Spinal Cord Injury Intensity Modifies the Expression of Inflammation-Related Gene Expression after Immunization with Neural Derived Peptides

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Abstract
Previous studies revealed that the intensity of Spinal Cord Injury (SCI) plays a key role in the therapeutic effects induced by Immunizing With Neural-Derived Peptides (INDP), as severe injuries abolish the beneficial effects induced by INDP. In the present study, we analyzed the expression of some inflammation-related genes (IL6, IL12, IL-1β, INFγ, TNFα, IL-10, IL-4, and IGF-1) by quantitative PCR in rats subjected to SCI and INDP. We investigated the expression of these genes after a moderate or severe contusion. In addition, we evaluated the effect of INDP by utilizing 2 different peptides: A91 and Cop-1. After moderate injury, both A91 and Cop-1 elicited a pattern of genes characterized by a significant reduction of IL6, IL1β, and TNFα but an increase in IL10, IL4, and IGF-1 expression. There was no effect on IL-12 and INFγ. In contrast, the opposite pattern was observed when rats were subjected to a severe spinal cord contusion. Immunization with either peptide caused a significant increase in the expression of IL-12, IL-1β, INFγ, (pro-inflammatory genes), and IGF-1. There was no effect on IL-4 and IL-10 compared to controls. After a moderate SCI, INDP reduced pro-inflammatory gene expression, and generated a microenvironment prone to neuroprotection. Nevertheless, severe injury elicits the expression of pro-inflammatory genes that could be aggravated by INDP. These findings correlate with our previous results demonstrating that severe injury inhibits the beneficial effects of protective autoimmunity.

Keywords: Gene Expression; Neural Derived Peptides; Spinal Cord Injury

Introduction
Several auto-destructive mechanisms arise after Spinal Cord Injury (SCI) including massive entry of calcium into the cellular compartment, neural fiber damage, metabolic disturbances, destruction of microvessels, and breakdown of the blood-spinal cord barrier. One of the most important subsequent events is the recruitment of immunological cells (neutrophils, hematogenous macrophages, and T lymphocytes) to the site of injury; accompanied by the activation of resident microglia that triggers an inflammatory reaction at the damaged area increasing the inflammatory response [1]. This inflammatory reaction is carried out by different cells and pro-inflammatory cytokines, which exacerbate lipid peroxidation, free radical production, and demyelination; leading to extensive secondary tissue damage [2,3]. The exacerbated inflammatory response can trigger a pathological auto-reactivity reaction that is mostly mediated by the activation of T lymphocytes. In these pathological circumstances, activation of T cells shifts towards a Th1 phenotype (pro-inflammatory), thereby promoting a higher demyelination and increasing injury size. Nevertheless, if the response is activated towards a Th2 phenotype (anti-inflammatory), it could regulate the secondary damage by creating a neuroprotective microenvironment [3–5]. The immune system plays a pivotal role in the pathophysiology secondary to SCI [6]. It has been demonstrated that modulation rather than inhibition of the immune response is beneficial and promotes neurological recovery after injury [7,8].

Protective autoimmunity (PA) is an innovative strategy based on the modulation of the immune response after trauma to the central nervous system (CNS) [9–11]. PA is boosted by immunizing with non-encephalogenic neural derived peptides (INDP) such as A91 or Cop-1 [4,12]. A91 is a myelin basic protein (MBP) -derived peptide (sequence of amino acids 87–99), originated by replacing lysine with alanine at residue 91 [12,14]. Another peptide capable of modulating PA is Cop-1, a random polypeptide synthesized from four amino acids (L-tyrosine, L-glutamic acid, L-alanine, and L-lysine) with an average molar fraction of 0.141, 0.427, 0.095 and 0.0338, respectively [15,16].

At the moment, the way through which PA exerts its beneficial actions is not entirely understood, as there is a broad spectrum of mechanisms that have not yet been explored. Gene expression is one of the main phenomena that could provide some interesting information about how PA exerts its protective effects. Inflammation-related genes like Interleukin (IL)-6, IL12, IL1β, Interferon (INFγ), Tumor Necrosis Factor Alpha (TNFα), IL-10, IL-4, and Insulin-like Growth Factor-1 (IGF-1) could influence cell function over minutes to hours - or a much longer period of time - in a pro or anti-inflammatory manner [6].

