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Short communication

Late blocking of peripheral TNF-α is ineffective after spinal cord injury in mice

Pía M. Vidal[1], Evi Lemmens[1], Lies Geboes, Tim Vangansewinkel, Sofie Nelissen, Sven Hendrix*

Department of Morphology & Biomedical Research Institute, Hasselt University, Belgium

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ABSTRACT

Spinal cord injury (SCI) is characterized by different phases of inflammatory responses. Increasing evidence indicates that the early chronic phase (two to three weeks after SCI) is characterized by a dramatic invasion of immune cells and a peak of pro-inflammatory cytokine levels, such as tumor necrosis factor-α (TNF-α) derived from the injured spinal cord as well as from injured skin, muscles and bones. However, there is substantial controversy whether these inflammatory processes in later phases lead to pro-regenerative or detrimental effects. In the present study, we investigated whether the inhibition of peripheral TNF-α in the early chronic phase after injury promotes functional recovery in a dorsal hemisection model of SCI. Three different approaches were used to continuously block peripheral TNF-α *in vivo*, starting 14 days after injury. We administered the TNF-α blocker etanercept intraperitoneally (every second day or daily) as well as continuously *via* osmotic minipumps. None of these administration routes for the TNF-α inhibitor influenced locomotor restoration as assessed by the Basso mouse scale (BMS), nor did they affect coordination and strength as evaluated by the Rotarod test. These data suggest that peripheral TNF-α inhibition may not be an effective therapeutic strategy in the early chronic phase after SCI.

Introduction

The pluripotent pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is synthesized by many cell types including neurons, glia, activated macrophages, T cells, astrocytes, Schwann cells and mast cells (Hopkins and Rothwell 1995). It is immediately upregulated in response to central nervous system (CNS) trauma such as spinal cord injury (SCI) (Oshima et al. 2009; Pineau and Lacroix 2007; Tracey and Lowry 1990; Wajant et al. 2003) deriving from the injured CNS itself, as well as from injured skin, muscles and bones. Besides the first peak of TNF-α mRNA levels in the acute and subacute phases after SCI (Pineau and Lacroix 2007; Bartholdi and Schwab 1997; Bethea et al. 1999; Koopmans et al. 2009; Streit et al. 1998), some authors have also reported a second TNF-α mRNA peak around 14–28 days after injury (Pineau and Lacroix 2007).

There is a lot of controversy about the *in vitro* and *in vivo* effects of TNF-α after CNS trauma, reported to be either detrimental (Lee et al. 2000; Neumann et al. 2002; Clarke and Branton 2002; Chertoff et al. 2011) or neuroprotective (Oshima et al. 2009; Chertoff et al. 2011; D'Souza et al. 1995; Sullivan et al. 1999) and pro-regenerative (Schmitt et al. 2010), depending on the animal model used. These contradicting results may be in part the result of TNF-α interaction with two types of receptors, namely TNFR-1 and TNFR-2, which are expressed on different cell types and are associated with different cellular effects (Wajant et al. 2003). In different models of SCI, TNF-α appears to have detrimental effects on spinal cord recovery, since treatment with TNF-α blockers (Koopmans et al. 2009; Genovese et al. 2006, 2007) and antibodies to the TNFR-1 (Lee et al. 2000; Neumann et al. 2002; Clarke and Branton 2002) improved spinal cord recovery substantially. In contrast, the life-long absence of TNFR-1 and TNFR-2 in knockout mice reduces NF-κB activation and functional recovery after SCI (Kim et al. 2001).

Increased TNF-α levels shortly after CNS trauma have been linked to a sequence of cellular dysregulations, accompanied by enhanced vascular permeability, impaired glutamate metabolism and clearance (Takahashi et al. 2003), and in some settings an excessive inflammatory reaction (Pineau and Lacroix 2007). Several studies indicate that it is important to regulate increased TNF-α levels immediately after injury to control the excitotoxic effect of TNF-α on AMPA and GABA receptors after injury (Stellwagen et al. 2005; Zhao et al. 2010), and to decrease TNF-α-induced apoptosis (Genovese et al. 2006). Consistently, a single dose of etanercept immediately after injury appears to be sufficient to improve hind limb locomotor function and reduce apoptosis of neurons and oligodendrocytes in the rat spinal cord (Chen et al. 2011).

However, in all functional studies TNF-α antagonists were administered immediately or 6 h after injury (Genovese et al. 2006,

* Corresponding author at: Department of Morphology & Biomedical Research Institute, Hasselt University, Agoralaan, Building C, B-3590, Diepenbeek, Belgium. Tel.: +32 11 26 9246; fax: +32 11 26 9299.
E-mail address: sven.hendrix@uhasselt.be (S. Hendrix).
[1] Contributed equally.

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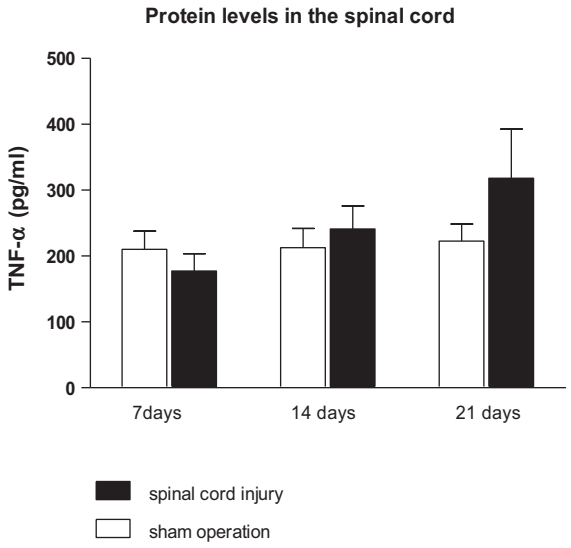

Fig. 1. TNF-α protein levels after SCI. Cytometric bead array analysis in spinal cord shows a slight but not statistically significant increase of local TNF-α levels in the spinal cord of mice at days 7, 14 and 21 after spinal cord injury compared to sham-operated mice ($n = 5$–12 mice per time point).

