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Plantar incision with severe muscle injury can be a cause of long-lasting postsurgical pain in the skin

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ABSTRACT

Aims: Although chronic local inflammation in deeper tissues after skin wound healing might produce chronification of acute postsurgical pain, its mechanisms have not been fully elucidated. We hypothesized that muscle injury and severe inflammation would prolong acute postsurgical pain by its central nervous system mechanisms.
Main methods: After approval of the Animal Care Committee, experiments were performed in Male Sprague-Dawley rats weighing 250–300 g. Plantar incision and plantar incision combined with cryoinjury of the plantar flexor digitorum brevis muscle were made in the plantar incision group and muscle injury group, respectively. Pain-related behaviors were assessed, and inflammatory cells were isolated from injured muscle and analyzed by flow cytometry. Spinal microglial activation was assessed with Iba-1 staining.
Key findings: Mechanical hyperalgesia from day 5 to day 8 and spontaneous pain-related behavior from day 3 to day 7 were significantly greater in the muscle injury group than in the plantar incision group ($P < 0.05$), whereas there was no significant difference between the two groups in thermal hyperalgesia. In the muscle injury group, the number of inflammatory cells on day 4 was significantly larger and spinal Iba-1 expression levels on days 4 and 7 were significantly higher than those in the plantar incision group ($P < 0.05$).
Significance: Surgical injury in deep tissues accompanying severe muscle inflammation induced prolonged postsurgical pain in the healing wound of the skin not by the persistence of muscle inflammation but by a central mechanism involving microglial activation at the level of the spinal cord.

1. Introduction

Postsurgical pain results from tissue injury in the skin, fascia, muscles and small nerves terminating in these tissues [1]. Findings using an animal model for plantar incision [2] have revealed that the mechanisms of postsurgical pain are different from those in other pain models in which exogenous algesic substances were used to produce tissue injury [1,3–6], leading to new insights into therapeutic mechanisms of postsurgical pain [7]. However, an incision model would not completely mimic the features of postsurgical pain in humans. One reason for this is that the duration of spontaneous pain in an incision model is relatively short (~several hours) compared to the duration of postsurgical pain in a clinical setting (~several days). Another reason is that chronic postsurgical pain (CPSP) occurs in 10% to 50% of postsurgical patients with various types of surgical injury including injury caused by minor surgery [8], but it is not known whether pain-related behaviors are prolonged in any animal plantar incision models.

It has recently been reported that subcutaneous muscle incision rather than skin incision is a factor associated with hyperalgesia [9]. It has also been reported that local chronic inflammation in muscles [10,11] and nerves [12] might be one of the causes of CPSP. Indeed, in addition to the skin, deep muscles are incised, excised, dissected, retracted and easily injured by common surgical procedures such as contusion or cauterization. Injury and subsequent inflammation in deep muscles may persist after healing of a skin wound, possibly resulting in prolongation and/or chronification of postsurgical pain.

We hypothesized that a severe and long-lasting inflammatory response in the injured muscle results in long-lasting postsurgical pain at the site of the healing skin wound after skin incision combined with deep tissue damage including damage to the muscle. We applied severe injury to the plantar muscle of the rat and investigated the features of this type of injury-induced pain compared with the features of pain induced by

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plantar incision alone.

2. Materials and methods

2.1. Experimental animals

All procedures were conducted with approval by the Institutional Animal Care and Use Committee of Shinshu University School of Medicine (No. 270020) and in accordance with the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals as issued by the International Association for the Study of Pain [13]. All efforts were made to minimize the number of animals used and to decrease the suffering of the animals used in the study. Male Sprague–Dawley rats weighing 250–300 g at the start of surgery were used. Rats were housed in groups of two or three per cage and were acclimatized to the laboratory conditions (12-h light/dark cycle) with food and water being available ad libitum.

2.2. Surgical procedures

A plantar incision was made according to a previously described method [2] (plantar incision group). Briefly, anesthesia was induced and maintained with 3% sevoflurane in oxygen, and the plantar aspect of the right hind paw was sterilized with a 10% povidone–iodine solution before and after surgery. Beginning 0.5 cm from the proximal edge of the heel, a 1-cm longitudinal incision was made through the skin and underlying fascia with a #11 surgical blade. To separate the flexor digitorum brevis muscle from surrounding tissue, small forceps were used to elevate the flexor tendon from the heel to the toes, and then a 1-cm longitudinal incision was made to the plantar flexor digitorum brevis muscle. The wound was then closed using three inverted 5.0 nylon mattress sutures.

Plantar muscle cryoinjury (muscle injury group) was added at the same plantar site after the plantar incision had been made by the same method as that described above. Cryoinjury was induced by using a metal probe of 2 mm in diameter after cooling in liquid nitrogen (−190 °C) for 2 min immediately before application. The metal probe was applied on the center of the incised plantar muscle for 3 min (Fig. 1A). This procedure was repeated twice. The wound was then closed using three inverted 5.0 nylon mattress sutures. After surgery, the wound was covered with 0.1% aminoglycoside ointment (Takata Pharmaceutical Co., Ltd., Saitama, Japan) and anesthesia was discontinued. In the sham group, rats were anesthetized with sevoflurane, but no surgical procedure was performed.

