Experimental Neurology 240 (2013) 205–218

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Selectively reducing cytokine/chemokine expressing macrophages in injured nerves impairs the development of neuropathic pain

Stefania Echeverry [a,c,1], Yichen Wu [a,b,1], Ji Zhang [a,b,c,*]

[a] The Alan Edwards Centre for Research on Pain, McGill University, 740, Dr. Penfield Ave. Montreal, Quebec, Canada H3A 0G1
[b] Faculty of Dentistry; McGill University, Canada
[c] Department of Neurology and Neurosurgery, Faculty of Medicine, McGill University, Canada

ARTICLE INFO

Article history:
Received 8 October 2012
Revised 9 November 2012
Accepted 14 November 2012
Available online 21 November 2012

Keywords:
Nerve injury
Neuropathic pain
Inflammation
Transforming-growth factor beta
Macrophages
T-lymphocytes
Proliferation

ABSTRACT

It has been well documented that Wallerian degeneration following nerve injury is associated with inflammatory reaction. Such local inflammation contributes to the development of chronic neuropathic pain. Macrophages are one of the major players in the process of either or both degeneration/regeneration and hypersensitivity. To elucidate whether cellular and molecular changes involved in Wallerian degeneration are simultaneously involved in the induction and maintenance of neuropathic pain, and to identify which subpopulation of macrophages can be responsible for the chronic pain following nerve injury, we investigated the peripheral effects of an anti-inflammatory cytokine TGF-β1 in neuropathic pain. Rat sciatic nerves were partially ligated. Macrophages accumulated in injured sciatic nerves displayed heterogeneity with two distinctive functional phenotypes. While MAC1$^+$ macrophages were able to express IL-6 and MIP-1α, ED1$^+$ macrophages were always devoid of signals of inflammatory mediators. Intraneural injection of TGF-β1 resulted in delayed and attenuated neuropathic pain behaviour. In parallel, we observed that exposure of the nerve to TGF-β1 dramatically reduced the number of MAC1$^+$ macrophages. Consequently, the expression of IL-6 and MIP-1α decreased in the injured nerve. Very interestingly, local TGF-β1 treatment had no effect on the population of ED1$^+$ phagocytic macrophages. In addition to its effect on selective subsets of macrophages, TGF-β1 also reduced T-lymphocyte infiltration. Our results revealed the critical roles of cytokine/chemokine secreting MAC1$^+$ macrophages in the development of neuropathic pain, and highlighted the needs and benefits of targeting specific populations of macrophages in alleviating neuropathic pain without delaying nerve regeneration.

Introduction

Peripheral nerve injuries occur frequently as a result of either trauma or damage from surgical procedures. These injuries can give rise to substantial disability and, in many patients, chronic pain because of the sensory deficit that is created. Despite attempts at repairing the nerve, functional recovery is sometimes incomplete and the chronic pain that results from it is extremely difficult to manage (Kehlet et al., 2006; Lee and Wolfe, 2000).

Injury to peripheral nerves causes deep disturbances in the nerve microenvironment. Axons undergo a stereotyped self-destruction known as Wallerian degeneration (Gaudet et al., 2011; Ramer et al., 1997). Non-neuronal cells such as glial and immune cells are also severely altered. They interact with injured axons and play active roles in the pathophysiology of degeneration/regeneration process and injury-associated functional disorders. Schwann cells and macrophages remove the cellular debris of the fragmented nerves, thereby generating an extracellular milieu conducive to axonal regeneration (Hirata and Kawabuchi, 2002; Vargas and Barres, 2007). In response to nerve injury, resident and infiltrated circulating immune cells, including mast cells, neutrophils, macrophages, and T-lymphocytes, become activated, eliciting a multicellular inflammatory response (Austin and Moalem-Taylor, 2010). Among these activated immune cells, the involvement of macrophages is the most investigated. In addition to their powerful phagocytic activities, macrophages secrete many cytokines and chemokines, such as TNF-α, IL-1β, IL-6 and MIP-1α, most of which are potential mediators of hyperalgesia (Lee and Zhang, 2012; Sommer and Kress, 2004). Macrophage depletion not only attenuates thermal hyperalgesia following nerve injury (Liu et al., 2000), but also delays the progression of neuropathic pain in diabetic animals (Mert et al., 2009). In Wld^s (slow Wallerian degeneration) mice where recruitment of macrophages to the site of nerve injury is delayed, the development of thermal hyperalgesia is also impaired (Myers et al., 1996; Sommer and Schafers, 1998). However, some other studies have shown that treatment with liposome-encapsulated clodronate reduces established hyperalgesia

