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Short communication

Intraplantar injection of sialidase reduces mechanical allodynia during inflammatory pain

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ABSTRACT

Sialic acids are highly charged glycoresidues that are attached to glycoproteins or glycosphingolipids, and they are associated with various biological functions. Gangliosides, sialic acid-containing glycosphingolipids, are abundant in neural tissues and play important roles in the nervous system. Previous studies revealed that peripheral gangliosides are involved in nociceptive behavior and hyperalgesia. These observations prompted us to determine whether the sialic acid-cleaving enzyme sialidase affects pain signaling. Intraplantar injection of sialidase reduced mechanical allodynia during complete Freund's adjuvant-induced inflammation. We also found that ganglioside induces mechanical allodynia in naïve mice. These results suggest that sialyl conjugates in subcutaneous tissues modify allodynia.

Sialic acids are acidic monosaccharides that confer a negative charge to glycoconjugates, such as glycosphingolipids and glycoproteins. Gangliosides are sialic acid-containing glycosphingolipids prominently found in neural tissue. In the nervous system, robust expression of sialic acids on gangliosides suggests they are involved in various neural functions, such as axonal elongation, neuron survival, and signal transduction. Gangliosides are synthesized from glucosylceramide via step-by-step glycosylations, resulting in o-, a-, b-, and c-series gangliosides based on the position and number of their sialic acids. Previous studies using genetically remodeled mice that have a deletion in enzymes related to the ganglioside synthetic pathway have revealed important insights into the roles of gangliosides in the nervous system (1–7).

Several studies have reported that gangliosides are involved in pain sensing, an important sensory nervous function that enables an organism to recognize harmful stimuli (8–11). Experiments using mice lacking GD3 synthase, an enzyme that catalyzes the production of the precursor of complex b-series gangliosides, revealed that gangliosides are involved in mechanical allodynia, thermal hyperalgesia, and formalin-induced nociception (8). Several studies have focused on the roles of a-series gangliosides in neuropathic pain and opioid induced-hyperalgesia using various spinal cord pain models in rodents. For example, intrathecal injection of the a-series ganglioside GM1 into the spinal cord reduced hyperalgesia in a rat model of experimentally induced neuropathy (9). Interestingly, cholera toxin, a GM1-binding protein, was found to ameliorate opioid-induced hyperalgesia (11).

Unlike a-series gangliosides, b-series gangliosides appear to enhance hyperalgesia. We previously demonstrated that the b-series ganglioside GT1b produces nociceptive behavior and enhances formalin-induced nociceptive behavior when injected into the hind paws of mice (10). We also observed that GT1b causes glutamate to accumulate in subcutaneous tissues and that intraplantar injection of glutamate receptor antagonists blocks GT1b-induced hyperalgesia. Since GT1b is a sialylated glycosphingolipid, we hypothesized that sialic acids might play an important role in pain signaling in skin. In the present study, we examined whether sialidase, which cleaves sialyl residues from glycoconjugates, reduces inflammatory pain in a complete Freund's adjuvant (CFA) mouse model of pain.

Male ICR mice (5–6 weeks old; CLEA Japan, Inc., Kyoto, Japan) were used. The mice were housed under a 12-h light/12-h dark cycle and had free access to food and water. Experiments were performed during the light phase of the cycle. All animal studies were approved by the Animal Care and Use Committee of Kitasato University.

Mice were subject to a von Frey test in which mechanical withdrawal 50% thresholds were measured using up-down methods (12). Briefly, mice were placed individually into transparent chambers (10 cm in diameter) and were allowed to adapt to

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the chambers for 1 h. Measurements were started by first applying a 3.22 filament. Eight filaments (2.36–4.17) were used.

After obtaining basal mechanical withdrawal thresholds, we injected 20 μl of CFA (Sigma Aldrich, St. Louis, MO, USA) into the unilateral plantar area of the mice. One day after CFA injection, mechanical allodynia was measured, and then mice were injected with either 40 μl solution of vehicle or sialidase derived from *Arthrobacter ureafaciens* (Nacalai Tesque, Kyoto, Japan). Mechanical withdrawal thresholds were then measured 40, 80, and 160 min after sialidase treatment. Sialidase was dissolved in a 40 μl solution of 50 mM sodium acetate buffer (pH 5.2) and saline. One unit of sialidase was defined as the amount of enzyme required to digest 1 μmol of N-acetylneuraminic acid (NANA)-lactose in 1 min at pH 5.0 at 37 °C, according to the manufacturer's specification sheets.

For experiments using heat-inactivated sialidase, sialidase was inactivated at 95 °C for 60 min. Any remaining activity was measured using the following colorimetric assay. Briefly, untreated or heat-inactivated sialidase was incubated with 1 mg/ml NANA-lactose in 50 mM acetate buffer (pH 5.2) for 10 min at 37 °C. The amount of NANA released into the reaction solution was measured using a thiobarbituric acid assay (13).

To examine the effects of gangliosides on the mechanical withdrawal thresholds of naïve mice, we measured mechanical allodynia 40 min after intraplantar injection of 10 nmol GT1b (Wako, Osaka, Japan), which was dissolved in 40 μl of phosphate-buffered saline (PBS).

Results are presented as means ± S.E.M. The statistical significance between groups was assessed by two-tailed unpaired Student's *t* tests, the Mann–Whitney test, or Kruskal–Wallis test followed by Dunn's multiple comparison test. GraphPad Prism software (San Diego, CA, USA) was used. P values of less than 0.05 ($P < 0.05$) were considered to be significant.

