MUSCLE INJURY IN RATS INDUCES UPREGULATION OF INFLAMMATORY CYTOKINES IN INJURED MUSCLE AND CALCITONIN GENE–RELATED PEPTIDE IN DORSAL ROOT GANGLIA INNERVATING THE INJURED MUSCLE

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ABSTRACT: *Introduction:* In this study we evaluated the relationships among the behavioral changes after muscle injury, histological changes, changes in inflammatory cytokines in the injured muscle, and changes in the sensory nervous system innervating the muscle in rats. *Methods:* We established a model of muscle injury in rats using a dropped weight. Behavior was assessed using the CatWalk system. Subsequently, bilateral gastrocnemius muscles and dorsal root ganglia (DRGs) were resected. Muscles were stained with hematoxylin and eosin, and inflammatory cytokines in injured muscles were assayed. DRGs were immunostained for calcitonin gene–related peptide (CGRP). *Results:* Changes of behavior and upregulation of inflammatory cytokines in injured muscles subsided within 2 days of injury. Repaired tissue was observed 3 weeks after injury. However, upregulation of CGRP in DRG neurons continued for 2 weeks after injury. *Conclusion:* These findings may explain in part the pathological mechanism of persistent muscle pain.

Muscle Nerve **54**: 776–782, 2016

Muscle injures are common in sports and account for 10%–55% of all sports-related injuries.[1,2] Although muscle can regenerate, recurrent muscle injuries could affect muscle performance. Skeletal muscle regeneration is well documented, and several studies have shown that regeneration takes place after contusion.[3–6] Contusions are the most common type of muscle injury. Despite their common occurrence, treatment is rarely required. However, it is well known that these injuries affect muscle structure and function, resulting in muscle atrophy, contracture, pain, and reinjury.[7]

Investigators have reported various models of muscle injury. The invasive freeze-induced model[8]

and contraction-induced model of muscle injury[9] are well known. However, these models do not closely match clinical observations. By contrast, several investigators have developed models of muscle injury using mechanical methods, including a spring-loaded hammer[10] and a dropped-weight technique.[4,5]

Upregulation of various inflammatory cytokines in injured muscle has been proposed as a pathomechanism of muscle injury pain.[8,11–13] Muscle injury induces upregulation of sensory neuropeptides in the sensory nervous system.[14]

The aim of this study was to evaluate the hitherto unreported relationships between behavioral changes observed after muscle injury in rats, histological changes in injured muscle fibers, levels of inflammatory cytokines in injured muscle, and changes in the sensory nervous system innervating muscle fibers.

METHODS

All protocols for animal procedures were approved by the ethics committee of Chiba University following the *Guidelines for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (1996 revision).

Animals and Surgery. One hundred 8-week-old male Sprague-Dawley rats weighing from 250 to 300 g were used. Rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally, with supplemental doses as required) and treated aseptically throughout surgery.

We used a model of muscle injury based on right gastrocnemius muscle contusion produced with a dropped-weight technique.[4] Using a custom-made frame, a 115-g weight was dropped from a 1-m height through an acrylic guide tube onto an impactor, driving the impactor into the muscle belly without injuring the overlying skin or Achilles tendon.

Experiment 1: Behavioral Analysis: CatWalk System. We used the CatWalk gait analysis system (Noldus Information Technology, Wageingen, The Netherlands) to evaluate the behavior of rats with muscle

injury. The CatWalk system provides a detailed analysis of gait, which has been described in detail elsewhere.[15] A detailed gait analysis was made using the CatWalk system at 3 hours before injury, and at 12 hours; 1, 2, and 3 days; and 1, 2, and 3 weeks after muscle contusion. The gait of 28 rats was recorded 3 times and analyzed using the CatWalk system. In this study, 18 paw variables (the mean of hind paws: 18 variables) were used. We calculated the ratio between the affected (right hind) side and the unaffected (left hind) side.

