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Pro and anti-inflammatory cytokine levels (TNF- , IL-1 , IL-6 andIL-10) in rat model of neuroma

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Abstract

Traumatic neuroma is neuronal tissue proliferation developed in a nerve injury site, often associated with increased sensitivity and spontaneous or evoked neuropathic pain. The mechanisms leading to the disorganized nerve proliferation are not completely understood, though inflammation in the injured nerve vicinity most likely has a role in the process. Inflammatory cytokines are also known to be involved in the maintenance and development of post-traumatic and neuropathic pain. The goal of this study was to quantify and compare pro and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-10) levels in nerves that formed neuromas and nerves that did not, following sciatic nerve transection.

A total of 30 rats were used in this study. Twenty rats underwent sciatic nerve transection and 10 underwent sham surgery. Six weeks post-surgery nerve sections were collected and histologically evaluated for neuroma formation. The samples were then classified as neuroma, non-neuroma and sham groups. TNF-α, IL-1β, IL-6 and IL-10 levels were measured in the nerves employing ELISA. TNF-α levels were significantly higher in both neuroma and non-neuroma-forming injured nerves compared to the sham group. IL-1β and IL-6 levels were significantly higher in the neuroma-forming nerves compared to the sham group. IL-10 levels were significantly higher in the non-neuroma group compared to the sham group. In conclusion IL-6, and IL-1 β may have a role in the formation of traumatic neuroma while IL-10 may inhibit neuroma formation.

Introduction

The exact prevalence of neuromas following nerve injury is not known, though it has been variously reported as 10% to as high as 80% [1, 2]. Neuromas may occur following various injuries to the nerve, such as pressure, inflammation, ischemia, stretching, partial laceration, or complete transection [3]. Neuroma formation usually occurs during the nerve's attempt at self-

repair [4]. Histologically, neuromas consist of irregularly interwoven bundles of axons, Schwann cells, fibroblasts and their collagen products [5]. Up-regulation of sodium channels and a decrease in their thresholds may partly explain spontaneous activity, hyperalgesia, and easily-evoked activity. This can result in the development of hyperalgesia, allodynia, and spontaneous pain [6, 7]. While inflammation clearly plays an important role in the process of nerve injury and repair, the role of inflammatory mediators such as cytokines in neuroma formation, if any, is unclear [8].

The management of neuromas may be challenging. Various modalities, such as surgery, nerve blocks, botox, and local and systemic medications have been used, often with limited success [9-13]. When detectable visibly or by palpation, neuromas can be surgically removed or reduced in size by electrocoagulation or freezing [4, 14]. In other cases the neuroma can be rerouted into bone or muscle [15-18]. However, the current treatment options are often ineffective, and also have the potential to increase pain [14].

One reason for this limited treatment effectiveness has been an incomplete understanding of the underlying mechanisms of neuroma formation. What is known is that in the period immediately following nerve damage, an inflammatory reaction takes place, which can have a significant role in neuroma development. The initial inflammatory process typically involves the accumulation of numerous bioactive molecules such as bradykinin, nerve growth factor, serotonin, and prostaglandins. This can sensitize peripheral receptors, which can present as hyperalgesia and allodynia [19, 20]. Ectopic discharge and hyper-excitability have also been shown to occur in neuroma axons via up-regulation of sodium and calcium channels and down-regulation of potassium channels [21-23]. Nerve injury may be followed by degeneration of the affected C-fibers. A-beta fibers that typically do not transmit noxious information may undergo

phenotypical changes to do so. Persistent nociceptive input from the neuroma site can eventually result in central sensitization, which can involve the activation of glial cells, sensitization of wide dynamic range neurons, and glutamate-induced excitotoxicity of inhibitory interneurons.

Pro inflammatory cytokines such as IL-1β, 1L-6 and TNF-α are elevated in various neuropathic pain conditions [24-30]. Inflammatory cells have been shown to be present in traumatic neuromas. TNF-α and IL-1β appear to disturb the regeneration of injured nerves [8, 31]. Anti-inflammatory cytokines like IL-10 have the potential to reduce neuropathic pain by the down-regulation of pro-inflammatory cytokines such as TNF-α, IL-1 β and IL-6 [32, 33]. In the central nervous system, the production of these key cytokines and glial cells plays a critical role in the processing of abnormal pain signaling [34, 35]. These cytokines have been studied extensively in nerve damage, inflammation and the pain associated with it. As the pathogenesis of neuromas often includes nerve damage, the cytokine profile may be the same for both these conditions.

The goal of this study was to quantify TNF-α, IL-1β, IL-6 and IL-10 levels in sciatic nerves six weeks following complete transection, and to compare their levels in nerves that did or did not develop neuromas. We hypothesized that pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) levels would be higher in nerves that developed neuroma, while anti-inflammatory cytokines (IL-10) level will be lower in the same nerves [36].