* Corresponding author at: The Alan Edwards Centre for Research on Pain, McGill University, 740, Dr. Penfield Avenue, Genome Building, suite 3200C, Montreal, Quebec, Canada H3A 2B2. Fax: +1 514 398 8121.
E-mail address: Ji.Zhang@mcgill.ca (J. Zhang).
[1] These two authors contributed equally to this work.

0014-4886/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.expneurol.2012.11.013

albeit with limited effect on mechanical allodynia (Barclay et al., 2007; Rutkowski et al., 2000). Our recent report demonstrates that in injured nerves, macrophages exhibit heterogeneity: while a subpopulation identified by the marker MAC1 (CD11b/CD18) is cytokine/chemokine expressing, another subpopulation labeled by ED1 (CD68) antibody is predominantly phagocytic; and the two populations have distinct spatial and temporal distributions (Lee and Zhang, 2012). Nevertheless, some key questions remain to be answered: which subpopulation of macrophages is responsible for the chronic pain following nerve injury? As phagocytic macrophages are necessary for the regeneration process (Dubovy, 2011; Liu et al., 2000; Myers et al., 1996), can selective removal of pain eliciting macrophages be an effective strategy to treat neuropathic pain without delaying the nerve regeneration?

Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine, having a wide variety of functions that are context dependent (Bottner et al., 2000). Its immune functions are, however, mostly anti-inflammatory. Binding of TGF-β1 to the constitutively expressed serine/threonine kinase receptor RII leads to the recruitment of RI and activation of the Smad family proteins, which translocate into the nucleus and regulate gene transcription (Dennler et al., 2000). TGF-β1 receptors are found in most immune cells including T-cells and monocytes/macrophages (Smith et al., 2011), and the evidence from both *in vitro* and *in vivo* data support the roles of TGF-β1 in modulating pro-inflammatory properties of monocytes and macrophages. TGF-β1 can block monocyte proliferation, free radical induction (Ding et al., 1990; Li et al., 2006b; Tsunawaki et al., 1988) and suppress IFN-γ induced NO production in macrophages (Takaki et al., 2006). TGF-β1 has been implicated in the differentiation of pro-inflammatory blood monocytes into non-inflammatory intestinal macrophages: by activating its receptor on blood monocytes and in mucosal macrophages, TGF-β1 can promote the expression of IκBα, the negative regulator of NFκB, leading to inhibition of NFκB-mediated activities and profound inflammatory reaction (Smythies et al., 2010). Therefore, we predict that TGF-β1 is a useful and valid tool to examine the impact of cytokine/chemokine expressing macrophages in the development of neuropathic pain.

In addition, a recent study reveals that "BMP and activin membrane-bound inhibitor homolog" (BAMBI)$^{-/-}$ mice whose endogenous TGF-β1 signalling activity is systemically increased have reduced acute pain behaviour and attenuated hypersensitivity in a model of neuropathy (Tramullas et al., 2010). From our previous study, we know that recombinant TGF-β1 delivered into the spinal cords of rats having nerve injury can partially relieve neuropathic pain by blocking microglial cell proliferation, inhibiting spinal microglial activation and reducing the expression of pro-inflammatory cytokines (Echeverry et al., 2009). It is however not clear how important TGF-β1 is in inflammation-mediated peripheral sensitization and the genesis of chronic pain following nerve injury.

