivo cell fusion and the functional consequences of these events remain largely unknown. Cell fusion transfers genetic and other cellular components from blood cells into cells of other tissues, a process that could contribute to rescuing cells that have suffered genetic damage. In lethal genetic liver and kidney deficiencies, transplantation of disease-free BMDCs that fused with mutant hepatocytes or renal tubular proximal cells rescued both cell types, resulting in increased survival.^{3,9}

Studies in brain have demonstrated that transplantation of bone marrow carrying green fluorescent protein (GFP) into radiated recipients is followed by the appearance of GFP-labeled Purkinje neurons. While initial studies suggested that these were new neurons formed by transdifferentiation,¹⁰ more recent work indicates that the labeled Purkinje neurons had two nuclei and were formed by fusion with BMDCs.^{1,2, 11–13} It has been suggested that these fusion events could be induced by cerebellar damage caused by whole body radiation, a procedure that depletes the host bone marrow in preparation for transplantation. Recent works suggest that while radiation is not required for the formation of binucleated Purkinje neurons,^{11,13} general inflammatory responses can increase the number of fusion events.^{11–13} Radiation-induced damage in the cerebellum is minimal in adults compared to neonates. Low doses of radiation have multiple effects on the development of the cerebellum: the number of granule and basket cells is significantly reduced,^{14–20} Purkinje neurons are misaligned and have abnormal dendrites,^{14–16,21,22} and the overall size of the cerebellum is decreased along with foliar and lobular malformations.^{14,15,21,22} Functional deficiencies following early postnatal radiation of the cerebellum include tremor, ataxia, hypoactivity, and cognitive deficits.^{16,21–25} It is not known if cell fusion is affected by radiation in neonatal animals.

In this study, we asked whether radiation increases fusion events between BMDCs and Purkinje neurons during early postnatal (P4) development. We grafted BMDCs carrying GFP and Cre recombinase into animals carrying a floxed LacZ gene. Upon cell fusion, the Cre in the BMDCs recombines the loxP sequences upstream of the reporter LacZ, allowing its activation and, therefore, the detection of fusion events.² Cell fusion was confirmed by the presence of two nuclei in a Purkinje neuron. We compared the number of fused Purkinje neurons after engraftment of donor bone marrow in radiated or non-radiated neonatal mice. We also determined the frequency of cell fusion events in head-radiated transgenic mice that carry Cre recombinase under a hematopoietic-specific promoter and floxed LacZ reporter

gene in all cells. The results indicate that radiation damage dramatically increases the incidence of fusion events between BMDCs and Purkinje neurons in the neonatal cerebellum. Cell fusion may be a consequence of BMDCs infiltration after radiation-induced damage or/and to direct or indirect radiation damage in Purkinje neurons. These observations are relevant to understand cerebellar damage following therapeutic radiation in young individuals.

MATERIALS AND METHODS

Animals

Animal care and all procedures were approved by the Institutional Animal Care Committees at UCSF. The transgenic mice in this study: R26R,26 β -actin-Cre,27 β -actin-GFP 28 and CD45-Cre were on a C57BL/6 background and have been described previously.

Bone marrow harvesting

Adult bone marrow from donor mice were flushed from femurs and tibiae with a 5-ml syringe and 27-gauge needle containing PBS.

Bone marrow transplantation

Adult and 4-day-old R26R reporter female mice were radiated with 9.5 Gy and 4 Gy, respectively, in two equal doses delivered 3 h apart. To deplete bone marrow in non-radiated newborn mice, 15 mg/kg of busulfan (Sigma) in 0.02% dimethyl sulfoxide (DMSO), was intraperitoneally injected into pregnant females on days 17 and 18 of pregnancy. For experiments with busulfan exposure and subsequent total body radiation, busulfan was injected in pregnant females as explained above. After birth, 1-day-old female mice were exposed to 4 Gy of radiation. Bone marrow cells (10×10^6) from donor male mice were injected into adult (retroorbitally) and neonatal (intrahepatically) recipient mice. The drinking water of the adult radiated and transplanted mice contained neomycin sulphate (1 g/l) and polymyxin B sulphate (1×10^6 U/l) to suppress pathogens. Hematopoietic engraftment was quantified 3 months after transplantation by flow cytometry, based on the frequency of cells expressing GFP in peripheral blood. After blood analysis, animals were killed.