Methods

All procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee at Rutgers, Newark, NJ (10077). Experiments were in accordance with the

National Institute of Health regulations, and the guidelines of the International Association for the Study of Pain [37].

Animals

Male adult Sprague-Dawley rats were ordered from a single lab (Charles laboratory). Rats weighed 300-325g at the time of surgery and were housed in the animal facility under veterinary supervision. Rats were allowed standard rodent chow and reverse osmotically-treated water ad libitum, and maintained on a twelve-hour day and night cycle.

Ten of the rats underwent sham surgery, and 20 rats underwent sciatic nerve transection. The sham-treated rats served as controls. Six weeks following the transection, the rats were euthanized. The transected nerves were evaluated histologically by a pathologist and categorized into neuroma and non-neuroma formation groups.

Surgery

For surgical procedures, rats were anaesthetized with a ketamine (50 mg/kg) and xylazine (7.5 mg/kg) solution administered intraperitoneally. The surgical site was shaved using a trimmer and sterilized with betadine and alcohol prior to the procedure. The eyes were lubricated and the rats were placed on a warming pad to maintain consistency of body temperature during surgery. A single, trained investigator performed the surgeries.

a. Sciatic Nerve Transection

The left common sciatic nerve was exposed via a mid-thigh incision proximal to the sciatic trifurcation, and transection was performed. Nerve tissue measuring approximately 1cm was removed distal to the incision. The incision was closed with stainless steel clips [38]

b. Sham Surgery

The common sciatic nerves were exposed at the mid-thigh level by blunt dissection through the biceps femoris, and gently separated from adjacent tissue. The incision was closed with stainless steel clips. The purpose of the sham surgery group was to control for the possibility that exposure of the nerve could cause the secretion of cytokines.

Histology and Neuroma evaluation

For the histological examinations, the tissue was immediately fixed using Zamboni's Fixative at 4^{o}C for 24 hours, after which time the tissue was processed and embedded in paraffin. Transverse sections (4μm) were cut from the proximal ends of the transected nerves, and also from the sham operated nerves. The sections were stained with hematoxylin & eosin (H&E) [8].

On gross examination, traumatic neuromas appeared as firm, white-gray nodules which were located in continuity with the proximal end of the injured or transected nerve. Histologically under intermediate power microscopy, traumatic neuromas showed extensive collagenization of the nerve tissue with residual chronic inflammation. Under higher power, histology revealed scarring and separation of collagen bundles and nerve fibers. The key finding in the histologic diagnosis of traumatic neuroma was the more extensive fibrosis, compared to that found in non-neuroma group (Figures 1 and 2)

Enzyme-Linked Immunosorbent Assay (ELISA)

Tissue samples were weighed, homogenized and centrifuged followed by collection of the medium. The medium and standards were pipetted and assayed with a site enzyme-linked immunoassay (ELISA, R&D Systems Inc., MN, USA) for IL-10, TNF-α, IL-1β and IL-6. After the binding of the primary and secondary antibodies, the substrate and stop solutions were applied, which changed the color of the bound substance. The intensity of the color reflected the amount of the cytokine bound. Using a curve plotted from standard solutions, cytokine levels were calculated as: pg. (cytokine)/ mL (medium)/mg (tissue) [33]

Data Analysis

Statistical tests were performed two-tailed, and the significance level was set to alpha 0.05. Data were tabulated and analyzed using JMP statistical analysis software (JMP, Version 10. SAS Institute Inc.). Mean values and standard deviations (SDs) were calculated for all continuous variables examined. All means were arithmetic means unless otherwise specified. Descriptive statistics are presented in the graphs as means and standard errors of the means.

In the neuroma, non-neuroma and sham group, data were collected for left sciatic nerve six weeks following the surgical procedures. ANOVA (RMANOVA), followed by Tukey's method for post-hoc analysis, was used to detect differences in cytokine changes.

Results

Results are expressed as cytokine levels measured in the surgically operated nerve for neuroma, non-neuroma and sham groups (pg/mL/mg ± S.D).

TNF α levels were significantly increased in both the neuroma (23.46 ± 1.4 pg/mL/mg) and the non-neuroma groups (17.942 ± 1.818 pg/mL/mg), compared to the sham group (1.26 ±

0.14 pg/mL/mg). The TNF α levels were also significantly higher in the neuroma group compared to the non-neuroma group ($P < 0.0001$) (Figure 3).

IL-6 levels were significantly increased in both the neuroma group (3,426.4 ± 237.6 pg/mL/mg) and the non-neuroma groups (703.470 ± 78.6 pg/mL/mg), compared to the sham groups (23.4 ±2.1 pg/mL/mg). IL-6 levels were also significantly higher in the neuroma group compared to the non-neuroma group ($P < 0.0001$) (Figure 4)

IL-1β levels were significantly elevated in the neuroma group (509.2 ± 118.5 pg/mL/mg, $p<0.05$) compared to both the non-neuroma (49.4 ± 9.6 pg/mL/mg) and sham group (17.9± 1.82 pg/mL/mg). There was no significant difference in IL-1β levels between the non-neuroma and sham groups (49.4 ± 9.6 pg/mL/mg and 17.9± 1.82 pg/mL/mg, $p>0.05$ respectively) (Figure 5).

