Longitudinal evaluation of the histological changes in a rat model of paravertebral muscle injury

（ラット傍脊柱筋損傷モデルを用いた経時的組織変化の検討）

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Abstract

Introduction: Thus far, few reports have described the time-series histological variation in injured paravertebral muscle tissues for longer durations, considering the type of pain.

Purpose: To evaluate histological changes in injured paravertebral muscle and dominant nerve considering the type of pain.

Methods: We used 59 eight-week old male Sprague Dawley rats. A 115-g weight was dropped from a height of 1 m on the right paravertebral muscle. FluoroGold (FG), a sensory nerve tracer, was injected into the paravertebral muscle. Hematoxylin and eosin (HE) staining and nerve growth factor (NGF) immunostaining of the muscle were performed for histological evaluation. L2 dorsal root ganglia on both sides were resected, and immunohistochemical staining for calcitonin gene-related peptide (CGRP, a pain-related neuropeptide) and activating transcription factor 3 (ATF3, a neuron injury marker) was performed. Each examination was performed at 3 days, 1-3 weeks, and 6 weeks after
injury.

Results: HE staining of the paravertebral muscle indicated infiltration of inflammatory cells and the presence of granulation tissue in the injured part on the ipsilateral side at 3 days and 1 week after the injury. Fibroblasts and adipocytes were present at 2-3 weeks. At 6 weeks, the injured tissue was almost completely repaired. NGF was detected at 2-3 weeks post injury and appeared to colocalize with fibroblasts, but was not observed at 6 weeks post injury. The percentage of cells double-labeled with FG and CGRP in FG-positive cells of the primary muscle was significantly higher in the injured side at 3 days and 1-3 weeks post injury (P < 0.05). However, at 6 weeks, no significant difference was observed. No significant expression of ATF3 was observed.

Conclusions: These results suggest that sensitization of the dominant nerve in the dorsal root ganglia, in which NGF may play an important role, can protract pain in injured muscle.
Introduction

The number of lumbar fusion surgeries in Japan has been increasing over the last 10 years\(^1\). It has been reported that use of the conventional midline posterior approach for lumbar spine surgery causes significant muscle injury, which is especially severe if powerful self-retaining retractors are used\(^2\)\(^3\). It was also reported that extended use of muscle retractors causes back muscle atrophy and postoperative low back pain at 6 months after surgery\(^4\). In contrast, another study of patients who underwent supine surgery for lumbar spinal stenosis reported that, despite worsened muscle atrophy, low back pain had improved significantly at 1 year post surgery\(^5\). We previously reported a time-series of the histological changes in injured paravertebral muscle and dominant nerves during the first 3 weeks post injury in rats\(^6\). In this study, the inflammatory phase at the injury site ended at 1 week post injury. However, activation of the dominant nerve in the dorsal root ganglia (DRG) continued throughout the experimental period. The cause of this gap between activation in the DRG and local injury and how long the agitation in the dominant nerve continues after inflammation of the injured muscle has ceased are unknown. Thus far, very few reports have described the relationship between histological variation in injured tissues and the neuropeptide transition in the dominant nerve for longer durations.

It has been reported that the use of gabapentinoids immediately decreases opioid consumption and pain intensity in patients following lumbar spine surgery\(^7\). Although many pathways, including neuropathic and nociceptive pathways, contribute to perioperative pain, it has not been clarified whether muscle injury causes emphatic neuropathic pain or not.

Therefore, the purpose of the current study was to determine the relationship between a
time-series of histological variations in injured back muscle and the production of calcitonin gene-related peptide (CGRP; a pain-related neuropeptide) and activating transcription factor 3 (ATF3, a neuron injury marker) in the dominant nerve over a 6-week period after experimental back muscle injury in rats.

We previously reported the expression of inflammatory mediators, such as nerve growth factor (NGF), tumor necrosis factor (TNF)-a, and interleukin (IL)-6, at 1 day after injury in a rat model of gastrocnemius muscle injury [8], and showed that administration of an antibody against NGF suppressed the inflammation in the DRG [9]. Therefore, here we focused on the role of NGF in the pain-producing mechanism in injured back muscle.