2.3. Pain behaviors

For baseline testing, the rats were first acclimated to the testing environment for two days. The following pain-related behaviors were assessed on the third day as baseline (day 0) and once daily from day 1 to day 14 after plantar incision or plantar muscle cryoinjury.

We assessed the cumulative pain score (CPS) on the basis of weight bearing as a measure of spontaneous pain-related behavior [14]. Rats were placed individually on a stainless-steel mesh floor (openings, 8 × 8 mm) under a clear plastic cage and allowed to habituate for 10 to 15 min before the testing began. Subsequently, the positions of both injured and non-injured paws were observed for a 1-min period every 5 min for 1 h in each rat. A score of 0, 1, or 2 was given according to the position in which the paw was found during most of the 1-min scoring period. A score of 0 was given when the paw was firmly placed on the mesh if the wound was blanched or distorted by the mesh. If the area of the wound was lightly touching the mesh floor, a score of 1 was given. If the paw was completely away from the mesh floor, a score of 2 was given. The sum of 12 scores (range: 0–24) was obtained during the 1-h testing period. The CPS was then obtained by subtracting the score for the injured paw from that for the non-injured paw.

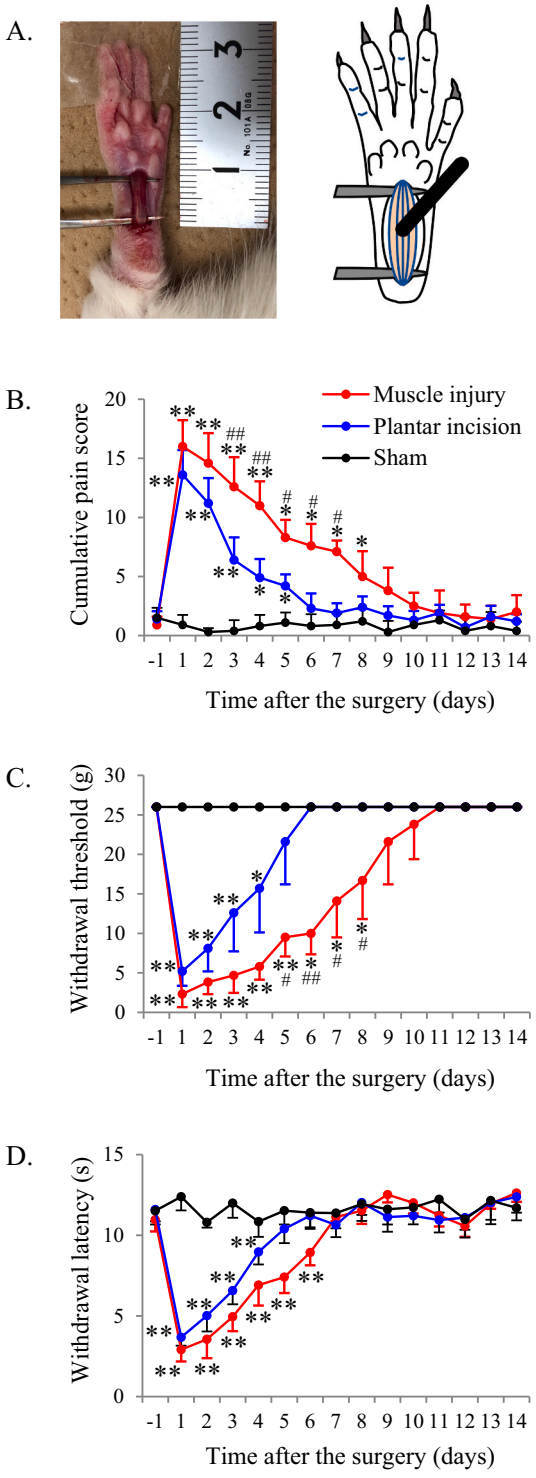

Fig. 1. Muscle injury and pain behaviors. **A:** Photograph of surgical exposure of the plantar muscle (left) and simplified cartoon of muscle injury of the rat plantar muscle (right). A nitrogen-cooled metal probe (black bar) was applied to the plantar flexor digitorum brevis muscle elevated by small forceps (grey instrument). **B:** Spontaneous pain assessed by cumulative pain score. **C:** Mechanical withdrawal threshold to stimulation with von Frey filaments. **D:** Withdrawal latency to heat stimulation. Data are expressed as means ± SDs. $n = 10$ in each group. * $P < 0.05$, ** $P < 0.01$ vs sham, # $P < 0.05$, # $P < 0.01$ vs plantar incision.

For mechanical withdrawal threshold (MT) measurement, unrestrained rats were placed on a stainless mesh floor (openings, 8 × 8 mm) covered with a clear plastic cage top. Calibrated von Frey filaments (Stoelting, Wood Dale, IL) with incremental degrees of stiffness (0.4–15 g) were applied adjacent to the wound in the plantar incision group and muscle injury group or the corresponding area in the sham group from least to greatest forces. Each filament was applied once until a withdrawal response was evoked in ascending order starting with 0.4 g. If there was no withdrawal response to a 15 g filament, 26 g was recorded as the threshold. Three tests were performed with 5-min intervals and the lowest force from the three tests was defined as the MT. [9]

Paw withdrawal latency (PWL) to noxious heat stimuli was measured by using a focused radiant heat source (model number 37370; Ugo Basil, Comerio, Italy). Each rat was placed in a clear plastic box on a glass surface and acclimatized for 10 to 15 min. The calibrated radiant heat source was focused on the injury area in the plantar incision group and muscle injury group or the corresponding area in the sham group [6]. The latency to evoke a withdrawal response was measured three times with intervals of 10 min and was determined with a cutoff value of 20 s to avoid tissue damage. The intensity of the heat was adjusted so that the basal PWL was 10 to 15 s in sham rats. The average of the three trials was recorded as the PWL.