On the other hand, previous studies have demonstrated that severe injury avoids the neuroprotective effect elicited by INDP [17]. In these instances, we hypothesize that the lack of a neuroprotective effect is related to the upregulation of pro-inflammatory and downregulation of anti-inflammatory genes. For that reason, we...
explored the expression of eight inflammation-related genes in two models of SCI (moderate and severe contusion) in rats immunized either with A91 or Cop-1 peptides.

Materials and Methods

Study design

The sample size for this experiment was calculated using an alpha of 0.05 and beta of 0.20. Two experiments were performed with twenty animals used in each experiment. In the first, fifteen rats were subjected to a moderate SC contusion and then were randomly allocated into 3 groups as described in experiment 1. Sham-operated rats (n=5); 2) Rats immunized with A91-peptide (n=5); 3) Rats immunized with Cop-1 (n=5). In the second experiment, fifteen rats were subjected to a severe SCI and then allocated into 3 groups as described in experiment 1. Sham-operated rats (n=5) were used to normalize the values of all groups in both experiments. Seven days after injury, animals of all groups were euthanized, and the spinal cord was analyzed for expression of inflammation related-genes.

Animals

Adult Fischer 344 (F344; 13-14 weeks old, 200-230 g) female rats (n=40) were used. Animals were supplied by Proyecto Camina A.C. and were handled according to the NIH guidelines for management of laboratory animals. All the procedures were carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals, and the Mexican Official Norm on Principles of Laboratory Animal Care (NOM 062-ZOO-1999). Also, all animal procedures were approved by the Animal Bioethics and Welfare Committee (ID:57204; CSNBTBIBAJ 090812960).

Spinal cord injury

Rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg; Probiomed, Mexico City) and xylazine (10 mg/kg, Fort Dodge Laboratories, Fort Dodge, IA), and their spinal cords were exposed by laminectomy at the T9 level. A 10 g rod was dropped onto the exposed spinal cord from a height of 25 mm for moderate injury and 50 mm for severe injury using the New York University impactor (Basso et al., 1996; Basso, Beattie, & Bresnahan, 1995). Subsequently, muscles and skin were closed in layers, and animals were placed in a temperature-controlled room. During the first 7 days post-surgery temperature was maintained at 23°C. Manual voiding of the bladder was performed twice per day. Antibiotic (Enrofloxacin 64 mg/kg/day; Marvel Mexico City) and analgesic therapy were given daily throughout the study to avoid complications.

Active immunization

Sixty minutes after SCI rats were immunized subcutaneously at the base of the tail with 150 μg of A91 or Cop-1 dissolved in 0.15M phosphate-buffered saline (PBS) (experimental groups), or only with PBS (control groups; SC injury + immunization with PBS and sham-operated animals). Both peptides and PBS alone were emulsified in an equal volume of complete Freund’s adjuvant (CFA) containing 0.5 mg/ml of Mycobacterium tuberculosis. A91 peptide was purchased from Invitrogen Life Technologies (San Diego, CA), and its purity (higher than 95%) was confirmed by reverse-phase HPLC. Cop-1 was purchased from Sigma (St. Louis, MO).

Quantitative polymerase chain reaction

A 3 cm long segment of the spinal cord was obtained 7 days after injury. The total RNA was then isolated using the phenol-chloroform extraction method with Trizol (Life Technologies, Carlsbad, CA). RNA concentration and purity was evaluated by UV spectrophotometry, integrity by electrophoresis, and complementary DNA (cDNA) was obtained by reverse transcription. The cDNA synthesis was performed with oligo (dT) at 55°C for 50 min in a final volume of 20 μl from 2 μg of total RNA, following the manufacturer’s instructions for Superscript reverse transcriptase-RNAse H (Invitrogen, Carlsbad, CA). The template cDNA was normalized to the ribosomal RNA. Real-time RT-PCR was performed using a Light Cycler 2.0 instrument (Roche, México D.F., MX). Three independent experiments for every set of RT-PCR analyses were performed. We assayed the expression of cDNA by quantitative PCR using the selected gene-specific primers pairs listed in Table 1. For the initial denaturation step, samples were heated up to 95°C for 10 min, followed by the first cycle consisting of a denaturation step (95°C, 10 sec), an annealing step (72°C, 10 sec), an extension step (72°C, 10 sec), a melting curve (65°C, 1 min), and a cooling step (40°C, 30 sec). The reaction was carried out in 40 cycles. Expression levels of individual genes were represented in arbitrary units after

