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Axonal degeneration in dorsal columns of spinal cord does not induce recruitment of hematogenous macrophages

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Abstract

It is generally accepted that there are two populations of macrophages that respond to neural injuries and successful recruitment of hematogenous macrophages has been shown to help the process of nerve repair in the peripheral nervous system (PNS). Meanwhile, the recruitment of circulating macrophages after central nerve system (CNS) injuries is considered mild and delayed. We compared the recruitment of circulating macrophages in peripheral nerves and spinal cord after dorsal root ganglionectomies, which induce selective and approximately similar extent of sensory fiber degeneration in PNS and CNS, in bone marrow chimeric mice. Our results showed that circulating macrophages were efficiently recruited in PNS but virtually no recruitment in CNS despite degeneration of peripheral and central sensory projections emanating from same dorsal root ganglion (DRG) neurons. The mechanisms that prevent recruitment of circulating macrophages in CNS after injury remain poorly elucidated.

Keywords

Hematogenous macrophages recruitment; Dorsal root ganglionectomy; Axonal degeneration; Cytokines; Chemokine; Bone marrow chimeric mice; CNS; PNS

Introduction

There are two macrophage populations, namely resident and hematogenous macrophages, which respond and participate in degenerative and regenerative responses after mammalian neural injury. Recruitment of hematogenous macrophages after peripheral nerve injuries is vital for Schwann cell proliferation and axonal regeneration (Beuche and Friede, 1984; Heumann et al., 1987; Horie et al., 2004; Perry and Brown, 1992; Perry et al., 1987; Stoll and Muller, 1999) and the kinetics of macrophage recruitment and microglial responses in experimental models of Wallerian degeneration in peripheral nerves are now quite well characterized (Bruck et al., 1996; George and Griffin, 1994; Mueller et al., 2003; Perry et

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al., 1987). In contrast to the injured PNS, the CNS axons have extremely limited capacity to regenerate due to a number of factors and it has been implied that delayed and limited macrophage response also contributes to poor regeneration and recovery after CNS insults such as traumatic spinal cord injury (Lazarov-Spiegler et al., 1998; Perry. et al., 1987; Shechter et al., 2009).

Most studies indicate that there is very limited recruitment of circulating macrophages along degenerating spinal tracts except at the site of injury (Bartholdi and Schwab, 1997; George and Griffin, 1994; Jander et al., 2001; Popovich and Hickey, 2001; Popovich et al., 2003). There are several studies that have compared recruitment of hematogenous macrophages after neural injury in both CNS and PNS but an injury paradigm quantifying recruitment of hematogenous macrophages in PNS and CNS after inducing selective and equivalent damage to a single neuronal pathway in PNS and CNS is not readily available.

Dorsal root ganglionectomies provide a convenient model that allows direct comparison of degeneration of peripheral and central axonal projections emanating from the same neurons into peripheral nerves and spinal cord afferent tracts, respectively. Since reliable immunologic and phenotypic markers that can differentiate between hematogenous and resident macrophage populations are not available, we used green fluorescent protein (GFP) donor mice to create bone marrow chimeric mice and examined and quantified the recruitment of hematogenous macrophages in dorsal columns of spinal cords and compared them to sciatic nerves after dorsal root ganglionectomies.

Material and Methods

Bone marrow chimeric mice

All animal procedures were approved by the Animal Welfare Committee at the University of Texas Health Science Center at Houston. GFP transgenic mice on a C57BL/6 background (Okabe et al., 1997) breeders were obtained (Jackson Laboratory, Bar Harbor, ME) and bred locally. Bone marrow chimeric mice were generated according to previously described methods (Hickey and Kimura, 1988; Mueller. et al., 2003). Briefly, C57BL/6 wild-type recipients were lethally irradiated with 700 cGray. Bone marrow cells were harvested from the long bones of GFP transgenic mice and 2×10^7 cells were transplanted by tail vein injections. In animals used for further studies the efficiency of chimerism was established by fluorescence-activated cell sorting (FACS) analysis on peripheral blood mononuclear cells (PBMCs) stained with PE conjugated anti-CD45 antibody (BD Pharmingen, San Diego, CA) with or without double labeling with rabbit anti-GFP antibody (Molecular Probes, Eugene, OR), and developed with anti-rabbit IgG conjugated with FITC (Jackson Immunoresearch, West Grove, PA). For the FACS analysis, PBMCs from GFP transgenic mice and wild type C57BL/6 were used as positive and negative controls respectively.