2.4. Plantar muscle edema, histological analysis, isolation of immune cells and flow cytometry in the muscle after surgery

For the assessment of muscle edema after surgery, the wet/dry ratio of rat plantar muscle was determined. After each rat had been deeply anesthetized and euthanized with 3–5% sevoflurane, the rat's plantar muscle was isolated and weighed as wet weight. After the isolated muscle had been dried at 80 °C for 48 h, the muscle was weighed as dry weight. The wet/dry ratio (%) was calculated as (wet weight − dry weight) / wet weight × 100 [15].

For hematoxylin and eosin (H&E) staining, anesthetized rats were transcardially perfused with 4% paraformaldehyde in 0.1-M phosphate buffer. The rat hind paw including the skin and underlying muscle was removed, postfixed overnight at 4 °C, and embedded in paraffin. Sagittal sections (20 μm in thickness) were cut and stained with H&E. The number of infiltrating leucocytes in an area of 900 × 300 μm in the injured site was counted.

To isolate immune cells from the rat plantar muscle, anesthetized rats were transcardially perfused with ice-cold PBS to exclude blood. The plantar muscle was dissected in RPIM-1640 medium and digested with 0.5 mg/mL of type 2 collagenase (Worthington, Columbus, OH, USA) at 37 °C for 30 min in a shaker. The digest was filtered through a 40-mm cell strainer and centrifuged. The pellet was suspended in a 40% percoll (GE Healthcare, Milwaukee, WI, USA) solution overlaid on a 70% percoll solution and purified by gradient centrifugation at 600*g* for 30 min. Immune cells were collected from the interface and washed in MACS buffer (Miltenyi Biotec, Auburn, CA, USA). After Fc receptor blocking with anti-CD32 (BD Pharmingen, San Jose, CA, USA) for 30 min, cells were stained with anti-CD45-FITC (e-biosciences, San Diego, CA, USA) as a marker of leukocytes and with anti-CD11-PE (e-biosciences, San Diego, CA, USA) as a marker of myeloid cells including neutrophils, monocytes/macrophages and dendritic cells. Flow cytometric analysis and cell counting were performed on a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA) and data were analyzed by using FlowJo software (TreeStar, Ashland, OR, USA).

2.5. Expression of cFos and Iba-1 at the dorsal horn of the spinal cord after surgery

Immunohistochemical analysis was performed as previously described [6]. In brief, after perfusion fixation with 4% paraformaldehyde in 0.1-M phosphate buffer, the L4 to L5 segments of the spinal cord were removed, postfixed for 2 h, and cryoprotected in 30% sucrose in phosphate buffer overnight at 4 °C. The spinal cord was cut into sections of 50 μm in thickness using a sliding cryostat. The sections were blocked with 10% normal donkey serum in 0.2% TritonX-100 (Sigma-Aldrich, St Louis, MO, USA) in PBS (PBS-T), and incubated overnight at 4 °C with the following primary antibodies: c-Fos antibody (1:20,000, rabbit, Merck, Darmstadt, Germany), protein kinase Cγ (PKCγ; 1:200, guinea pig, Frontier Institute, Sapporo, Japan), or Iba-1 (1:500, rabbit, Wako Pure Chemical Industries, Osaka, Japan). After rinsing with PBS-T, the sections were incubated with Alexa Fluor 488- and Alexa 594-conjugated species-specific secondary antibodies (1:500, Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. The stained sections were examined with a confocal laser scanning microscope (Zeiss LSM5 EXCITER; Zeiss, Jena, Germany). Because the inner lamina II of the spinal dorsal horn in rats contains PKCγ-positive interneurons [16], the spinal dorsal horn was divided into the superficial dorsal horn (laminae I and II) and deep dorsal horn (laminae III and IV) by the PKCγ-labeled area. The number of c-Fos–positive neurons was counted in six randomly selected sections from each animal, and the mean of the six sections was calculated.

The activation of microglia at the spinal dorsal horn was assessed by measuring the immunodensity within a fixed area of the dorsal horn of the lumbar spinal cord [17,18] using Image J software (National Institutes of Health, Bethesda, MD, USA). The threshold density was obtained from the background. A box (100 × 100 μm) was placed onto areas of the lateral, central, and medial dorsal horn and the total pixels of these areas were measured. These measurements were performed on two randomly selected sections and the immunodensity was averaged for each animal.