**Materials and Methods**

**Animal models**

All animal procedures and protocols were approved by the ethics committee of our university, and were performed in accordance with the United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (1996 revision). The methods used to generate the models and obtain specimens were described previously [9]. For the model, we used 59 8-week-old male Sprague-Dawley rats. Each rat weighed 250–300 g at the time of muscle injury. Before injury, the rats were anaesthetized with ketamine hydrochloride and xylazine. Then, a muscle contusion was induced without making a skin incision by dropping a 115 g weight from 1 m onto an impactor placed on
the right medial paraspinal musculature (Fig. 1).

**Histology**

Hematoxylin-eosin (HE) staining

For HE staining, the muscle injury site (i.e., the right side) was dissected from the back muscle under anesthesia with sodium pentobarbital (40 mg/kg, intraperitoneally) at 3 days and 1, 2, 3, and 6 weeks post muscle injury (4 rats per time point, 20 rats total), and perfused transcardially with 0.9% saline, and then with 500 mL of 4% paraformaldehyde in phosphate buffered saline (PBS; 0.1 M, pH 7.4). After dehydration for 14 h in a graded series of ethanol, each tissue specimen was formalin-fixed and embedded in paraffin with a Tissue-Tek VIP (M1500; Sakura Finetek Japan Co., Ltd., Tokyo, Japan). Subsequently, 4-µm-thick sections were generated from these paraffin blocks using a sliding microtome (LS113; Yamato Kohki Industrial Co., Ltd., Saitama, Japan). The sections were then placed on glass slides (#5116; Muto Pure Chemicals Co., Ltd., Tokyo, Japan) and stained with Mayer’s hematoxylin (Muto Pure Chemicals Co., Ltd.) for 5 min after deparaffinization with xylene and ethanol. After washing with distilled water, these sections were dipped in 0.1% ammonium solution several times, washed, and stained with a mixture of 1% eosin and phloxine (equal parts) in ethanol for 20 s. The sections were
finally overlaid with Entellan mounting medium (Merck KGaA, Darmstadt, Germany) after dehydration in a graded series of ethanol and xylene. A professional animal pathologist scored the slides under a microscope (BH20; Olympus Corp., Tokyo, Japan), and each finding was semiquantitatively evaluated. The slides were evaluated for the presence or absence of histological degeneration, bleeding, and neutrophil recruitment.

NGF immunostaining

The muscle injury site (i.e., the right side) was dissected from the back muscle under anesthesia at 2, 3, and 6 weeks post injury (3 rats per time point, 9 rats total).

Paraffin blocks were made with a Tissue-Tek V. I. P "6 (Sakura Finetek Japan). The center portion of each tissue block was cut into 4-µm-thick sections with a sliding microtome. Each section was mounted on a glass slide coated with 3-aminopropyltrimethoxysilane (APS) and dried. Then, the slide was immunostained for NGF.

Deparaffinized specimens were immersed in 10 mM citrate buffer (pH 6.0) and autoclaved at 121 °C for 10 min to induce antigen activation. After the treatment, peroxidase activity and nonspecific proteins were blocked in a refrigerator. Then, the sections were incubated with an anti-NGF rabbit polyclonal antibody (ab 6199; Abcam, Cambridge, UK; 1:250) overnight at 4 °C. Next, the sections were incubated with a horse
radish peroxidase (HRP)-labeled anti-rabbit IgG goat polyclonal antibody (#424144; Nichirei Corp., Tokyo, Japan) at room temperature for 30 min. Finally a chromogenic substrate, 3,3′-diaminobezidine-4-hydrochloric acid (DAB), was added. Antibody-positive signals were detected as a brown color, and nuclei were counterstained with hematoxylin (light purple).

**Immunohistochemistry for CGRP and ATF3**

Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA), a retrograde neuronal tracer, was injected into both sides of the paravertebral muscle to label the afferent sensory nerves. The L2 DRG of both the injured and uninjured side were removed at 3 days and 1, 2, 3, and 6 weeks after injury. According to a previous report, the paravertebral musculature is under a significant degree of L2 control. The proportion of FG-labeled neurons also showing immunoreactivity for CGRP, a marker of inflammatory pain, was determined. Endogenous peroxidase activity was quenched by soaking sections with 0.3% hydrogen peroxide in 0.01 M PBS for 30 min. The specimens were then incubated in a blocking solution (0.3% Triton X-100 and 3% skim milk in 0.01 M PBS) for 90 min at room temperature. The sections were incubated a primary rabbit antibody against CGRP (Chemicon, Temecula, CA, USA; 1:1000 in blocking solution), and then incubated for 20
h at 4°C. To detect CGRP immunoreactivity in the DRG, the sections were then incubated with an Alexa Fluor 488-conjugated goat anti-rabbit fluorescent antibody (Molecular Probes, Eugene, OR, USA; 1:400). The sections were examined under a fluorescence microscope, and the numbers of FG-labeled neurons, CGRP-immunoreactive (ir) neurons, and FG-labeled and CGRP-ir labeled neurons were determined in five randomly selected areas of each DRG section.