Table 1: PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference sequence number</th>
<th>Sequence</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s Ribosominal</td>
<td>NM_0010076</td>
<td>Forward5’-ACATTGGGAAGCCTCACTGTCG-3’</td>
<td>157 pb</td>
</tr>
<tr>
<td>Interleukin6 IL6</td>
<td>NM_012589</td>
<td>Forward5’-TGTTGAGGACAACATGTTGC-3’</td>
<td>117 pb</td>
</tr>
<tr>
<td>Interleukin12 IL12</td>
<td>NM_053390</td>
<td>Forward5’-TGGCCATCTGTTAAACCACTC-3’</td>
<td>111 pb</td>
</tr>
<tr>
<td>Interleukin-1β</td>
<td>NM_031512.2</td>
<td>Forward5’-CACTTGTTGCCTCTGTCG-3’</td>
<td>120 pb</td>
</tr>
<tr>
<td>Interferon gamma IFNy</td>
<td>NM_138880</td>
<td>Forward5’-CAACAGCCATGCAAAAC-3’</td>
<td>128 pb</td>
</tr>
<tr>
<td>Tumor necrosis factor alpha TNF α</td>
<td>NM_012675</td>
<td>Forward5’-CTCTTTGCTCATGGATCCGGG-3’</td>
<td>115 pb</td>
</tr>
<tr>
<td>Interleukin 4 IL4</td>
<td>NM_201270</td>
<td>Forward5’-GGCTCCAGGCTGTCGGCAAA-3’</td>
<td>150 pb</td>
</tr>
<tr>
<td>Interleukin10 IL10</td>
<td>X60675</td>
<td>Forward5’-GGGGTGCAATACATCTGCA-3’</td>
<td>216 pb</td>
</tr>
<tr>
<td>Insulin growth factor -1 IGF-1</td>
<td>NM_001082477</td>
<td>Forward5’-GCTGAGGGCTTCTTATTAG-3’</td>
<td>171 pb</td>
</tr>
</tbody>
</table>

PCR primers.
normalization with ribosomal RNA. All experiments were performed per triplicate. Each reaction was subjected to melting curve and melting temperatures to confirm single amplified products using the Light-Cycler software (build 4.1.1.21). The crosspoint value was used to obtain the delta 1 and delta 2 analyses, which reported the relative expression for each group.

**Statistical analysis**

Data is displayed as the mean ± Standard Deviation (SD), and statistical significance was established when p < 0.05. Graph Pad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA, USA) was employed in statistical the analysis. All data were analyzed using Kruskal-Wallis or Mann-Whitney U test.

**Results**

First, we analyzed pro-inflammatory gene expression subsequent to immunization with A91 or Cop-1 (INDP). INDP was performed in rats with moderate or severe SCI. Immunization either with A91 or Cop-1 caused a significant reduction of IL-6, IL-1β (Figure 1A and 1E) and TNFα (p < 0.05, Kruskal-Wallis followed Mann Whitney U test; Figure 2C) in rats with moderate SCI. In contrast, immunization with any of these peptides induced a significant upregulation of IL-12 and IL-1β genes after severe SCI.

In the case of anti-inflammatory genes, INDP provoked a significant increase of IL10 and IL4 after a moderate SCI (Figure 3A and C). Meanwhile, there was no significant effect on these cytokines when animals were subjected to a severe SCI (Figure 3B and 3D). Finally, IGF-1 was significantly increased in both moderately and severely injured rats (p < 0.05, Kruskal-Wallis followed Mann Whitney U test; Figure 3E and 3F).

**Discussion**

Changes in gene expression have been documented in diverse in...
vivo models as an early response to SCI [18-20]. The up regulation of diverse transcription factors and molecules involved in signaling pathways suggests that changes in the expression of many genes can develop as a result of the traumatic insult. Several in vivo studies have been carried out to analyze the post-traumatic gene expression in an attempt to establish the biological and functional after math of SCI [21,22]. Here, we analyzed changes in the expression of eight different genes: IL6, IL12, IL1β, IFNγ, TNFa, IL10, IL4, and IGF-1, in moderate and severe SCI. It was evident that moderate injury allows INDP to create a microenvironment where cytokines like IL4 and IL10 prevail; which could play an important role in protecting and restoring neural tissue [23,24].