2.6. Statistical analyses

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). For continuous data, a normal distribution of values was determined by the Kolmogorov-Smirnov test. Cumulative pain scores and withdrawal latencies were compared by two-way ANOVA followed by the Tukey post hoc test. For withdrawal threshold, the Kruskal-Wallis test for between-group comparison followed by Dunnett's post hoc test and Friedman's test for within-group comparison were used. The numbers of c-Fos-positive cells and infiltrating immune cells and fluorescence intensities of Iba-1 staining were compared by the Kruskal-Wallis test. Data are presented as means ± SDs. In all cases, a P value less than 0.05 was considered statistically significant.

3. Results

3.1. Pain-related behaviors after surgery

Spontaneous pain-related behaviors assessed by CPSs were increased after surgery in both the plantar incision group and muscle injury group (Fig. 1B). CPSs in the plantar incision group were significantly greater than those of the sham control from day 1 to day 5 after surgery ($P < 0.05$). Rats in the muscle injury group also showed significantly higher CPSs from day 1 to day 8 after surgery than those in the sham group ($P < 0.05$). Rats in the muscle injury group showed significantly greater and longer CPSs than those for rats in the plantar incision group from 3 days to 7 days after surgery ($P < 0.05$).

MTs by von-Frey filaments were significantly lower from day 1 to day 4 after surgery in the plantar incision group ($P < 0.05$) and from day 1 to day 8 after surgery in the muscle injury group ($P < 0.05$) than those in the sham group (Fig. 1C). The decrease in MTs was significantly greater and longer from day 5 to day 8 after surgery in the muscle injury group than in the plantar incision group ($P < 0.05$). PWLs to radiant heat stimulation were significantly lower from day 1 to day 4 after surgery in the plantar incision group ($P < 0.05$) and from day 1 to day 6 after surgery in the muscle injury group ($P < 0.05$) (Fig. 1D). However, there

was no significant difference between the plantar incision group and the muscle injury group.

3.2. Local inflammation and infiltrating immune cells after surgery

From the findings of behavior analysis, we decided to assess the involvement of surgical site inflammation in postsurgical pain in a relatively late phase, and we isolated and examined the plantar muscle on day 4 and day 7 after surgery. The surgical wound in the skin healed by day 7 in both groups, and the appearance of plantar muscle after surgery is shown in Fig. 2A. To evaluate the swelling of plantar muscle, we analyzed the wet/dry ratio of plantar muscle. Muscle swelling was induced in both the plantar incision group and muscle injury group at day 4 after surgery, and the swelling in the muscle injury group was more severe than that in the plantar incision group. The muscle swelling remained until day 7 in the muscle injury group (Fig. 2B). Leukocyte infiltration was seen on day 4 in both the plantar incision group and muscle injury group, and the number of infiltrating leukocytes in the muscle injury group was larger than that in the plantar incision group at 4 days after surgery (214 ± 58 vs 51 ± 27, $P < 0.05$) (Fig. 2C, D). On day 7, the number of infiltrating leukocytes in the muscle injury group had greatly decreased and there was no significant difference between the plantar incision and muscle injury groups (34 ± 15 vs 17 ± 8) (Fig. 2C, D).

3.3. Changes in the number of infiltrating myeloid cells and non-myeloid cells after surgery

Flow cytometry analysis was performed after isolation of infiltrating cells by collagenase digestion. Most of the infiltrating cells were myeloid cells ($CD45^{+}CD11^{+}$ cells; Fig. 3A) and the numbers of myeloid cells and non-myeloid cells on day 4 were larger in the muscle injury group than in the plantar incision group (53,505 ± 9030 vs. 19,897 ± 4240, $P < 0.01$; 10,108 ± 4997 vs. 3274 ± 1375, $P < 0.05$) (Fig. 3B, C). On day 7, the numbers of myeloid cells and non-myeloid cells had decreased and there was no significant difference between the plantar incision and muscle injury groups (9571 ± 2416 vs. 7715 ± 2420, 3669 ± 2277 vs. 2083 ± 893) (Fig. 3B, C). These results showed that surgical site inflammation was greater in the muscle injury group than in the plantar incision group on day 4, whereas the degrees of inflammation were comparable in the two groups on day 7.