Experiment 2: Histology. We used a model of muscle contusion as described for Experiment 1. After rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally), they were perfused transcardially with 0.9% saline, followed by 500 ml of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4), and gastrocnemius muscles were resected bilaterally at 3 days ($n = 12$), and 1, 2, and 3 weeks after muscle injury ($n = 4$ each). The specimens were immersed in 4% phosphate-buffered paraformaldehyde at 4°C for 3 days, followed by immersion in 70% ethanol for 12 hours. The specimens were immersed in paraffin after dehydration in an automatic embedding machine (Tissue-Tek VIP M1500; Sakura FineTek, Tokyo, Japan). The specimens were continuously immersed in paraffin for 5 days, and embedded in paraffin blocks. The samples in paraffin blocks were sectioned serially in an axial (transverse) plane in 8-μm steps. Sections were deparaffinized in xylene, dehydrated using a graded series of ethanol, stained using hematoxylin and eosin (HE), and assessed by light microscopy.

Experiment 3: Response of Inflammatory Mediators. We used a model of muscle contusion as described for Experiment 1. After the rats were further anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally), gastrocnemius muscles were resected bilaterally at 12 hours, and 1, 2, and 3 days after muscle injury ($n = 10$ each). The trimmed samples of gastrocnemius muscle tissue were cut into pieces about 5 mm × 5 mm × 5 mm in size, rinsed well in phosphate-buffered saline (PBS), frozen in liquid nitrogen, and then crushed into powder and homogenized in a 1,000-μl lysis/ extraction reagent (CelLytick; Sigma-Aldrich, St. Louis, Missouri). The samples were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were extracted for assay. The cytokine concentrations were measured using enzyme-linked immunoassay (ELISA) kits optimized for each cytokine according to each manufacturer's protocol as follows: interleukin (IL)-6 (R&D Systems, Minneapolis, Minnesota); tumor necrosis factor (TNF)-α (R&D Systems); and nerve growth factor (NGF;

Millipore, Billerica, Massachusetts). The limits of sensitivity for the cytokines measured were 14, <5, and 10 pg/ml, respectively. The total protein concentration in all samples was measured using the Lowry method with a DC protein assay kit (Bio-Rad Laboratories, Hercules, California). After measurement with a microplate reader (Iwaki, Tokyo, Japan), we converted the absolute cytokine concentrations into corresponding concentrations per milligram of total protein.

Experiment 4: Neurotracing and Immunohistochemistry for CGRP. Rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally, with supplemental doses as necessary) and treated aseptically throughout surgery. Retrograde Fluoro-Gold (FG; Fluorochrome, Denver, Colorado) labeling was used to detect dorsal root ganglion (DRG) neurons innervating the gastrocnemius muscle. In brief, FG was applied to the surfaces of gastrocnemius muscle bilaterally.

Rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) 1 week after application of FG and treated aseptically throughout surgery. We used a model of muscle injury as described for Experiment 1.

At 3 days and 1, 2, and 3 weeks after muscle injury ($n = 5$ each) rats were anesthetized and perfused as described for Experiment 2. Next, DRGs from the L1 to S3 levels were resected bilaterally, the specimens were immersed in the same fixative overnight at 4°C, and then stored in 0.01 M PBS containing 20% sucrose for 20 hours at 4°C.