Head Radiation

For radiation-induced cerebellar injury, 4-day-old and adult CD45-Cre/R26R mice were anesthetized and the body was shielded with a lead plate. The head was radiated with increasing doses of x-rays (Phillips orthovoltage X-ray system): 2 and 4 Gy for neonates, and 5, 10, and 20 Gy for 2-month-old mice. Animals were killed 3 months after radiation.

Tissue collection

Mice were anesthetized and transcardially perfused with 0.9% saline followed by 30 ml of 2% paraformaldehyde plus 0.25% glutaraldehyde. Cerebella were dissected, serially cut in 50- μ m vibratome sections, and stained with X-gal or by immunohistochemistry.

X-gal staining and immunohistochemistry

Cerebella were placed in phosphate buffer containing 10mM $K_3Fe(CN)_6$ and 10mM $K_4Fe(CN)_6$ along with the β -gal substrate X-gal (1 mg/ml) (Molecular Probes) at 37 °C for 8–12 h.

Sections were permeabilized and blocked in 10% goat serum with 0.1% Triton X-100, and overnight stained with primary and secondary antibodies at 4 °C. Primary antibodies: chicken anti-GFP (Aves laboratory, 1:500), rabbit anti-calbindin (Swam, 1:3000), mouse

anti-NeuN (Chemicon 1:200), mouse anti-Parvalbumin (Chemicon 1:4000), rat anti-ED-1 (Serotec 1:400), mouse anti-myelin basic protein (Convance 1:2000). Secondary antibodies: goat anti-chicken conjugated with Alexa Fluor-488, goat anti-rabbit conjugated with Alexa Fluor-594, goat anti-mouse conjugated with Alexa Fluor-594 and goat anti-rat conjugated with Alexa Fluor-594 (Molecular Probes). Nuclei were stained with Hoechst 33342 (10 μ g/ml) (Molecular Probes).

Pictures were acquired either on a microscope (Axiovert 200M LSM 510; Carl Zeiss, Inc.) with a 20X Plan-Apochromat objective with a NA of 0.75 and 63X Plan-Apochromat objective with a NA of 1.05 in oil; or on a microscope (Leica CTR 6500) with 20X HCX PL APO CS objective with a NA of 0.7 and 63X HCX PL APO CS objective with a NA of 1.2 in water.

Pictures were taken using Zeiss LSM510 software (Carl Zeiss, Inc.) or LAS AF software (Leica Microsystems).

Brightfield pictures were taken with a microscope (Olympus AX70) using 20X UPlanApo objective with a NA of 0.70 and a 100X PlanApo objective with a NA of 1.4 in oil. Pictures were acquired with a camera (Retiga 2000R; Q-Imaging) using Openlab software (Improvision). Images were processed using Adobe Photoshop.

Cell counting

Granular cells, basket cells and activated microglia were counted using a confocal microscope (Leica CTR 6500) in the lobulus simplex of the anterior cerebellum. The number of cells was established for three random fields, 25 $\times$ 25 μ m in optical sections 8 μ m thick, using a 63X objective for granular cells, 80 $\times$ 80 μ m in optical sections 20 μ m thick, using 20X objective for basket cells and 7 random fields, 25 $\times$ 50 μ m in optical section 20 μ m thick, for activated microglia.

RESULTS

Radiated -- bone marrow transplanted neonatal (P4) mice have more fused Purkinje cells than adults

To investigate the effect of radiation on the formation of fused Purkinje neurons, adult and neonatal R26R-LacZ reporter mice 26 were exposed to 9.5 Gy and 4 Gy of x-rays respectively. Following radiation, both groups were transplanted with bone marrow from β -actin-Cre/GFP mice; these are transgenic mice that express Cre and green fluorescent protein (GFP) in most cell types, including BMDCs. 27-28 Three months after bone marrow transplantation (Fig 1A), the contribution of donor cells to the peripheral blood of the recipients was determined by flow cytometry. Mice were then killed, the cerebella were serially sectioned, and fused Purkinje cells were identified by GFP and X-gal stainings. Prior to X-gal staining, cell fusion was confirmed by the presence of two nuclei using Hoechst 33342.