IL-10 levels were significantly elevated in the non-neuroma group (338.9± 58.3 pg/mL/mg, $p<0.01$) compared to the neuroma group (179.5 ± 19.7 pg/mL/mg). No difference was found between the sham (222.2± 48.3 pg/mL/mg) and neuroma group (179.5 ± 19.7 pg/mL/mg, $p>0.05$) (Figure 6).

Discussion

The most noteworthy findings of this study were (1) all three pro inflammatory cytokines evaluated in this study were elevated in the neuroma group compared to the non- neuroma and sham groups, (2) IL-1β increase, relative to the sham group, was exclusive to the neuroma-

formation group, and (3) TNF-α and IL-6 were increased in both the neuroma and non- neuroma groups. The levels for the anti-inflammatory cytokine evaluated in this study, IL-10, were significantly higher in the non-neuroma group.

TNF-α is produced by activated macrophages and other cells such as T and B lymphocytes, Natural Killer cells, lymphokine activated cells, astrocytes, endothelial cells or smooth muscle cells [39]. TNF-α levels are elevated in inflammatory conditions and its presence can indicate an ongoing inflammatory process. It also appears to be a key player at both central and peripheral nerve degenerative pathologies, and in maintenance of neuropathic pain after following nerve injury [40]. In this study, as was expected, it was elevated in both neuroma and non-neuroma nerves that underwent transection.

IL-6 and IL-1β levels were significantly elevated only in the neuroma-forming nerves. IL-1β is a pro inflammatory cytokine, produced primarily by monocytes and macrophages, as well as by astrocytes, oligodendrocytes, Schwann cells, T cells, fibroblasts and other cells [41]. IL-1β, is involved in the immune response and signal transduction both in the peripheral and the central nervous systems. It plays an important role in inducing and maintaining neuropathic pain. Moreover, peripheral or central administration of IL-1β is usually followed by hyperalgesia [27, 42]. The pain accompanying neuromas may be associated to the increased IL-1β levels. Treatment with neutralizing antibodies to IL-1 receptor type I have been shown to reduce the development of neuropathic pain [28]. One reason for elevated IL-1β levels in the neuroma group in this study could have been increased neural activity in that group. Ectopic firing of sensitized nociceptive neurons, and the increased density of nociceptors nerve sprouts, may increase quantities and secretion of IL-1β. Studies have also reported IL-1 to attenuate neuropathic pain

and an increase in IL-1 levels in neuromas, while this study found an increase in IL-1β in neuromas [8, 43].

IL-6 is a multifunctional cytokine, with pro inflammatory and regulatory effects, and is induced during acute inflammation associated with injury, infection and neuronal death. IL-6 levels were significantly increased in both neuroma and non-neuroma nerves compared to the sham group, but the neuroma-forming nerves had significantly higher levels compared to the non-neuroma group. This may also be for the same reasons previously mentioned. Human studies in acoustic neuromas have also shown an association with elevated IL-6 levels [44].

IL-10 was only increased in the non-neuroma group and may suggest a protective effect. IL-10 is an anti-inflammatory cytokine produced by a wide variety of adaptive and innate immune cells such as CD4 and CD 8 T-lymphocytes, dendritic cells mast cells, natural killer cells, eosinophils macrophages and non-hematopoietic cells (35-36). IL-10 is considered primarily to have an anti-inflammatory effect and does so by suppressing the production of inflammatory cytokines [45, 46]. Recent studies have shown that IL-10 may also reduce neuropathic pain. Moreover, the efficacy of certain neuropathic pain medications is in part associated with IL-10 [33, 47]. Other studies have demonstrated an upregulation of IL-10 in response to nerve injury [48]. Future studies focusing on the administration of IL-10 and neuroma formation should be explored.

One limitation of this study was our lack pain behavior data. This absence was due to the fact that the nerve supplying the target organ (paw) was severed completely. Further studies could be done to evaluate the pain behavior using the neuroma-in-continuity model, and correlate the same with the quantity of cytokines. Another method that could be considered is

electrophysiology studies to measure neural activity as a marker for the presence of a neuroma [49]. Knowledge of the precise agents involved in traumatic neuromas will help in understanding the underlying mechanisms in neuroma formation and the development of treatment. Further studies should explore neuroma formation and cytokines in the injured nerve vicinity followed by medication efficacy and safety studies. Animal models of neuroma formation can help increase our understanding of these underlying mechanisms, and possibly lead to enhanced treatment in future. In summary TNF- α, IL-6, and IL-1 β may play a strong role in the formation of traumatic neuromas, while IL-10 may serve a protective function.

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