L4-5 Dorsal root ganglionectomies

To avoid significant replacement of resident microglial cells in nervous tissues of chimeras with GFP-expressing donor bone marrow derived cells, these studies/surgeries were done 3 weeks after the generation of chimeric animals. Animals were anesthetized, and under sterile conditions, left L4-5 hemilaminectomies were done. Dorsal root ganglia were removed with minimal damage to ventral roots. At post-surgical days 7 (n=5) and 21 (n=5) mice were perfused with PBS and sciatic nerves and lumbar, thoracic, and cervical spinal cords were harvested and processed for immunohistochemistry and pathological studies. These studies were repeated to confirm the results (n = 3 each) at 7 and 21 days after dorsal root ganglionectomies.

Immunohistochemical and pathological analysis

Sciatic nerves (mid-thigh level), lumbar, thoracic, and cervical spinal cords were studied because these sites are remote from the surgical site. Unfixed tissues were cryosectioned, 10-12 μ m cross sections were fixed on glass slides, double labeled with rat anti-mouse CD68 (Serotec, Raliegth, NC) and rabbit anti-GFP antibodies (1:100-200, overnight at 4°C), and developed with specific secondary antibodies conjugated with Texas red and FITC respectively (Jackson ImmunoResearch, West Grove, PA) (1:200, 1hr at room temperature). The slides were coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) to stain nuclei. These tissues were analyzed by fluorescent microscopy. Quantitative analysis for resident microglia and hematogenous macrophage numbers was done on 15-30 of each sciatic nerve and thoracic spinal cord/section/animal. For this analysis, only cells at the level of nuclei (DAPI positive) were counted and considered hematogenous macrophages if they were double labeled for CD68 and GFP and resident microglia if singly stained by anti-CD68 antibody. Right sciatic nerve and right side of the spinal cord in each animal were used as uninjured controls. Lumbar and cervical spinal cords were analyzed qualitatively only.

Fixed sciatic nerves, cervical, and thoracic spinal cords were embedded in epoxy resin, as described (Sheikh et al., 1999; Zhang et al., 2011). Toluidine blue stained 1- μ m cross sections were examined by light microscopy.

Preparation of conditioned media

Sciatic nerves and thoracic spinal cord were rapidly harvested from 10-12 week-old C57BL/6 mice. Under sterile conditions, the sciatic nerves were cut into 1 mm pieces, and spinal cords were sectioned transversely at 500 μ m intervals with a McIlwain tissue chopper (Mickle Laboratory Engineering, UK). The sciatic nerves pieces and spinal cord slices were placed in DMEM supplemented with 5% FBS and kept at 37 °C in a humidified environment of 5% CO₂ for 48 hours. The conditioned media were then collected at 2, 6, 12, 24, and 48 hours after the placement, and pooled for cytokine analysis.

Cytokine assay

Cytokine levels in culture supernatants were determined using mouse cytokine antibody array (RayBiotech, Norcross, GA), according to manufacturer's protocol. Each value shown in the quantitative densitometry results represents the mean of triplicate values.

Results

Bone marrow chimeric mice

The FACS analysis at post-transplantation day 20 showed that in chimeric animals circulating blood had more than 70% leucocytes and 90% monocytes that were GFP positive without GFP immunostaining (data not shown), whereas more than 90% leucocytes and monocytes were GFP positive when stained with an anti-GFP antibody (Fig. 1C and 1D). The frequency of GFP positive cell in chimeric animals was comparable to those of bone marrow donor GFP mice (Fig. 1B-1D).

Loss of peripheral and central projections after dorsal root ganglionectomy in bone marrow chimeras

The pathological analysis of plastic sections confirmed the presence of axonal degeneration in posterior columns of spinal cords (Fig. 2A) and ipsilateral sciatic nerves (Fig. 2B) of chimeric animals that underwent L4-5 dorsal root ganglionectomies. The peripheral nerves showed the presence of many post-phagocytic cells, some of which contain myelin debris,

whereas myelin removal and macrophage recruitment were not appreciated in spinal cord at 7 or 21 days after dorsal root ganglionectomies (Fig. 2).