Binucleated, GFP⁺ Purkinje neurons were observed in all mice that were transplanted at P4 (Fig 1B). Subsequent staining of these sections with X-gal revealed similar numbers of β -gal⁺ cells (Table 1). The activation of the LacZ reporter in the R26R Purkinje neuron resulted, presumably, from fusion with BMDCs and the successive transfer of the Cre-recombinase to the Purkinje neuron. Consistently, GFP⁺ and β -gal⁺ Purkinje cells had 2 nuclei (Fig 1B). As previously described,^{1,2} Purkinje nuclei can be distinguished from BMDCs nuclei as the former have more dispersed chromatin and irregular shape while the latter have spherical nuclei (Fig 1B-C). Purkinje neurons in mice radiated as neonates had poorly developed dendritic trees and aberrant positions within the cerebellum (Fig 1C). 14-

16:21:22 When radiation and transplantations were done in adult animals (2-month-old) and analyzed three months later, only 4 fused cells were observed in 2 of the 4 animals (Table 1). In these mice, the dendrites of Purkinje neurons and the position of their cell bodies were not significantly affected (Fig 1C). Mice that were radiated as neonates contained $\approx 50\%$ less donor cells in their peripheral blood when compared to adult-transplanted mice, but the number of fused Purkinje cells was significantly higher (Table 1). Similar increased incidence of GFP⁺ Purkinje neuron has been observed in mice that were radiated and transplanted with GFP expressing bone marrow as neonates than as adults.²⁹ We conclude that under the conditions used, the number of Purkinje cells that fuse with BMDCs is increased in neonatal animals compared to adults. Note that increased level of cell fusion observed in P4 animals was not dependent of the level of chimerism in the blood. The increased number of fusion events may be triggered by exposure of the developing cerebellum to radiation.

The number of BMDCs that fused with Purkinje neurons increases with radiation dose

We have previously shown that cells of the hematopoietic lineage (CD45⁺) fuse with Purkinje neurons.² CD45-Cre mice, transgenic mice that express Cre under the control of the CD45 gene promoter, were mated with R26R-LacZ reporter mice (CD45-Cre/R26R-LacZ). These mice allowed us to confirm that Cre is expressed in BMDCs and also to study cell fusion in the cerebellum without exposing the mice to radiation. Since Cre is not expressed in Purkinje neurons, presence of reporter positive Purkinje neurons in CD45-Cre/R26R-LacZ mice is likely due to cell fusion. Under normal conditions, very few binucleated β -gal⁺ Purkinje neurons were observed in these mice and only when they were 3–6 months old. CD45-Cre/R26R-LacZ mice, therefore, provide a model system for testing the effects of radiation on the number of fused Purkinje neurons.

We determined whether increasing doses of radiation to the cerebellum affects the number of fused Purkinje neurons in CD45-Cre/R26R-LacZ mice. We shielded the bodies of neonatal (P4) and adult mice (2-month-old) and exposed the heads to different doses of radiation (2 and 4 Gy in P4 animals, and 5, 10, and 20 Gy in adults). Three months after radiation, mice were sacrificed and the number of β -gal⁺ Purkinje neurons were determined in one hemisphere of the cerebellum (Fig 2A). Binucleated, β -gal⁺ Purkinje cells were observed in both animal groups (Fig 2B), but in mice radiated as neonates, there were many more β -gal⁺ binucleated cells than in adults. The number of fused Purkinje neurons was highly variable, but within the P4 group, the number of fused cells increased with increasing doses of radiation (Table 2). Interestingly, although we used higher radiation doses in adult mice, we did not observe an effect of radiation on the incidence of fused Purkinje neurons. Similar to non-radiated controls, fused Purkinje neurons in adult-radiated CD45-Cre/R26R-LacZ mice were very rare. These results suggest that radiation induces a dose-dependent increase in the incidence of fusion between BMDCs and Purkinje neurons in the developing cerebella.

Radiation damage is required for cell fusion between BMDCs and Purkinje neurons

In order to confirm the link between cell fusion and radiation damage using the transplantation of Cre-expressing BMDCs into the R26R mice, we needed a method of bone marrow engraftment that did not expose the animals to radiation. We transplanted bone marrow from β -actin-Cre/GFP adult mice into newborn R26R-LacZ reporter mice treated with busulfan, a myeloablative alkylating agent that, like radiation, has been shown to induce death in bone-marrow stem cells.³⁰ Pregnant females were injected with busulfan at E17.5 and E18.5, and newborn (P1) R26R-LacZ reporter female mice were then transplanted with bone marrow from adult β -actin-Cre/GFP male mice. One group of mice

(busulfan + radiation, n=4) received radiation and another group (busulfan, n=4) was not radiated as a control (Fig 3A).