To detect ATF3, we used an anti-ATF3 antibody to (Chemicon; 1:200). The experiment to detect ATF3 was performed as described above for CGRP.

**Statistical analysis**

A t-test was used to compare the proportion of CGRP- and FG-positive neurons and ATF3- and FG-positive neurons in the DRG between the injured and uninjured sides at 3 days and 1, 2, 3, and 6 weeks after injury. A P value less than 0.05 was considered significant.

**Results**

**Histology**

The histological changes in the muscle samples from the injured side over time are shown
in Figure 2. HE staining of the paravertebral muscle showed infiltration of inflammatory cells and the presence of granulation tissue in the injured site, as well as neovascular hyperplasia at 3 days and 1 week post injury. Absorption of degenerated cell tissues, fibroblasts, and fat cells, were observed at 2 and 3 weeks post injury. Muscle atrophy was observed at 6 weeks post injury (Fig.2).

NGF was detected at 2–3 weeks post injury, and appeared to colocalize with the fibroblasts, but was not observed at 6 weeks post injury (Fig.3).

Immunohistochemistry for CGRP and ATF3

The results of the L2 DRG immunohistochemistry assay are shown in Figure 4. The percentage of cells double-labeled with FG and CGRP in the primary muscle was significantly higher on the injured side than on the uninjured side at all time points (3 days, injured side: 47.1 ± 6.1% [mean% ± SD], uninjured side: 29.2 ± 4.1%, P < 0.05; 1 week, injured side: 51.9 ± 9.4%, uninjured side: 25.6 ± 4.4%, P < 0.05; 2 weeks, injured side: 41.1 ± 7.9%, uninjured side: 21.3 ± 6.2%, P < 0.05; 3 weeks, injured side: 40.5 ± 5.4%, uninjured side: 20.4 ± 6.1%, P < 0.05). However, at 6 weeks, no significant difference was observed (injured side: 24.1 ± 6.9%, uninjured side: 25.1 ± 8.7%). In contrast, although a few ATF3-labeled cells were detected, we did not detect a significant
difference between the injured and uninjured sides at any tested time point (Fig.5).

**Discussion**

Huard described the healing of injured muscle as three phases: The first phase is degeneration and inflammation, which lasts for 1–2 weeks post injury. Next is the muscle regeneration phase, which starts in the first week and peaks at 2 weeks post injury. Finally, the fibrosis phase occurs at 2–4 weeks post muscle injury.

In the present study, inflammation at the injured site peaked at 3 days post injury and ended during the first week post injury; scarring developed by the third week, and although no fibroblasts were detected, muscle atrophy was detected at 6 weeks post injury. Although these transitions occurred a little earlier in the present study than in Huard’s study, the appropriate cells were detected in the corresponding order (Fig.6).

However, it should be noted that the proportion of CGRP-labeled neurons on the injury side remained high for more than 3 weeks, which is during the regeneration or fibrosis phase, even though the inflammatory cells had disappeared from the site before 2 weeks post injury; a similar gap was observed in our previous study. This discrepancy may provide insight into the mechanism underlying the prolonged pain experienced after muscle injury. NGF was detected on fibroblasts at 2–3 weeks post injury, during the
regeneration phase, in which neuronal cells in the DRG were inflamed. NGF disappeared at 6 weeks post injury, and at this time point, the elevated levels of CGRP decreased. These results suggest that NGF on fibroblasts may maintain activation of the DRG. Some reports that showed production of NGF at satellite cells or in the vascular smooth muscle in the early phase of regeneration and in fibroblasts in the early inflammatory phase also supported this hypothesis. Although we did not conduct a quantitative evaluation of NGF at the injury site or DRG, we cannot exclude the idea that NGF on fibroblasts may stimulate the neuron cells in the DRG. Further experiments, such as an evaluation of the dorsal horn of the spinal cord and behavioral studies, may elucidate the role of NGF in prolonged pain after back muscle injury.

It was reported that Tanezumab®, an anti-TNF agent, was very effective for chronic low back pain. Thus, this anti-TNF agent may be useful for low back pain after surgery.