Subsequently, the DRG specimens were embedded in OCT TissueTek (Sakura FineTek) and frozen in liquid nitrogen. Each ganglion was sectioned at a 10-μm thickness on a cryostat, and sections were mounted on poly-L-lysine–coated glass slides. Endogenous tissue peroxidase activity was quenched by soaking the mounted sections in 0.3% hydrogen peroxide solution in PBS for 30 minutes. Sections were then incubated for 90 minutes at room temperature in a blocking solution of PBS with 0.3% Triton X-100 and 3% skim milk. To stain the DRG sensory neurons, the sections were processed for CGRP immunohistochemistry using a rabbit antibody to CGRP (1:1,000; Chemicon, Temecula, California) diluted in blocking solution and incubated with the section for 20 hours at 4°C. To detect CGRP immunoreactivity in the DRGs, sections were incubated with goat anti-rabbit fluorescent antibody conjugate (1:400; Alexa 488; Molecular Probes, Eugene, Oregon). Sections were examined using a fluorescence microscope and, for each DRG section, we counted the numbers of FG-labeled (only) neurons and the numbers of FG-labeled and CGRP-immunoreactive (IR)
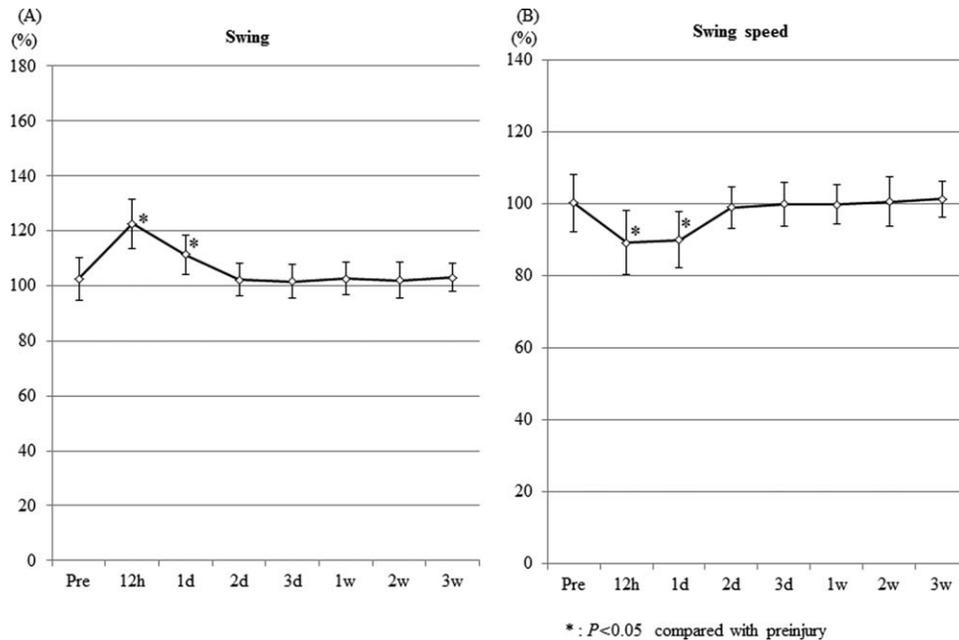
FIGURE 1. CatWalk analysis ($n = 28$). **(A)** The ratio of swing time of the injured (right hind) paw to the uninjured (left hind) paw (%). **(B)** The ratio of swing speed of the injured (right hind) paw to the uninjured (left hind) paw (%). *$P < 0.05$ difference between ratio of injured (right hind) side to uninjured (left hind) side before and after injury, with nonrepeated ANOVA followed by Bonferroni correction.

neurons per 0.45 mm^2 in 10 randomly selected fields at $400\times$ magnification using a counting grid. The proportion of CGRP-IR DRG neurons among all FG-labeled neurons was then calculated.

Statistical Analyses. Gait variables from CatWalk data between the injured (right hind) side and uninjured (left hind) side were compared before and after injury using a non-repeated analysis of variance (ANOVA) followed by Bonferroni correction. The levels of inflammatory mediators in the gastrocnemius muscle between the injured and uninjured side were compared using an unpaired t-test. The proportion of FG-labeled and CGRP double-labeled neurons among FG-positive neurons in the DRG from the L1–S3 levels between injured and uninjured sides was compared using an unpaired t-test. $P < 0.05$ was considered statistically significant.

RESULTS
Experiment 1: CatWalk Analysis. Swing, the duration of no contact between a paw with the glass CatWalk plate, and swing speed, the speed of the paw during the swing, were 2 variables that showed significant mean differences between the right hind paw (injured side) and the left hind paw (uninjured side).

The ratio of swing of the injured side to uninjured side 12 hours and 1 day postinjury was significantly larger than that preinjury ($P < 0.05$) (Fig. 1A).