To rule out that the busulfan treatment could affect cerebellar cells, we did a morphometric and histological analysis in busulfan and busulfan + radiation groups. We found no significant differences in the size or cell density of busulfan-only treated animals and intact controls (Fig 3B–C). However, consistently with previous radiation studies,^{14,15,21,22} the cerebella of busulfan + radiation treated mice was significantly smaller and showed slight folia digenesis compared to animals that only received busulfan (Fig 3D). We counted the density of neuronal cell bodies in the granule cell layer (granule neurons) and basket cells in the molecular layer using NeuN and parvalbumin staining respectively. We found no difference in the number of granule cells and basket cells on busulfan-only and non-treated controls. However, busulfan + radiation significantly reduced the density of granular neurons (Fig 3F–H) and basket cells (Fig 3I–K). The thickness of the molecular layer (Fig 3F–G) was also reduced compared to the busulfan-only treated group or to non-treated controls. As previously described for radiated rats,¹⁸ these effects were more severe in the anterior lobes of the cerebellum (Fig 3B–E). Cranial radiation increases the number of activated microglia in the rat brain.³¹ Similarly, we observed a 58.9%, $P < 0.02$ increase in number of ED-1 positive activated microglia in busulfan + radiation mice compared to busulfan-only and to non-treated controls (Fig 3L–N).

As previously observed in radiated rats,³² myelin staining (myelin basic protein immunocytochemistry) in white matter tracks showed similar staining in radiated-busulfan treated mice compared to controls (data not shown). These observations suggest that busulfan did not significantly alter the response of the cerebellum to radiation.

We next compared the number of Purkinje neuron heterokaryons in busulfan + radiation animals that received GFP-labeled bone marrow cells at P1 compared to animal that received busulfan and bone marrow at P1, but with no radiation. Three months after transplantation, flow cytometry analysis demonstrated 23–73% blood chimerism in busulfan-treated, non-radiated mice (Table 3). In the cerebella of these animals, a few GFP⁺ cells were present in the cerebella (Fig 4A) but no GFP⁺ or β -gal⁺ Purkinje cells were observed (Fig 4B). Busulfan-treated mice that were subsequently radiated had an increased number of GFP⁺ cells in the cerebellum (Fig 4A). In contrast to the non-radiated mice, numerous binucleated GFP⁺ or binucleated β -gal⁺ Purkinje neurons were observed in the busulfan-treated and radiated, β -actin-Cre/GFP bone marrow grafted R26R-LacZ animals (Fig 4B and Table 3). These observations indicate that cell fusion between BMDCs and Purkinje neurons in the developing cerebellum requires radiation. Increased radiation damage to Purkinje neurons, indirect effects to the population of basket or granule neurons, and/or higher numbers of recruited BMDCs may increase number of fusion events in cerebellum.

DISCUSSION

We have demonstrated that cerebellar radiation during neonatal stages in rodents significantly increase the number of BMDC - Purkinje neuron heterokaryons. Specifically, we have shown that fused Purkinje cells are significantly higher in mice that received whole body-radiation as neonates than in adulthood. The difference in the number of Purkinje neuron heterokaryons observed between neonates and adults was not due to lower levels of chimerism in the adult. To the contrary, with the sublethal dose of radiation we used, the level of blood chimerism in newborns was half of that in adults. Therefore, our results demonstrate that the fusion events that occur between BMDCs and Purkinje neurons were

not only dependent on the engraftment of donor cells, but also on other factors associated with the exposure of the developing cerebellum to radiation.

We have shown that fusion events in the cerebellum of newborn, but not adult mice, were increased with radiation dose. These mice were radiated only in the head, suggesting that fusion was associated with the level of damage to the juvenile cerebellum.

Analysis of mice that were bone marrow-depleted at birth using busulfan, showed that neonatal radiation is necessary to induce fusion between BMDCs and Purkinje neurons. Notably, mice treated only with busulfan had very few donor cells in cerebellum, despite showing substantial chimerism in blood. Thus, radiation also increases the number of BMDCs that enter the cerebellum.

Our results are in good agreement with recent reports showing increased number of GFP⁺ and binucleated Purkinje cells in chimeric mice with chronic inflammation.^{11–13} Our findings of fused Purkinje cells in non-radiated CD45-Cre/R26R-LacZ mice also support recent studies showing binucleated Purkinje neurons in unmanipulated and parabiotic mice.^{11,13}

Purkinje cells are postmitotic neurons at birth. Cell fusion could serve as a potential mechanism of cell repair, allowing postmitotic differentiated neurons to import genetic material and other cellular components from BMDCs. While this could be an important mechanism of cell repair, the number of fused cells after neonatal radiation or other pro-inflammatory insults that have been shown to increase the number of heterokaryons in the cerebellum,^{11–13} is extremely small compared to the entire population of Purkinje neurons.