We previously observed that certain sialylated glycoconjugates, such as b-series gangliosides, produce nociceptive behavior and enhance formalin-induced hyperalgesia in the paws of mice (10), prompting us to hypothesize that sialic acids might be involved in pain signaling. To test this hypothesis, we treated naïve mice or CFA-injected mice with sialidase, an enzyme that cleaves sialyl residues in glycoconjugates, and then we assessed how sialidase treatment affected their pain responses.

One day after intraplantar CFA injection, when mice displayed mechanical allodynia (Fig. 1A), sialidase (8 mU/paw) was injected into the paw receiving the CFA injection. Forty minutes after intraplantar injection of sialidase, CFA mice displayed increased mechanical withdrawal thresholds (Fig. 1A). CFA mice receiving vehicle injections were unaffected. In sialidase-treated CFA mice, the elevated thresholds persisted for at least 160 min. In sialidase-treated naïve mice, however, mechanical withdrawal thresholds remained unchanged for 160 min (Fig. 1B). These data showed that intraplantar injection of sialidase affected the mechanical withdrawal thresholds of only CFA mice but not that of naïve mice. Since mechanical withdrawal thresholds at 80 min after sialidase injection increased slightly but not significantly compared with those at 40 min, we examined the dose-dependent experiment at 40 min. Furthermore, at higher doses (>2 mU/paw), sialidase further elevated the mechanical withdrawal thresholds of CFA mice 40 min after intraplantar injection (Fig. 1C). Mice injected with 8 mU/paw sialidase displayed significant anti-allodynic effects (Fig. 1C).

It is possible that the analgesic effects of sialidase that we observed were due to its interaction with endogenous mouse proteins, not due to its enzymatic activity on sialic acid-conjugated compounds such as gangliosides. To test this possibility, we examined the anti-allodynic effects of sialidase that has lost its sialic acid-cleaving activity. As shown in Fig. 2A, heating sialidase

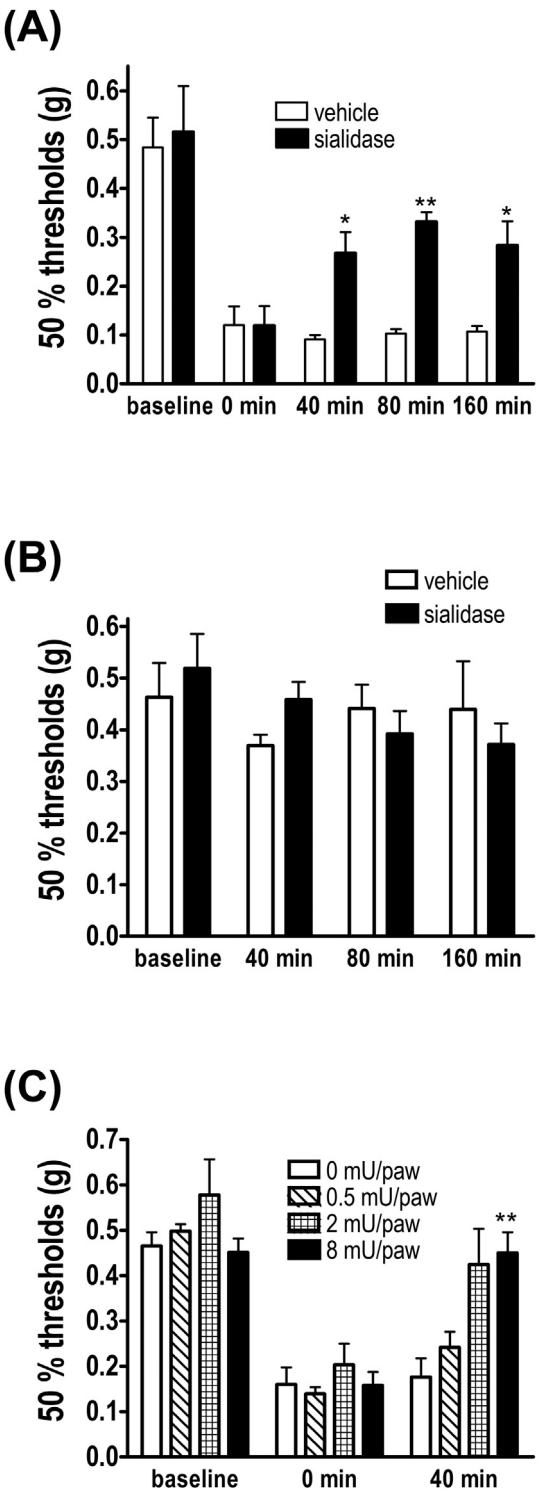

Fig. 1. Intraplantar sialidase injection reduces CFA-induced mechanical allodynia. Sialidase (8 mU/paw) attenuated mechanical allodynia 40 min after being injected into the paw (A). Sialidase treatment had no effects on naïve mice (B). Sialidase at 8 mU/paw, but not at lower doses, exerted significant anti-allodynic effects (C). Asterisks denote significance levels in comparison with vehicle: *P < 0.05, **P < 0.01; Mann–Whitney test (A, B); Kruskal–Wallis test followed by Dunn's multiple comparison test (C). Data are expressed as means ± S.E.M. of 6 mice.

(95 °C for 60 min) completely eliminated its cleaving activity. Intraplantar injections of inactivated sialidase had no effect on mechanical withdrawal thresholds during inflammation (Fig. 2B). This result suggests that sialidase enzymatic activity is crucial for