By contrast, the ratio of the swing speed of the injured side to uninjured side at 12 hours and 1

day postinjury was significantly lower than that preinjury ($P < 0.05$) (Fig. 1B).

Experiment: Histology. Specimens from day 3, and 1, 2, and 3 weeks after injury were examined, and the injured and uninjured sides were compared (Fig. 2A). Slides were examined grossly and microscopically at $40\times$ and $400\times$ magnifications. At day 3, marked edema and hematoma formation, an intense inflammatory response, and necrotized myofibers were observed in all specimens (Fig. 2B and C). Accumulation of eosinophils and neovascularization were also observed (Fig. 2D). The injury was localized near the surface of the gastrocnemius muscle, adjacent to the site of impact (Fig. 2E). From there, reparative and granulation tissues were observed deep in the gastrocnemius muscle (Fig. 2F).

At 1 week (Fig. 3A), HE staining revealed an intense cellular infiltration of myoblasts and fibroblasts (Fig. 3B). Accumulation of collagenous fibers and giant cell macrophages was observed (Fig. 3C). Residual inflammatory elements were rare. Hematoma formation or edema were present (Fig. 3D).

At 2 weeks (Fig. 4A), HE staining revealed intense cellular proliferation of lymphocytes on the surface layer of the injured side (Fig. 4B). Accumulation of moderate neovascularization was observed; the process of tissue repair followed (Fig. 4C).

By 3 weeks (Fig. 5A), HE staining of injured specimens was indistinguishable from controls
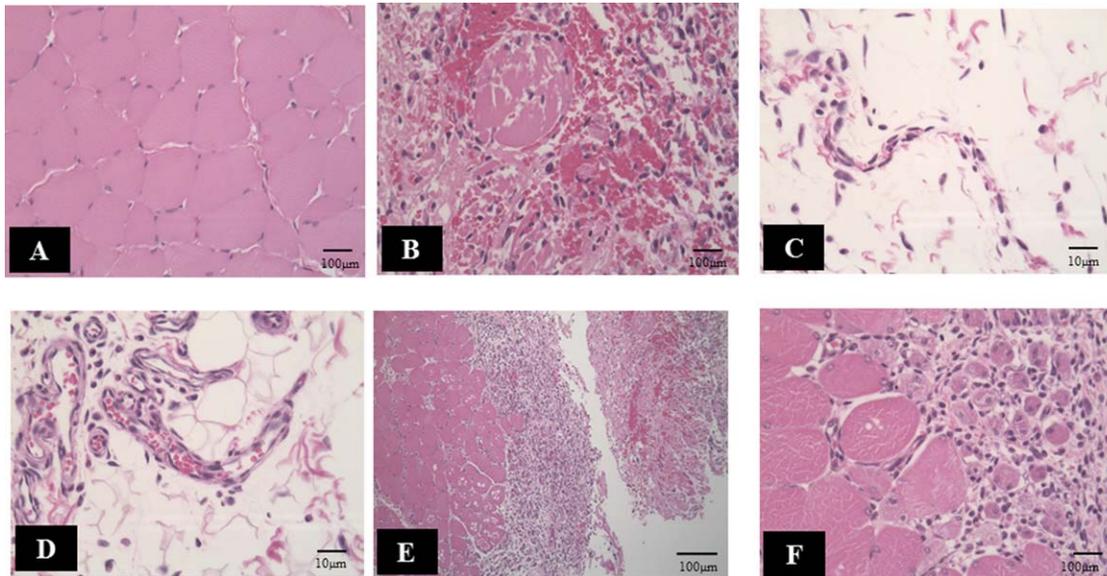
FIGURE 2. Hematoxylin and eosin (HE) staining ($n = 12$). Transverse sections showing gastrocnemius muscle fibers at 3 days and at 1–3 weeks. **(A)** Uninjured side appears normal. **(B)** Injured side shows edema and hematoma formation. **(C)** Injured side shows macrophages and fibroblasts ($400\times$ magnification). **(D)** Injured side shows neovascularization, neutrophils, and eosinophils. **(E)** Injured side, showing the superficial layer ($40\times$ magnification). **(F)** Injured side, showing the deep layer ($40\times$ magnification).