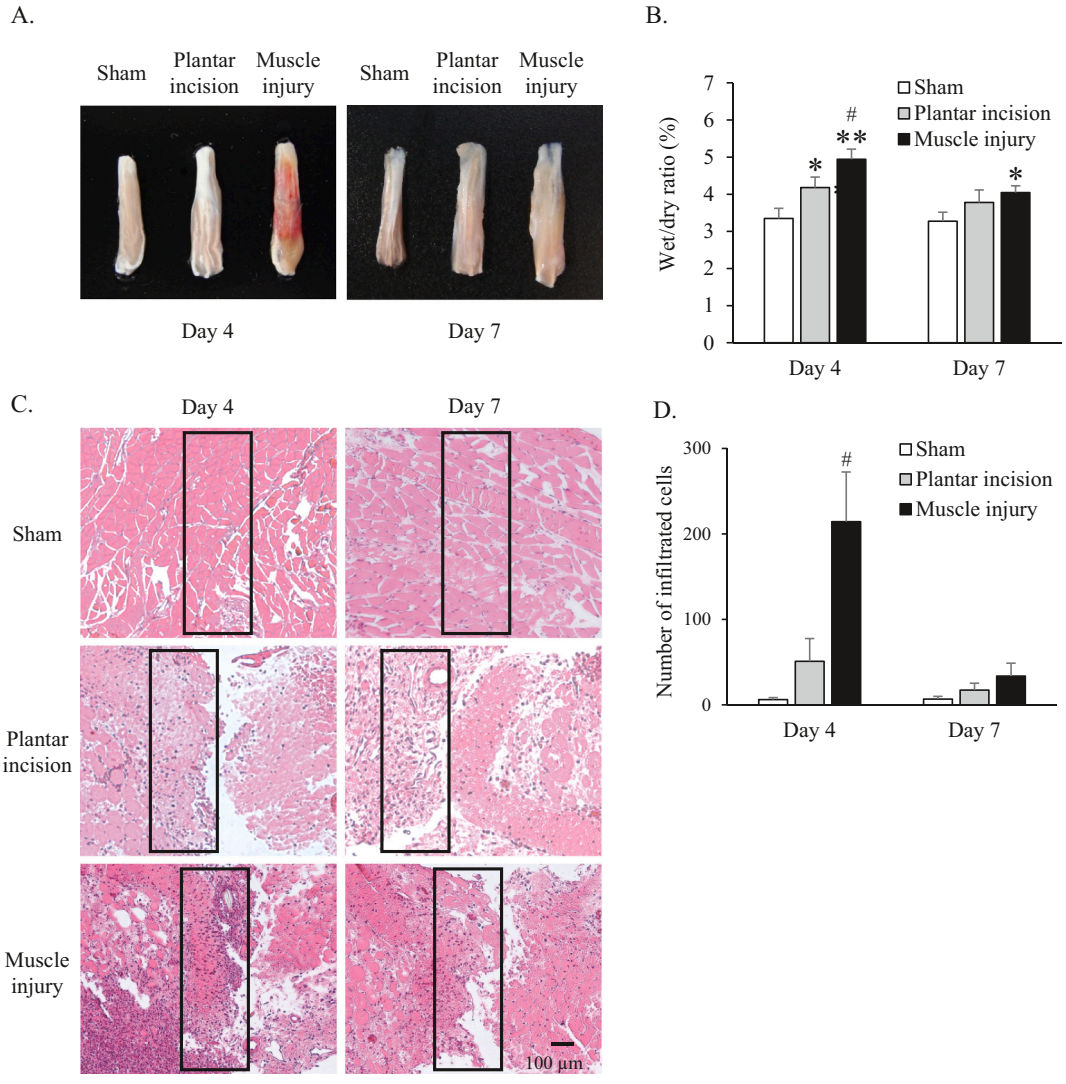

Fig. 2. Local inflammation after muscle injury. **A:** Representative images of the rat plantar muscle on day 4 and day 7 after surgerry. B: Wet/dry ratio of rat plantar muscle on day 4 and 7 after surgery. **C:** Representative image of hematoxylin and eosin staining in sagittal sections of the rat plantar muscle. The number of infiltrating immune cells in a 900 × 300 μm rectangle (black line) in the injured site was counted. **D:** Numbers of infiltrating immune cells in sections of the injured muscle on day 4 and 7 after surgery. Data are expressed as means ± SDs. $n = 6$ in each group. * $P < 0.05$, ** $P < 0.01$ versus sham, # $P < 0.05$ vs plantar incision.

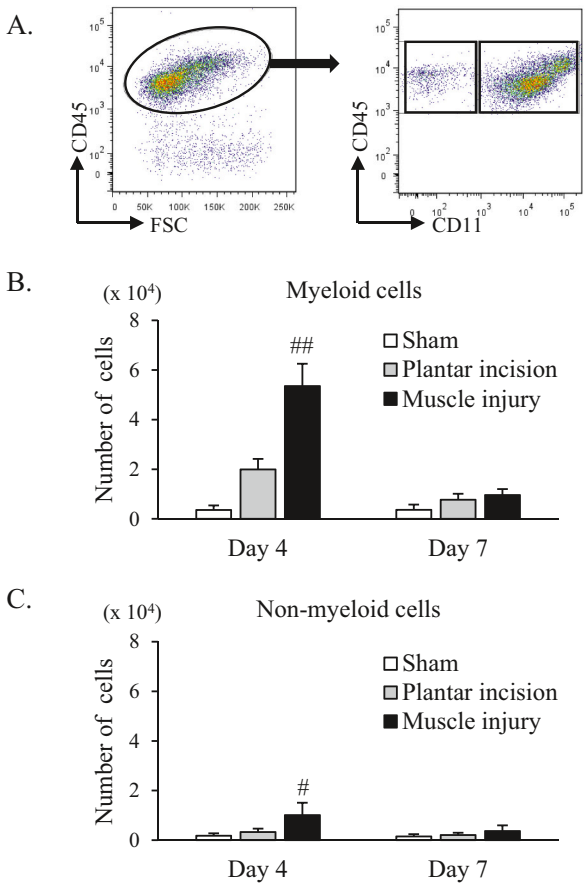

Fig. 3. Infiltrating immune cells after muscle injury. **A:** Gating strategy of flow cytometry. Numbers of myeloid ($CD45^+CD11^+$) cells **(B)** and non-myeloid ($CD45^+CD11^-$) cells **(C)** isolated from injured muscles on day 4 and day 7 after surgery. Data are expressed as means ± SDs. n = 6 in each group. # $P < 0.05$, ## $P < 0.01$ vs plantar incision.