The cytokine profile induced by INDP in the present SCI model is capable of collaborating with the modulation of the inflammatory response, due to its necessary role in activation, differentiation, and proliferation of Th2 lymphocytes [25]. Likewise, this cytokine microenvironment promotes an M2 macrophage differentiation, increases the macrophage expression of the Major Histocompatibility Complex class II (MHC-II), and decreases IL1, IL6, and TNFa production [26 – 30].

The latter was corroborated in the present work since INDP-treated rats with moderate SCI presented a significant reduction of inflammatory cytokines. The relevance of this effect is evidenced with the regulation of TNFa, a pro-inflammatory cytokine that stimulates a variety of factors that aggravate inflammation, such as IL8, IL6, IL1, nitric oxide, peroxides, and prostaglandins [31,32]. Previous studies in our laboratory have demonstrated that INDP is capable of reducing inflammation and lipid peroxidation [33]. In contrast, after a severe contusion, INDP was not capable of inducing the same effect [34]. In this case, INDP did not induce any sign of motor improvement. In the present work, we intended to delve into the cause of this lack of effect and found a prevailing inflammatory environment that could be inhibiting the beneficial effects induced by INDP. After severe SCI, immunization with any peptide (A91 or Cop-1) significantly increased the expression of inflammation-related genes and reduced the amount of those related to the anti-inflammatory response.

Of note, an interesting pattern of gene expression was observed in this SCI model, where INDP produced a higher expression of IL12, IL1β, and IFNγ. The reason why INDP induced the expression of this gene pattern is not clear yet. Nevertheless, it is well known that inflammatory gene expression could be increased by the action of the NFkB signaling pathway, which is activated by high concentrations of tissue protein-like DAMPs (Damage-Associated Molecular Patterns) [35,36]. With this in regard, when compared to moderate SCI, severe injury causes a more pronounced release of DAMPs and neural constituents, in such a way that the high concentration of these molecules along with INDP could be shifting the immune response towards a Th1 encephalitogenic phenotype [37]. This predominant phenotype (Th1) – which could be directed against other immunogenic determinants- could also inhibit the proliferation of Th2 protective lymphocytes, and thereby its beneficial actions [34]. Previous investigations in our laboratory have proven that severe injuries or the excessive administration of INDP inhibit the beneficial action of protective autoimmunity [17,34]. On the other hand, we found a significant increase of IGF-1 in A91 and Cop-1-immunized groups in severe SCI. This finding may be due to the arrival of peripheral and resident macrophages, as these cells produce pro-fibrotic mediators, including insulin growth factor 1 (IGF-1), transforming growth factor (TGF)-β, and Platelet-Derived Growth Factor (PDGF). IGF-1 is also produced by astrocytes and endothelial cells, it down regulates several pro-inflammatory cytokines, such as TNFa, IL1 β, and IL6 [38]. The increase of IGF-1 concentrations in treated animals - especially in those with severe injury -could be the response to the high concentrations of pro-inflammatory cytokines (TNFa, IL1 β, and IL6), that result from a failed effort to modulate the hostile microenvironment. Finally, IL10 -a cytokine involved in the anti-inflammatory response, by altering immune cell activities-is able to reduceIL1α, IL1β, IL8, IL12, IFNα, iNOS, and TNFa production [39-41]. Evidence suggests that application of IL10 after SCI could decrease the inflammatory response and augment neural survival [42,43]. Here, animals with INDP subjected to severe SCI did not present any significant production of this cytokine; however, INDP-immunized rats with moderate injury showed a significant production of IL-10. These results support our hypothesis, as the effect of INDP was avoided and an inflammatory microenvironment prevailed in severe SCI; while after a moderate SCI, INDP was capable of promoting its beneficial effect mediated by a microenvironment propitious for neuroprotection and neuroregeneration.

The present study provides evidence supporting the fact that neuroprotection induced by INDP could be the result of an upregulation of IL10 and IL4 accompanied by a down regulation of TNFa, IL1β, and IL-6 genes. This information correlates with previous studies from our laboratory that elucidated some of the protective mechanisms induced by INDP; for instance, the reduced expression of the Inducible Nitric Oxide Synthase (INOS) and decreasing nitric oxide production. INOS expression is down regulated by IL10 and IL4 [7]. Further studies on the effect of INDP are warranted in order to completely understand how this strategy provides its neuroprotective effects after SCI.

References