(Fig. 5A and B). Superficial remnants of bands of scar tissue were noted in some specimens. Accumulation of granulation tissue and myofibrillogenesis from repaired tissue and muscle development were observed on the injured side (Fig. 5C and D).

Experiment 3: Response of Inflammatory Mediators.
Levels of NGF on the injured side were significantly higher than those on the uninjured side ($P < 0.05$) by 12 hours and remained significantly higher for at least 1 day after the injury, with a maximum level seen at 12 days. However, by 2 days after injury, levels of NGF between the injured and uninjured sides were no longer significantly different (Fig. 6A). Levels of TNF-α on the injured side were significantly higher than those on the uninjured side ($P < 0.05$) by 12 hours, with a maximum level seen at that time. However, by 1 day after injury, the levels of TNF-α between the injured and uninjured sides were no longer significantly different (Fig. 6B). Levels of IL-6 on the injured side were significantly higher than those on the uninjured side ($P < 0.05$) by 12 hours, with a maximum level seen at that time. However, by 1 day after injury, levels of IL-6 between the injured and uninjured sides were no longer significantly different (Fig. 6C).

Experiment 4: Neurotracing and Immunohistochemistry for CGRP. FG-labeled DRG neurons, indicating where FG was transported from the gastrocnemius muscle, were present in bilateral DRGs from L4 through S1. FG-labeled (Fig. 7A) and CGRP-IR (Fig. 7B) neurons were present in bilateral DRGs

from L4 through S1 (Fig. 7). For up to 2 weeks after injury, the proportion of CGRP-immunoreactive and FG-positive neurons (Fig. 7A and B) among all FG-labeled DRG neurons on the injured side (3 days, 41.8%; 1 week, 39.1%; 2 weeks, 33.9%) was significantly higher than the corresponding proportion on the uninjured side (3 days: 21.6%; 1 week: 23.5%; 2 weeks: 22.6%) ($P < 0.05$). However, at 3 weeks, the proportion of CGRP-immunoreactive and FG-positive neurons
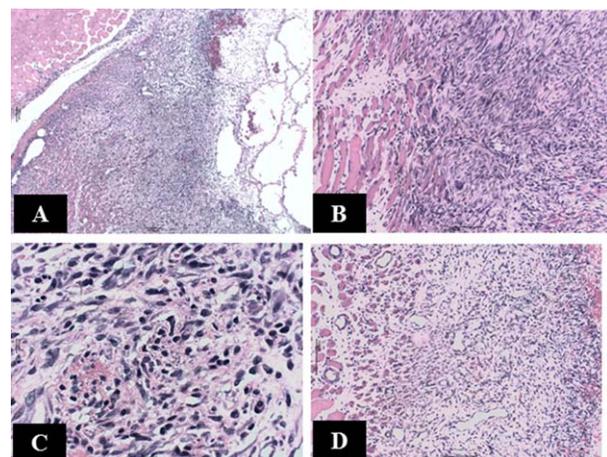