3.4. Changes in activation of nociceptive neurons in the spinal dorsal horn after surgery

Next, we examined the involvement of cell activation in the spinal dorsal horn. The number of c-Fos–positive neurons in the superficial and deep dorsal horn on the ipsilateral side to injury was increased 6 h after surgery in both the plantar incision group and muscle injury group (Fig. 4A, B). At 24 h after surgery, c-Fos–positive neurons had almost disappeared in the superficial dorsal horn, while the number of c-Fos-positive neurons had decreased but remained larger than that in the sham group in the deep dorsal horn in both the plantar incision and muscle injury groups (Fig. 4A, B). Changes in c-Fos-positive neurons within 24 h after surgery were comparable in the plantar incision and muscle injury groups.

3.5. Microglial activation at the level of the spinal cord after surgery

Microglial activation at the level of spinal cord after surgery was assessed by Iba-1 expression. In the plantar incision group, Iba-1 expression was increased on day 4 after surgery ($P < 0.05$ vs. sham) and it was decreased to the same level as that in the sham group on day 7. In the muscle injury group, Iba-1 expression was greatly increased on day 4 after surgery ($P < 0.01$ vs. sham, $P < 0.05$ vs. plantar incision) and the high expression level persisted until day 7 ($P < 0.05$ vs. sham and plantar incision) (Fig. 5A, B). Muscle injury induced significantly greater and longer activation of microglia than did plantar incision.

4. Discussion

The main findings of this study are 1) spontaneous pain and mechanical hyperalgesia were significantly greater and longer after surgery in the muscle injury group than in the plantar incision group ($P < 0.05$), 2) there was no significant difference in thermal hyperalgesia between the plantar incision group and muscle injury group, 3) the number of infiltrating immune cells at the injured site was larger at 4 days but not at 7 days after surgery in the muscle injury group than in the plantar incision group, 4). The number of c-Fos-positive neurons increased in the dorsal horn of the spinal cord until 24 h after surgery in both the plantar incision and muscle injury groups, but there was no significant difference between the groups, and 5) spinal Iba-1 expression was significantly greater and longer in the muscle injury group than in the plantar incision group ($P < 0.05$).

Severe postsurgical pain occurs not only after major surgical procedures including long incisions and a great extent of tissue trauma such as that caused by open lung resection, cardiac surgery, total gastrectomy and mastectomy but also after minor surgical procedures including small incisions and a small extent of tissue trauma such as that caused by laparoscopic approaches, appendectomy, cholecystectomy and tonsillectomy [19]. This is partly because patients undergoing 'minor surgeries' do not receive appropriate analgesic treatment to reduce postsurgical pain. On the other hand, pain signals originating from the skin and deeper tissues and/or organs could temporally and spatially summate and produce much pain after surgery. Indeed, skin incision combined with deep muscle incision causes severe and long-lasting spontaneous pain and mechanical hyperalgesia (9). In addition, referred pain from deeper tissues and organs may affect the degree of postsurgical pain itself [20]. If an inflammatory response persists in deeper tissues such as muscle, from which pain signals converge on the same spinal dorsal horn neurons predominantly receiving pain signals from the skin, pain around the healed wound will still exist after wound healing in the skin. Thus, the intensity of postsurgical pain may not be less after some 'minor surgeries' in which multiple tissues and/or organs are injured by surgical procedures.

To investigate whether severe inflammation due to greater damage in deep tissue causes long-lasting pain, we applied cryoinjury to the rat plantar muscle to induce severe muscle injury. Cryoinjury in which the tissue structure is severely disrupted has been used to study the pathogenesis and regeneration after tissue injury such as traumatic brain damage [21] and myocardial infarction [22,23]. Muscle appearance and inflammation induced by cryoinjury were quite different from those after plantar incision. The number of inflammatory cells that infiltrated the surgical site in the muscle injury group on day 4 after surgery was much larger than that in the plantar incision group. These inflammatory cells are known to produce inflammatory mediators of pain including bradykinin, adenosine triphosphate, prostanoids, and cytokines that act on nociceptive primary afferent nerves and to induce inflammatory pain [24]. Among these cells, myeloid cells are thought to play a crucial role in the development of inflammatory mechanical hypersensitivity [25] and their contribution is thought to be different depending on the type of inflammation. We showed that muscle injury induced more severe inflammation since the number of myeloid cells was greatly increased in the muscle injury group compared to that in the plantar incision group on day 4 after surgery; however, we could not demonstrate that long-lasting pain was due to long-lasting local inflammation at the surgical site because the numbers of myeloid cells on day 7 after surgery were comparable in the plantar incision and muscle injury groups. These results suggest that persistent pain behavior in the muscle injury group cannot be explained only by persistence of inflammation.