Macrophage responses and the recruitment of circulating macrophages in injured PNS and CNS

The generation of bone marrow chimeric mice allowed us to distinguish the infiltrating hematogenous macrophages (CD68 and GFP double positive cells) from resident macrophages (CD68 positive and GFP negative cells). Immunocytochemistry showed that one week after ganglionectomies left sciatic nerves had 583.2 ± 89.6 CD68 positive and 476.6 ± 68.2 CD68 and GFP double positive cells /section/animal. The contralateral right sciatic nerves had 15.6 ± 3.7 CD68 positive and 2.6 ± 1.6 CD68 and GFP double positive cells/section/animal (Fig. 3B). The quantitative studies on thoracic spinal cord showed 12.2 ± 6.3 and 2.7 ± 0.8 CD68 positive cells/ section/animal on the left and right side, respectively (Fig. 4B). Three weeks after the ganglionectomies, left sciatic nerves had 692.7 ± 79.8 CD68 positive and 567.2 ± 53.1 CD68 and GFP double positive cells/section/animal, whereas the right sciatic nerves had 17.9 ± 6.2 CD68 positive and 3.9 ± 1.9 CD68 and GFP double positive cells/section/animal (Fig. 3C). In spinal cords, at 3 weeks after the surgeries, quantification showed 23.8 ± 5.9 and 3.3 ± 1.2 CD68 positive cells/ section/animal on the left and right side, respectively (Fig. 4C). At one and three week time point, virtually no GFP positive cells were seen at cervical, thoracic, and lumbar levels of the spinal cords.

Cytokine/chemokine profile of injured PNS and CNS cultures

The cytokine array showed that approximately 9 cytokines/chemokines were upregulated in CNS and PNS conditioned media including MCP-1, RANTES, IL-6, and GCSF, etc. The chemokine and cytokine profile was similar in CNS and PNS conditioned media (Supplementary Fig. 1). Interestingly, higher levels of GCSF and RANTES were present in CNS conditioned medium compared to PNS conditioned medium (Supplementary Fig. 1). The significance of this finding is unclear.

Discussion

We showed that after dorsal root ganglionectomies, selective and similar extent of degeneration of sensory nerve fibers in sciatic nerves and spinal cords have distinctly different effects on the recruitment of macrophages in PNS and CNS. Quantitative studies show that there was massive recruitment of circulating macrophages in PNS within 3 weeks after the neural injury, whereas there were virtually no hematogenous macrophages found in the injured CNS. The advantage of generating chimeric animals is that immunologic and phenotypic markers are not necessary to differentiate between two populations of macrophages (resident and circulating macrophages). Notably, the macrophage recruitment is not affected by the irradiation (Shechter et al., 2009). The short interval between generation of GFP chimeras and surgical procedures was intentional and it allowed successful study of recruitment of circulating macrophages without significant contamination/chimerism of neural resident microglial pool (Mueller et al., 2003). The microglial activation responses in PNS and CNS, as assessed by CD68 staining, were low compared to a previous study (George and Griffin, 1994), which is most likely due to short interval between radiation (used for the generation of chimeric animals) and neural injury.

To our surprise there were hardly any GFP positive cells (the cells recruited from circulation) seen in the spinal cord sections. These results are consistent with previous studies reporting that either in the optic nerve crush or dorsal radiculotomy models, the anterograde or retrograde axonal degeneration, remote from the site of injury, induces a very modest and delayed macrophage response compared to the peripheral nerves (George and

Griffin, 1994; Perry. et al., 1987). The investigators have favored the explanation that round macrophages seen in degenerating optic nerves or dorsal columns of the spinal cord were derived from local microglia and not recruited from circulation (George and Griffin, 1994). Another study using bone marrow chimeric rats in spinal cord injury model noted that in dorsal columns rostral and caudal to the injury site the macrophage response was predominated by microglia but some monocyte infiltration was seen between 3-7 days (Popovich and Hickey, 2001). We believe that the virtual absence of circulating macrophage recruitment in our model is likely due to different injury paradigms as our model had no direct trauma to the spinal cord and very short interval between radiation and neural injury.