FIGURE 3. Transverse sections showing gastrocnemius muscle fibers with HE staining ($n = 4$) at 1 week. **(A)** Injured side, showing strong edema around muscle fibers, but moderate hematoma or muscle injury is not seen in the surface or deep layers ($40\times$ magnification). **(B)** Injured side, showing myoblasts and fibroblasts. **(C)** Injured side, showing giant cell formation and macrophage deposition ($400\times$ magnification). **(D)** Injured side, showing edema and hematoma formation ($40\times$ magnification).
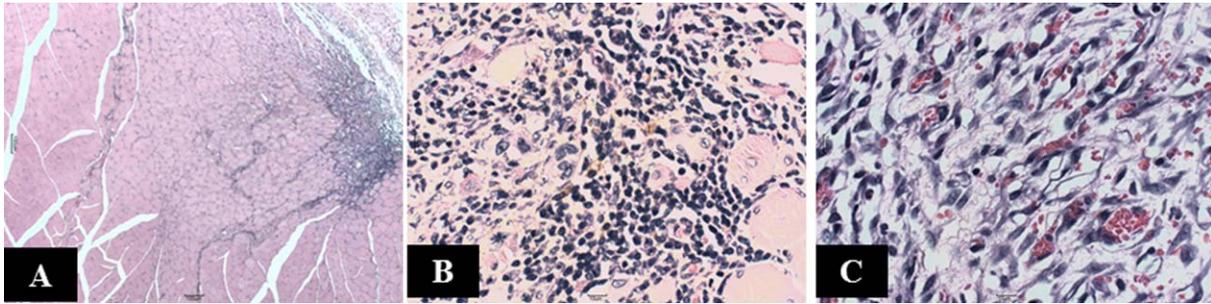
FIGURE 4. Transverse section showing gastrocnemius muscle fibers at 2 weeks with HE staining ($n = 4$). **(A)** Injured side. A group of converging lymphocytes around the muscle surface can be seen. Neovascularization is moderate, and continued muscle tissue repair process is noted (40× magnification). **(B)** Injured side, showing lymphocytes and the superficial layer (400× magnification). **(C)** Injured side, showing neovascularization (400× magnification).

among all FG-labeled DRG neurons was not significantly different between the injured (25.0%) and uninjured (21.1%) sides (Fig. 7C).

DISCUSSION

In this study, we found that the gait disturbance, upregulation of pain-related neuropeptides, and increase in inflammatory cytokine levels seen in this model of muscle injury recovered almost immediately. However, pain-related neuropeptide levels remained upregulated for up to 2 weeks. Histological findings indicate that tissue repair had completed at 3 weeks, but scar tissue remained.

Muscle injury induced gait changes, including a long swing time and slow swing speed. However, the gait changes normalized 2 days after muscle injury. Consistent with our findings, Iwata *et al.* reported that muscle injury induced a larger angle of ankle dorsiflexion, and the height of the calcaneus was lower in the stance phase during the acute phase after muscle injury, but these gait changes normalized during the subacute phase after muscle injury.[16] Here, we evaluated gait change after a contusion injury using the CatWalk system.

We found marked edema and hematoma histologically, suggesting an intense inflammatory response, and we observed necrotic myofibers through 3 days. By contrast, 3 days after injury, a process of tissue repair followed. By 3 weeks, tissue repair was indistinguishable from controls on HE staining, although scar tissue remained.

Okubo *et al.*[17] reported that 5 days after contusion, hematomas were present with sites of basophilia, probably representing muscle fibers. In addition, Iwata *et al.* reported that myotubes were detected in the regenerative area, and myotubes were still clearly observed at day 21 postinjury.[16] These findings indicate that muscle injury induces edema and hematoma during the acute phase of injury, but as time passes the injured muscle fibers are repaired. Accumulation of granulation tissue and myofibrillogenesis, evidenced by the regenera-

tion of new myofibers from repair tissue and muscle development, were observed on the injured side.

We found upregulation of TNF-α, IL-6, and NGF levels in gastrocnemius muscle for up to 1 day on the injured side compared with the uninjured side. Two days or more after injury, the levels had normalized. Similarly, Warren *et al.*[8] reported that traumatic skeletal muscle injury is accompanied by an early increase in TNF-α expression characterized by a single peak that is maximal at 24 hours after injury, as seen using real-time polymerase chain reaction. The time course of IL-6 expression in muscle postinjury was similar to that seen for TNF-α expression, and the authors concluded that upregulation of these inflammatory cytokines in injured muscle may play a role in muscle pain. NGF is generally reported to be involved in chronic inflammatory or neuropathic pain states.[18] In our study, muscle injury produced upregulation of NGF, as well as TNF-α and IL-6. These findings,
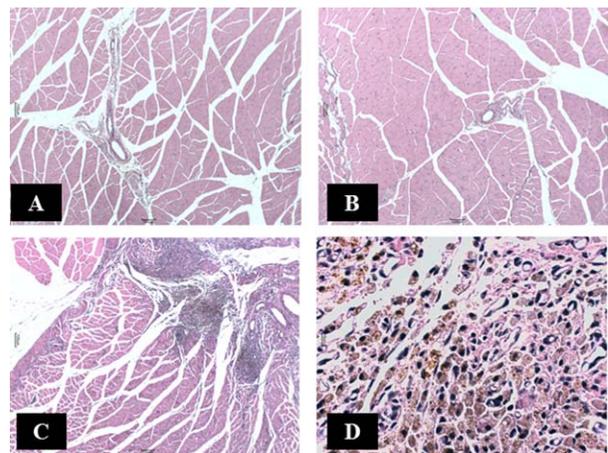