2007; Chen et al. 2011) ignoring the second peak of TNF-α in the later phases after injury. There is still much controversy whether the inflammatory response in the chronic phase after CNS trauma promotes or inhibits regeneration (Hendrix and Nitsch 2007). In addition, a modulation of peripheral inflammation may influence substantially the CNS immune status even if the blood brain barrier (BBB) is intact (Teeling et al. 2010). Thus, a modulation of peripheral TNF-α with inhibitors such as etanercept that do not cross the BBB may be a promising approach during the chronic phases after CNS trauma when the BBB is already repaired. In the present study, we investigated the effects of different doses and administration routes of the TNF-α inhibitor etanercept in the early chronic phase after SCI. Our data, however, indicated that all three therapeutic approaches were ineffective in modulating the functional outcome after SCI.

Materials and methods

Spinal cord hemisection injury

Dorsal hemisection SCI was performed as described previously (Boato et al. 2010). Briefly, 10-week old C57BL/6 mice (Harlan, the Netherlands) were anesthetized and underwent a partial laminectomy at thoracic level T8, and were subjected to a bilateral dorsal hemisection consisting of a complete transection with iridectomy scissors of all parts of the corticospinal tract: left and right dorsal funiculus, dorsal horns and the ventral funiculus. The muscles were sutured and the back skin closed with wound clips. After surgery, the mice were placed in a recovery chamber (32 °C) until they were well awake and could be returned to their home cage. The animals' bladders were manually voided daily until the animals were able to urinate independently.

Experimental groups

The treatment protocols are summarized in Fig. 2A. For each experiment, mice were distributed equally among the groups according to their Basso mouse scale (BMS) score after SCI, and treatment was started at day 14 after injury. For the first experiment, mice were injected i.p. every two days for 12 days with etanercept (Enbrel®, Amgen, Pfizer; 125 μg/mouse) (Genovese et al. 2006), or with saline solution (0.9%, w/v NaCl) or IgG from human serum (Sigma; 125 μg/mouse) as a control. For the second experiment, two groups of animals were injected daily i.p. with saline solution or etanercept (125 μg) for seven days. Finally, for the third experiment, the mice were deeply anesthetized with isoflurane, a small incision was made in the back skin to implant s.c an osmotic minipump (Alzet 2004; 0.25 μl/h) filled with etanercept or saline, and after implantation the back skin was closed with wound clips.

Locomotion tests

Animals were scored daily for functional recovery after SCI using the BMS (Basso et al. 2006) starting at day 1 after injury or with the Rotarod starting at day 7. The BMS is a 10-point scale (9, normal locomotion; 0, complete hind limb paralysis) which is based on hind limb movements made in an open field during a 4 min interval, provided the mouse is moving for at least three body lengths using a consistent speed. After allowing mice to recover for 6 days, Rotarod performance (Sheng et al. 2004) was determined daily up to the end of the observation periods. The mice were placed on an accelerated rolling rod (Ugo Basile, Comeris VA, Italy) and the latency to jump off from the rod was automatically recorded by the action of the mouse dropping onto a trigger plate. For BMS analysis we used the mean of the left and right hindlimb scores for each animal.

Protein expression in spinal cord

Animals were transcardially perfused with ringer solution at selected time points 7, 14 and 21 days after injury. After perfusion, spinal cord tissue was dissected out in a standardized area (0.5 cm proximal and 0.5 cm distal from the lesion site) and homogenized using the Procarta lysis buffer (Panomics, Italy). Homogenized tissue was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected and protein levels were determined by flow cytometry using the cytometric bead array mouse flex set system (BD Biosciences) according to manufacturer's instructions. Analysis was performed using FACS arrays Bioanalyzer and FCAP software (BD Biosciences). Data represent mean values ± SEM.

Statistical analysis

Rotarod and BMS data were analyzed using a two way ANOVA as previously described (Basso et al. 2006) and represent mean values for all the animals of each experimental group (±SEM). The analyses were performed using GraphPad Prism 5.0 software (GraphPad software Inc., CA, USA). Every experimental approach was performed three times.

Results and discussion

Etanercept is an FDA-approved TNF-α inhibitor for treating rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis, which has also been tested preclinically in rodents as a therapy for SCI (Genovese et al. 2006; Chen et al. 2011). Interestingly, large molecules, such as etanercept, cannot cross the BBB when delivered systemically (Zhou et al. 2011). However, blocking TNF-α signaling by etanercept confers improved functional recovery after SCI, if it is administered immediately after injury (Genovese et al. 2006; Chen et al. 2011). These findings suggest that either the damage to the BBB must be sufficient to reach an adequate concentration of etanercept at the lesion site or that a modulation of the peripheral TNF-α levels results in beneficial outcome, or both. The rationale of our study is based on results published on TNF-α mRNA levels in the injured mouse spinal cord using *in situ* hybridization (Pineau and Lacroix 2007), showing a second peak of TNF-α in the early chronic