These studies did not aim to elucidate the mechanisms underlying the dichotomous recruitment of circulating macrophage in response to nerve fiber/tract degeneration in PNS and CNS, respectively, originating from the same set of neurons. Chemotactic molecules mainly produced by denervated schwann cells, such as MCP-1, and LIF (Siebert et al., 2000; Tofaris et al., 2002), and serum factors including complement component C5 (Liu et al., 1999), have been suggested to play a role in attracting the monocyte derived-macrophages from circulation after PNS injury. Although, previous studies have suggested that lack of blood tissue barrier breakdown might contribute to the limited and delayed macrophages response in CNS (George and Griffin, 1994), the factors limiting the hematogenous macrophages influx after CNS injury remain largely unknown. One possibility contributing to the poor macrophage recruitment in CNS is the lack of certain cytokines/chemokines, which are chemotactic for macrophage, in injured CNS. Additionally, previous work indicates that pro-inflammatory cytokines/chemokines including TNF- α , IFN- γ , and MCP-1, could induce the breakdown of blood tissue (blood brain- and blood spinal cord)-barriers, which facilitate the infiltration of hematogenous macrophages (Echeverry et al., 2011; Lopez-Ramirez et al., 2012). In the current study, we compared expression levels of cytokines/chemokines secreted by injured PNS and CNS in culture. To our surprise, significant differences were not found in cytokine/chemokine profiles to explain dichotomous macrophage recruitment in injured PNS and CNS in our animal model. However, tissue culture studies may not be representative of in vivo situation.

Macrophages are essential innate immune cells and play pivotal role in immune defense to pathogens, inflammation, inflammation-resolution, and repair. The activated forms of macrophages include the “classic activated” pro-inflammatory M1 macrophages and “alternatively activated” anti-inflammatory M2 macrophages (Gordon, 2003; Martinez et al., 2009). Monocyte-derived macrophages recruited after nerve injury consist of both M1 and M2 populations (Shechter et al., 2013). It has been proposed that the recruited macrophages with M2 phenotype, and not the resident macrophage/microglia which mostly express pro-inflammatory markers (M1-like phenotype), enhance the repair of CNS after injury (Kigerl et al., 2009; Rapalino et al., 1998; Shechter. et al., 2009). Therefore, strategies that boost monocyte-derived macrophage recruitment in the injured nervous systems can potentially enhance neural repair. Elucidating the molecular and signaling mechanisms that prevent macrophage recruitment in CNS could allow development of strategies modulating trafficking of circulating macrophages. The animal model reported here provides a tool to examine these mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PNS	peripheral nerve system
CNS	central nerve system
DRG	dorsal root ganglion
GFP	green fluorescent protein
FACS	fluorescence-activated cell sorting
PBMCs	peripheral blood mononuclear cells

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Highlights

- An animal model comparing macrophage recruitment in CNS and PNS after DRG injury.
- Hematogenous macrophages (Mø) were efficiently recruited in injured PNS.
- There was virtually no Mø recruitment in CNS despite similar extent of axon injury.
- Useful model to evaluate the mechanisms underlying limited Mø recruitment in CNS.

Fig. 1.

The efficiency of chimerism in bone marrow chimeric mice. (A-C) The frequency of GFP positive leucocytes and monocytes in PBMCs of wild type mice (A), GFP mice (B), and bone marrow chimeric animal (C), were examined using FACS analysis and are described in the text. (D) Quantified data show that the number of GFP expressing leucocytes and monocytes are comparable in GFP and bone marrow chimeric mice.

Fig. 2.

Axonal degeneration in sciatic nerves and dorsal columns of the spinal cords.

(A) Representative light micrographs of toluidine blue-stained sections from chimeric mice 3 weeks after dorsal root ganglionectomies showing corresponding axonal loss and various stages of myelin degeneration/debris (arrows) and absence of phagocytic cells in the injured spinal cords (left side). (B) Sciatic nerves also show axonal loss but myelin degeneration and clearance by phagocytic cells (arrows) is much advanced compared to spinal cords. (C) Micrograph showing intact myelinated axons in the uninjured/control dorsal column (right side) of spinal cords. (D) Micrograph of uninjured/control right sciatic nerves. Scale bar, 10 μ m.

Fig. 3.

Macrophages recruitment in sciatic nerves. (A) Micrographs showing large numbers of CD68 positive, GFP positive, and CD68/GFP double positive cells (Merged) in sciatic nerves 3 weeks after injury. (B-C) Quantified analysis showed that majority of CD68 positive cells are recruited from circulation (CD68/GFP double positive) and minority are resident macrophages (CD68 positive/GFP negative) at one week (B) and three (C) weeks after surgery. Scale bar, 10 μ m.

Fig. 4.

Macrophages recruitment in dorsal columns. (A) Micrographs showing some CD68 positive, very rare GFP positive and CD68/GFP double positive cells (Merged) in ipsilateral dorsal column 3 weeks after injury. (B-C) Quantified analysis showed that all CD68 positive cells were resident macrophages (CD68 positive/GFP negative) at 1 week (B) and less than 1% cells are circulating macrophages (CD68+/GFP+) at 3 weeks after injury (C). Scale bar, 10 μ m.