FIGURE 5. Transverse section showing gastrocnemius muscle fibers at 3 weeks with HE staining ($n = 4$). **(A)** Injured side. Tissue repair indistinguishable from controls, but scar tissue remains visible (40× magnification). **(B)** Uninjured side (40× magnification). **(C)** Injured side, showing repaired tissue and muscle (40× magnification). **(D)** Injured side, showing granulated tissue and myofibrillogenesis (400× magnification).
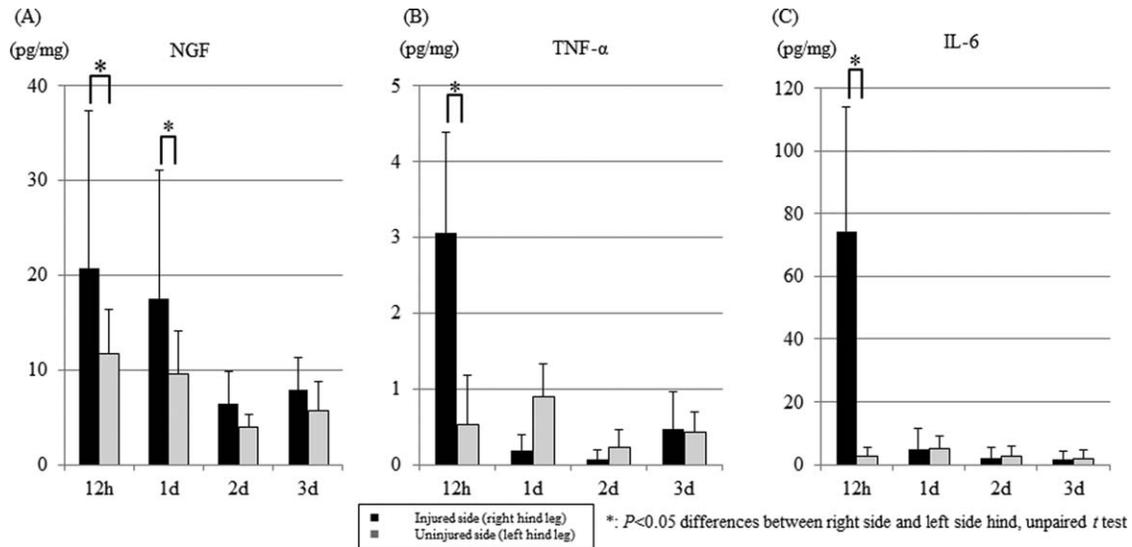
FIGURE 6. Expression of inflammatory mediators ($n = 40$). NGF **(A)**, TNF-α **(B)**, and IL-6 **(C)** production in the injured right gastrocnemius muscle and uninjured left gastrocnemius muscle. *$P < 0.05$ difference between injured (right) side and uninjured (left) side (unpaired t-test).

combined with the temporal gait data, suggest that upregulation of TNF-α, IL-6, and NGF in injured muscle may play a role in muscle pain.