Although the numbers of c-Fos-positive neurons were increased in the spinal dorsal horn at 6 h after plantar incision and muscle injury, they were decreased at 24 h after surgery, and there was no significant difference between the numbers of c-Fos-positive neurons in the plantar incision group and muscle injury group. Expression of c-Fos is a good

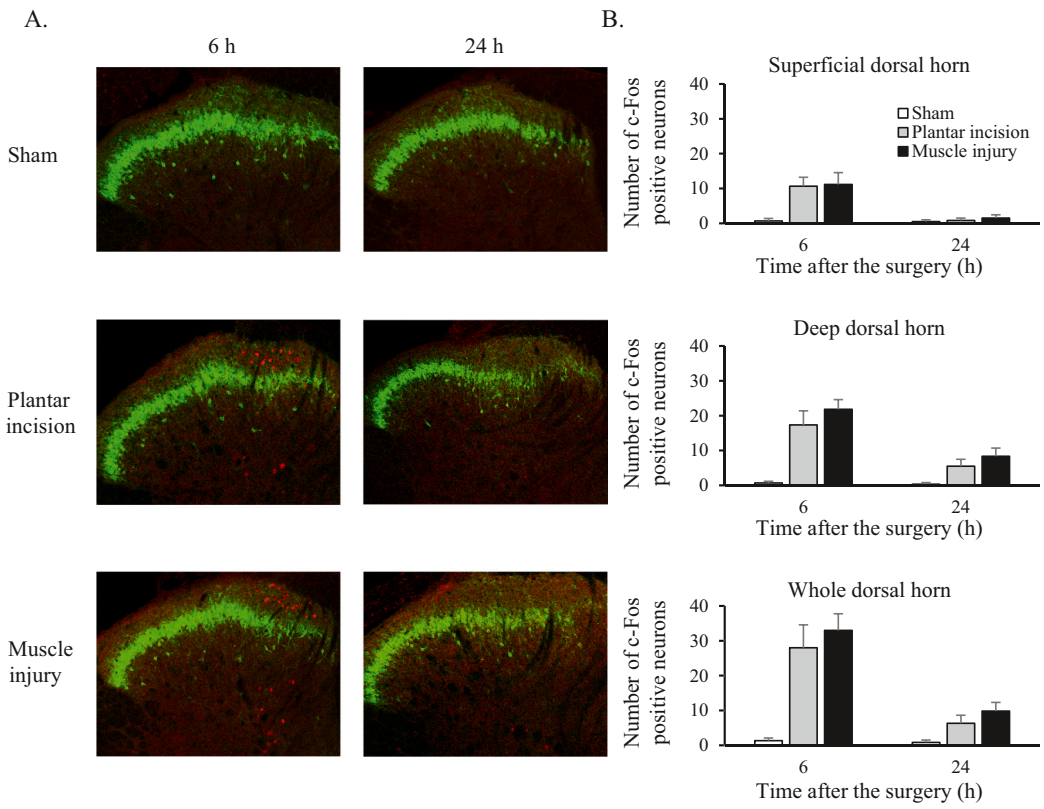

Fig. 4. Changes in activation of nociceptive neurons in the spinal dorsal horn with c-Fos expression. The spinal dorsal horn was divided into the superficial dorsal horn (laminae 1 and 2) and deep dorsal horn by the protein kinase Cγ-labeled area (green) that corresponds to the ventral part of lamina 2. Representative images of c-Fos expression (red) in the ipsilateral spinal cord after muscle injury (**A**) and numbers of c-Fos-positive neurons in the ipsilateral superficial dorsal horn (**B, upper panel**), deep dorsal horn (**B, middle panel**), and whole dorsal horn (**B, lower panel**). n = 6 in each group. Data are expressed as means ± SDs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tool for extensively examining activation of nociceptive neurons in the spinal cord, and it has been used in the pain research field for almost 30 years [26]. Since c-Fos expression is observed in spinal dorsal horn neurons, the degree of painful signal transmission from peripheral nociceptors to spinal nociceptive neurons can be evaluated by quantifying c-Fos-positive neurons. However, c-Fos expression and nociceptive behavior are not closely correlated, and c-Fos induction usually subsides even when nociceptive behavior still persists, suggesting that c-Fos has relatively low sensitivity, although c-Fos is a good marker of interaction between peripheral and central neurons. In addition, there was no significant difference between the degrees of thermal hyperalgesia in the plantar incision and muscle injury groups, and long-lasting thermal hyperalgesia was not observed in either group. A previous study showed that thermal hyperalgesia was induced by skin incision alone [27]. In the present study, the degree of skin injury in the muscle injury group was identical to that in the plantar incision group. Therefore, our results indicate that sensor proteins and transduction pathways involved in thermal hyperalgesia are different from those involved in mechanical hyperalgesia.

The prolonged mechanical hyperalgesia in the muscle injury group observed in this study is thought to be derived from hypersensitivity of the skin, not from sensitivity of the injured muscle. This is because more than fifty grams of force from the surface of the skin is required for noxious stimulation in the muscle even if muscular hyperalgesia is present [28,29]. While the concept of sensitization of nociceptive pathways has long been considered as a pain enhancement mechanism, it has become increasingly recognized that spinal glia, particularly microglia, play a crucial role in various types of pain [30] by modulating the neuronal nociceptive network (5). In this study, spinal Iba-1 expression was significantly greater and longer in the muscle injury group than in the plantar incision group, being consistent with spontaneous pain and mechanical hyperalgesia. Similar to our results, previous studies showed that plantar incision induced activation of spinal microglia on day 3 and that the activation disappeared on day 7 [31,32]. It has also been reported that activation of spinal microglia occurred and was sustained by prolonged inflammation induced by subcutaneous injection of complete Freund's adjuvant into the rat plantar surface [33,34]. In contrast, despite the fact that local inflammation in the muscle injury group was decreased on day 7 after surgery in our study, microglial activation was prolonged until day 7. These results indicate that activation of microglia is one of the important factors that cause long-lasting pain-related behavior after muscle injury and that there is little contribution of local inflammatory mediators to peripheral nerves.