ATF3 was not detected at any time point, which is a limitation of the present model. Tesarz reported that peripheral nerves only run along the surfaces of the thoracolumbar fascia. Furthermore, on the ventricle side of paravertebral muscle, only soft tissue is present, such as intra-abdominal organs and fat. Therefore, the dropped weight may not have caused sufficient damage to the nerves around the fascia of the back muscle. Kawaguchi et al. had reported that regeneration began at 1 week post injury and was complete by 6
weeks in a rat model of back muscle injury caused by a self-retractor for 3 hours \textsuperscript{16)}. Although the type of injury was different, we found that the repair process was nearly the same as in the present model.

**Conclusions**

In the present study, inflammation at the injured site peaked at 3 days post injury and terminated at 1 week post injury. However, the sensory nervous system in the DRG was still activated for more than 3 weeks post injury. This result provides a clue to resolving refractory pain after back muscle injury. NGF, which was detected at 2–3 weeks, may play an important role in pain mechanism after back muscle injury. No significant expression of ATF3 was observed.
References


Figure 4
Huard's three phases of muscle healing

| Inflammation phase | Regeneration phase | Fibrosis phase |

Muscle injury site

- Infiltration of inflammatory cells
- Production of NGF on fibroblasts

Nerve cells in DRG

- Elevation of positive rate for CGRP

Figure 6
Figure legends

Figure 1.
We defined the right-side back muscle as the injury site and the left-side back muscle as the non-injury site. Under anesthesia, 8-week-old male Sprague Dawley rats were injured on their right-side back muscle with a 115-g weight. The weight was dropped from 1 m high and was controlled by a pipe-shaped device. This figure used the schematic representation in our previous report as a reference.

Figure 2
Histological images of injury sites.
Transverse section showing injured muscle at 3 days (A), 1 weeks (B), 2 weeks (C), 3 weeks (D), and 6 weeks (E) post injury with hematoxylin-eosin staining (100× magnification). (A2), (B2), and (C2) are enlarged views of black-framed areas in (A2), (B2), and (C2), respectively. White arrowheads indicate red blood cells. Black arrowheads indicate macrophages. White arrows indicate neutrophils. Notably, fat cells were observed at 3 weeks post injury (D). Muscle atrophy was observed at 6 weeks post injury (E).
Figure 3

Histological evaluation for nerve growth factor (NGF).

Transverse sections of injured muscle at 2 weeks (A), 3 weeks (B), and 6 weeks (C) post injury immunostained for NGF (100× magnification). NGF was detected at 2–3 weeks post injury, and appeared to colocalize with the fibroblasts, but was not observed at 6 weeks post injury. Arrows indicate antibody-positive signals, which were detected as a brown color.

Figure 4

Immunohistochemical assessment of dorsal root ganglion (DRG) neurons.

(A) and (B), (C), and (D) are the same sections. Fluoro-Gold (FG)-labeled DRG neurons innervating injured muscle (A)(C). (B) Calcitonin gene-related peptide (CGRP)-immunoreactive DRG neurons. (D) Activating transcription factor-3 (ATF-3)-immunoreactive DRG neurons.

Arrows indicate FG and CGRP double-labeled neurons. Arrowheads indicate FG and ATF3 double-labeled neurons.

Figure 5
Change of activation in DRG neurons over time.

(A) The proportion of Fluoro-Gold (FG) and calcitonin gene-related peptide (CGRP) double-labeled dorsal root ganglion (DRG) neurons among all FG-labeled DRG neurons.

(B) The proportion of FG and activating transcription factor-3 (ATF-3) double-labeled DRG neurons among all FG-labeled DRG neurons. (*P<0.05, unpaired t-test).

Figure 6

Flow of events at the local injury site in paravertebral muscle and dominant neurons in the dorsal root ganglion (DRG). The proportion of CGRP-positive neurons remained high for more than 3 weeks, i.e., during the regeneration or fibrosis phase, even though the inflammatory cells had disappeared from the site by 2 weeks post injury. NGF was detected in fibroblasts at 2–3 weeks post injury, during the regeneration phase, in which neuronal cells in the DRG were inflamed. NGF disappeared at 6 weeks post injury, and at this time point, the elevated levels of CGRP decreased. Transitions occurred slightly earlier in the present study than in Huard’s study. This figure used Huard’s schematic representation as a reference.
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副論文
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