We found that muscle injury induced upregulation of CGRP for 2 weeks. Increased expression of sensory nerve fibers (substance P, CGRP) in particular was observed after 3 days, along with a significant reduction in CGRP-positive fibers from 3 to 21 days. CGRP has been identified in small, NGF-dependent DRG neurons involved in pain perception related to inflammation,[19] suggesting that

CGRP is a marker of inflammatory pain. Thus, upregulation of CGRP may explain the persistence of muscle injury pain. Despite the gait disturbance and increase in inflammatory cytokine levels that subsided almost immediately, pain-related neuropeptide levels remained upregulated for up to 2 weeks. Clinically, some patients have persistent muscle pain, although muscle function practically recovers.[2] We previously reported that, in a rat model of intervertebral disk injury, the experimental disk injury produced a persistent increase in
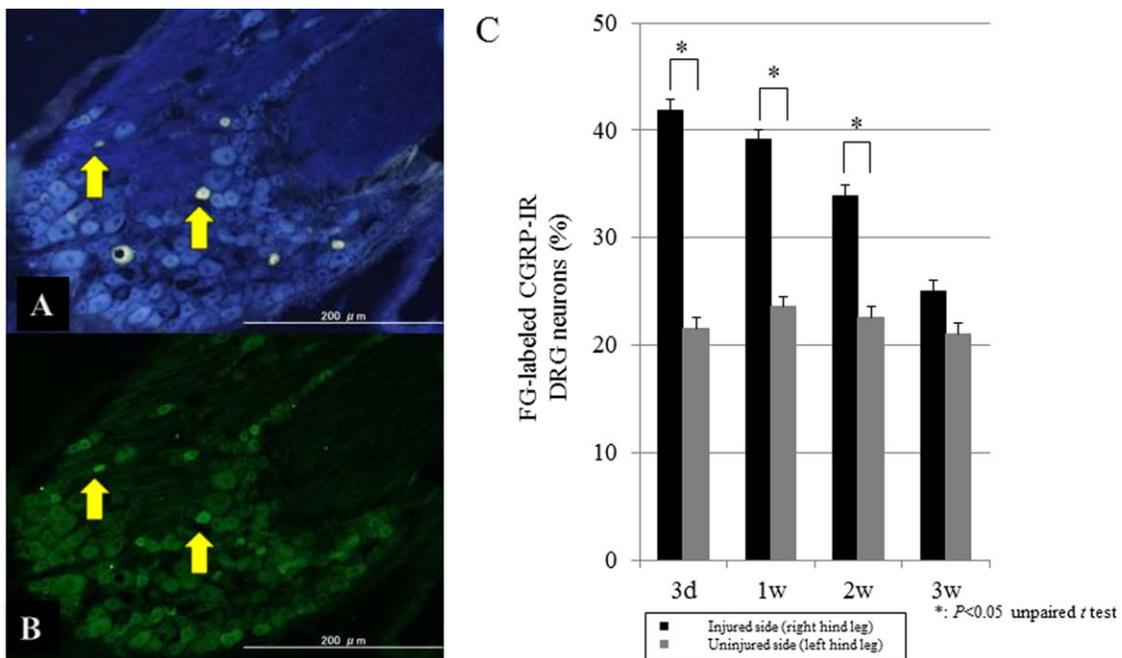

FIGURE 7. Immunohistochemical analysis of DRG neurons ($n = 20$). **(A)** FG-labeled and **(B)** CGRP-immunoreactive DRG neurons. Same section shown in **(A)** and **(B)**. Arrows indicate double-labeled neurons. **(C)** The proportion of FG-labeled CGRP-immunoreactive double-labeled neurons among all FG-labeled DRG neurons (*$P < 0.05$, unpaired t-test).

neuropeptides in DRGs and glia in the spinal cord, but only a transient increase in inflammatory mediators in disks.[20] Although the reasons for these discrepancies remain unclear, the findings may at least partially explain the pathomechanism of chronic pain.

One inherent limitation to this study was the histological evaluation of injured muscle by HE staining only. To identify myoblasts accurately, immunohistochemical staining would be needed.

In conclusion, using a model of muscle injury, we found that gait disturbance and increases in the levels of inflammatory cytokines in injured muscle subsided almost immediately after injury. However, pain-related neuropeptides remained upregulated in DRG for up to 2 weeks. This observation may explain the pathological mechanism of persistent muscle pain.

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