To address the questions mentioned above, in the current study, we delivered TGF-β1 directly into the nerve having a partial ligation. We observed that without affecting ED1^{+} phagocytic macrophages, TGF-β1 was able to inhibit local inflammatory reaction. It dramatically reduced the number of MAC1^{+} macrophages recruited to the site of injury, co-incidental with a significant decrease of cytokine and chemokine production. As a consequence, following the injury to the nerve, the development of neuropathic pain was delayed and attenuated.

Methods

Animals

Adult male Sprague–Dawley rats (Charles River, Quebec, Canada) were used in this study. They weighed 250–275 g at the time of surgery. Before surgery, they were habituated to standard laboratory conditions (14 h light, 10 h dark cycle) and had free access to rat chow and water. All protocols were performed in conformity with the guidelines from the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee.

Peripheral nerve injury and behavioural studies

Partial sciatic nerve ligation

Rats were deeply anaesthetized with isoflurane. Under aseptic conditions, the left common sciatic nerve was exposed at high-thigh level *via* blunt dissection through the biceps femoris muscle. The nerve was isolated from surrounding tissue and approximately 4–6 mm of the nerve was elevated minimally. Partial sciatic nerve ligation (PSNL) was conducted as described by Seltzer et al. (Seltzer et al., 1990). The dorsum of the nerve was carefully freed from surrounding connective tissue at the site near the trochanter. A 6-0 suture was inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle and tightly ligated so that the dorsal one-third to one-half of the nerve thickness was trapped in the ligature. The muscle and skin layers were closed with sutures. Sham operated animals went through the same procedure where the nerve was exposed yet left intact. Animals were allowed to survive 4 to 12 days after surgery.

Behavioural analysis

Mechanical allodynia and thermal hyperalgesia were assessed as index of pain behaviour. Baseline values were obtained before surgery. Upon injury, animals were tested daily until they were euthanized for histological studies. The investigator was blinded to the treatment conditions of the rats. Animals were habituated to the testing environment daily for at least two days before baseline testing. Mechanical hypersensitivity was assessed using calibrated von Frey Hairs as described by Chaplan et al. (1994). Animals were placed in boxes on an elevated metal mesh floor and allowed 40 to 60 min for habituation before testing. A series of von Frey filaments with logarithmically incrementing stiffness (Stoelting) was applied perpendicular to the mid-planter region of the hind paw. The mean paw withdrawal threshold (in grams) was determined using Dixon's up-down method (Dixon, 1980). Thermal hyperalgesia was measured by paw withdrawal latencies to a radiant heat source (IITC Model 336). Animals were placed on a glass floor within Plexiglass cubicles. After habituation, a focussed high-intensity projector lamp was directed to the mid-plantar surface of the hind paw from below the glass floor. The withdrawal latency (in seconds) of the hind paw was recorded automatically (Hargreaves et al., 1988). Twenty seconds was used as a cut-off time to avoid damage to the animal's skin. The measurements were repeated four times, at 3 min intervals on each paw, and the initial pair of readings was dismissed. The average of the three remaining pairs was taken.

TGF-β1 treatment paradigms

Intraneural injection to the ligation site was performed to the left (injured) sciatic nerve without piercing it through using a method adapted from previous works (Bastos et al., 2012; Zelenka et al., 2005). We attached a 30 G needle to a Hamilton syringe and inserted the tip of the needle beneath the epineurium into the immediate proximal region to the ligation. Ten microlitre of either 0.9% saline or human recombinant TGF-β1 at different doses (Peprotech, Rocky Hill, NJ) was slowly administered with special attention to avoid leakage. To ensure the accuracy of the injection, a histological dye was injected into the nerve of another group of animal using the same method, and only minimal leakage was found. No significant tissue damage was found in sham operated rats injected intraneurallly with saline (Figs. 4–7). Animal pain behaviour was not altered neither by the procedure of intraneural injection (Figs. 2–3).

Experiment 1: preventive paradigm

In the preventive paradigm, we examined the impact of TGF-β1 on the development of neuropathic pain by delivering a single shot of