Radiation does not affect the total number of Purkinje cells in radiated neonatal animals.¹⁵ However, the number of granule and basket cells is severely reduced in these mice.^{14–20} It is therefore intriguing that Purkinje neurons are the target of cell fusion. We did not observe instances of fused granule neurons or basket cells. However, although we did not find more fused Purkinje cells in anterior lobules, where the effect of radiation is more severe,¹⁸ we cannot rule out that changes in the number of other cerebellar neurons may indirectly alter the physiology of Purkinje cells and this could increase the number of fusion events.

The larger numbers of Purkinje heterokaryons in radiated neonates may have resulted from a general inflammatory response induced by radiation damage. Consistently, we observed a significant increase in the number of activated microglia (Fig 3N). Recent work using myelin oligodendrocyte glycoprotein (MOG) to induce experimental autoimmune encephalitis in mice, a disease characterized by white matter track degeneration and Purkinje cell death, reported a dramatic increase in the number of Purkinje heterokaryons.¹³ Interestingly, in the same study, lipopolysaccharide-induced inflammation resulted in greatly increased number of infiltrating BMDCs but no significant increase in the number of fused Purkinje neurons. White matter track are susceptible to radiation damage³³ and it is possible that in our mice, the radiation-induced increase in fused Purkinje neurons was due indirectly to myelin damage and immune responses. While we cannot exclude this possibility, staining for myelin basic protein in our busulfan+ radiation group was similar to that in control mice, yet busulfan + radiation animals had fused Purkinje neurons while controls did not.

Cell fusion and formation of multinucleated cells are essential for the normal development of some tissues like muscle, bone and placenta.³⁴ There is no evidence that heterokaryons play any physiological role in the development of the cerebellum or other brain regions. In non-radiated animals, a small number of fused Purkinje neurons was observed. Interestingly,

fused Purkinje neurons in radiated or normal animals were not observed before P90, suggesting that these events are not involved in cerebellar development. It remains to be determined if fused Purkinje neurons derive some physiological benefit, or if this is purely a nonspecific by-product of an ensuing immune recognition mechanism. Independent of its physiological role, this phenomenon should be taken into consideration when radiation is carried out in the young brain. It will be interesting to determine if children that have been exposed to radiation have increased number of binucleated Purkinje neurons. Formation of heterokaryons could serve as a readout of radiation damage.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1

Analysis of fused Purkinje cells in adult mice that were radiated and BM transplanted at P60 or P4

	Mouse	Blood chimerism ^a	GFP ⁺ and/or β -gal ⁺ Purkinje cells ^b
Adults (P60)	1	82.21%	2
	2	81.10 %	2
	3	77.94%	0
	4	82.83%	0
Newborns (P4)	1	42.29%	30
	2	37.88%	38
	3	45.10%	21
	4	32.87 %	31

^aBlood chimerism indicates the percentage of GFP⁺ cells in the peripheral blood

^bNumber of GFP/ β -gal positive cells represents the total number of fused Purkinje neurons in the entire hemisphere serially sectioned (50 μ m).

BM = bone marrow

Table 2

Analysis of fused Purkinje cells in adult mice that were head radiated at P4 or P60

	Mouse (n)	β -gal ⁺ Purkinje cells ^a
Control (non-radiated)	5	2, 0, 1, 0, 0
Newborns (P4)		
2 Gy	3	14, 6, 17
4 Gy	3	91, 30, 23
Adults (P60)		
5 Gy	3	0, 4, 2
10 Gy	3	2, 1, 2
20 Gy	3	2, 0, 0

^aNumber of β -gal positive cells represents the total number of fused Purkinje neurons in the entire hemisphere serially sectioned (50 μ m).

Table 3

Analysis of fused Purkinje cells in adult mice that were BM transplanted at P1 (after treatment with busulfan or busulfan + radiation)

	Mouse	Blood chimerism ^a	GFP ⁺ and/or β -gal ⁺ Purkinje cells ^b
Busulfan	1	60.87%	0
	2	59.03 %	0
	3	23.13%	0
	4	73.20%	0
Busulfan and Radiation	1	71.96%	28
	2	64.25%	33
	3	83.40%	18

^a Blood chimerism indicates the percentage of GFP⁺ cells in the peripheral blood.

^b Number of GFP/ β -gal positive cells represents the total number of fused Purkinje neurons in the entire hemisphere serially sectioned (50 μ m).

BM = bone marrow