Greater and longer activation of microglia in muscle injury is considered to be attributed to the difference in severity of inflammation in the early phase of tissue injury. On day 4, activation of microglia was already upregulated compared to that in the plantar incision group in our study. Peripheral nerves are known to be degenerated by inflammatory mediators in various pathological conditions [35–38], and injured peripheral nerves sometimes cause neuropathic pain [35,36]. Injured nerves also secrete proinflammatory molecules in the surrounding tissue and recruit circulating inflammatory cells, leading to peripheral sensitization, that is, 'hyperexcitability of nociceptors'. It has been reported that peripheral inflammatory monocytes/macrophages influence hypersensitivity after peripheral nerve injury [39,40]. Taken together, the findings suggest that long-lasting hyperalgesia in deep muscle injury is caused by peripheral nerve injury due to severe inflammation in the early phase of tissue injury followed by spinal microglial activation.

From the findings, pharmacological inhibition of myeloid cells including macrophages and/or macrophage-derived cytokines and chemokines may be a new target for postsurgical pain management as a novel treatment, although careful investigation is required for the wound-healing process [41] because inflammatory cells play various roles in wound healing, and the type, number and function of inflammatory cells change as the tissue damage and wound healing proceeds. Not only cells in peripheral tissue but also activated microglia in the spinal cord may be a new target for long-lasting postsurgical pain,

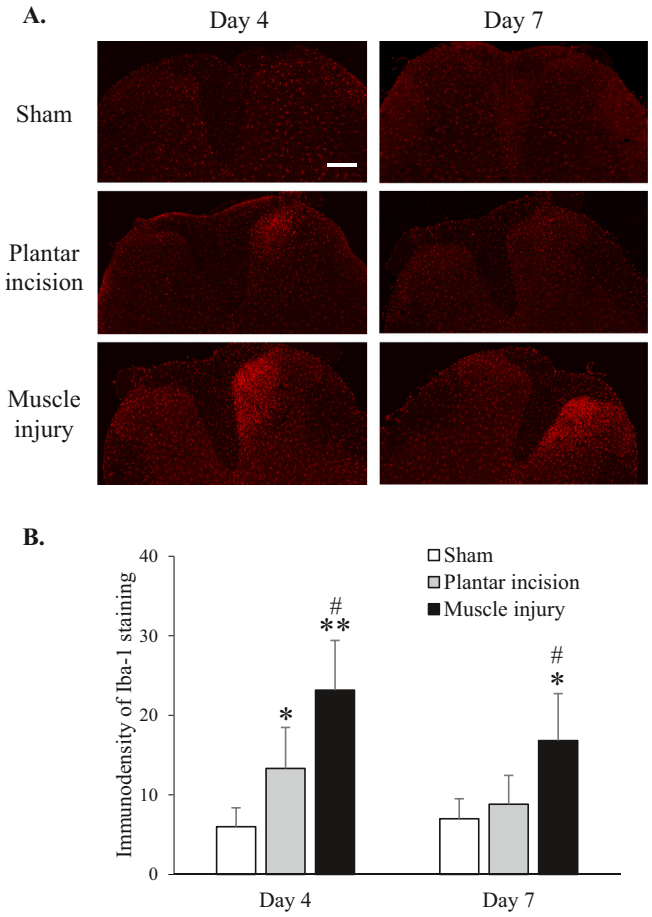

Fig. 5. Microglial activation after muscle injury assessed by Iba-1 reactivity. **A:** Representative images of Iba-1 immunofluorescence staining at the dorsal horn of the L4–5 spinal cord on day 4 and day 7 after surgery. **B:** Quantitative assessment of ipsilateral Iba-1 immunodensity at the dorsal horn of the L4–5 spinal cord on day 4 and day 7 after surgery. Data are expressed as means ± SDs. n = 6 in each group. * $P < 0.05$, ** $P < 0.01$ versus sham, # $P < 0.05$ vs plantar incision.

although we need to investigate the involvement of spinal astrocytes, which are known to be important for the generation and maintenance of neuropathic pain [42]. Since this study was the first stage of investigation of long-lasting postsurgical hyperalgesia due to multiple organ or tissue injury induced by a surgical procedure in a clinical setting, the animal model and investigation of peripheral tissue and the spinal cord were simplified. Further study is required to elucidate the mechanisms underlying the relationship between peripheral inflammation and neuroinflammation-associated postsurgical pain, and the development of an animal model that reflects the characteristics of postsurgical pain more closely is needed.

5. Conclusion

In this study, we demonstrated that plantar incision with muscle cryoinjury induced severe inflammation in the muscle in the early phase of tissue injury and that long-lasting postsurgical hyperalgesia was induced by its central mechanisms at the level of the spinal cord.

CRediT authorship contribution statement

Yuki Yoshiyama: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing-Review & Editing, Funding acquisition.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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