

Involvement of Tachykinins and NK₁ Receptor in the Joint Inflammation with Collagen Type II-Specific Monoclonal Antibody-Induced Arthritis in Mice

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Abstract

Rheumatoid arthritis (RA) is a chronic multisystem disease characterized by persistent joint inflammation associated with severe pain. Because RA is an immune-mediated joint disease and because type II collagen is considered an autoantigen, rodent models of arthritis using collagen type II-specific monoclonal antibodies are valuable for studying the pathogenesis of autoimmune arthritis and for evaluating therapeutic strategies. The tachykinin family peptides, substance P (SP) and hemokinin-1 (HK-1), are expressed in the nervous systems and in many peripheral organs and immunocompetent cells and activate tachykinin NK₁ receptors with similar affinities. NK₁ receptors are involved in the inflammation and hyperalgesia associated with a variety of inflammatory diseases. In the present study, we examined the involvement of SP and HK-1 in the joint inflammation and hyperalgesia in a collagen antibody-induced arthritis (CAIA) model in mice. The messenger RNA expression levels of the TAC1 gene encoding SP and of the TAC4 gene encoding HK-1 were decreased in the dorsal root ganglia and spinal cord at the peak of the inflammatory symptoms in CAIA. Systemic injection of an NK₁ receptor antagonist, WIN 51708, significantly inhibited the joint swelling, but not the mechanical allodynia, on day 7 in CAIA mice. The messenger RNA expression levels of TAC1 and TAC4 in the dorsal root ganglia and dorsal spinal cord were unaffected by treatment with WIN 51708. These findings suggest that tachykinins and NK₁ receptors play a key role in joint inflammation, rather than in nociceptive sensitization, in CAIA.

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Key words: allodynia, collagen antibody-induced arthritis, hemokinin-1, NK₁ receptor, substance P

Introduction

Rheumatoid arthritis (RA) is a systemic disease associated with debilitating joint inflammation causing severe ongoing pain. It is characterized by autoimmune responses promoted by imbalances in proinflammatory and anti-inflammatory immunomodulatory pathways. These immune-mediated responses include constitutive activation of immune surveillance cells and abnormal recognition of self antigens as nonself¹. In addition to immune cells, peripheral sensory neurons play a significant role in the persistent joint inflammation². Such neurogenic inflammation is caused by the release of inflammatory mediators, including substance P (SP) from peripheral dorsal root ganglion (DRG) axons².

Tachykinin NK₁ receptor, a cognate receptor for SP, is involved in the inflammation and hyperalgesia associated with a variety of inflammatory diseases^{3,4}. Activation of the NK₁ receptor causes vasodilation and plasma extravasation⁵⁻⁷ and regulates immune cell functions and inflammatory responses^{8,9}. In adjuvant- and carrageenan-induced arthritis, intra-articular injection of NK₁ receptor antagonists attenuates the joint swelling and hyperalgesia¹⁰⁻¹⁴. However, the involvement of NK₁ receptors in the inflammation and hyperalgesia in patients with RA remains less clear, whereas SP has been reported to be increased in the joint fluid and serum of patients with RA¹⁵⁻¹⁸.

The most recently identified tachykinin, hemokinin-1 (HK-1), also activates NK₁ receptors with similar affinity and potency to SP^{19,20}. HK-1 was originally identified as a regulator of B lymphopoiesis²¹. HK-1 is expressed in the nervous systems and in many peripheral organs and immunocompetent cells, such as microglial cells, macrophages, and dendritic cells^{19,22,23}, and has been suggested to be involved in immune function and inflammation^{20,21,24-27}. HK-1 also induces nociceptive behavior in the spinal cord, but in a different mode from SP^{28,29}. Furthermore, HK-1 messenger (m) RNA expression in the dorsal spinal cord is upregulated in neuropathic pain³⁰ and might, therefore, induce central sensitization.

Collagen type II is a major component of the articular cartilage matrix, and autoantibodies against it can induce arthritis^{1,31,32}. Therefore, rodent models of arthritis using collagen type II-specific monoclonal antibodies (collagen antibody-induced arthritis [CAIA]) are suitable for studying the pathogenesis of RA and for evaluating therapeutic strategies in a short time period³³⁻³⁶. However, it remains unknown whether tachykinins play roles in autoantibody-induced RA models similar to their roles in other arthritis models.

In the present study, we examined the expression of the HK-1 and SP at message levels and the involvement of NK₁ in inflammation and hyperalgesia in CAIA mice.

Materials and Methods

Animals

All experimental procedures were approved by the Nippon Medical School Animal Care and Use Committee (Approval number: 22-054) and carried out in accordance with the guidelines of the International Association for the Study of Pain³⁷. Male DBA/1 mice (8–10 weeks of age; Japan SLC, Hamamatsu) were used for all experiments. The mice were housed at 5 per cage for at least 4 days before the experiments. Water and food were available *ad libitum*.

CAIA

As a model of RA, CAIA was produced in the mice^{31,32}. Briefly, the mice received intraperitoneal injections of a mixture of different monoclonal antibodies against mouse type II collagen (10 mg/mL; Chondrex, Redmond, WA, USA) at a dose of 0.15 mL. Three days after antibody injection, 50 µg of lipopolysaccharide (0.5 mg/mL; Chondrex) was injected intraperitoneally. In the control mice, the same volume of phosphate-buffered saline was injected in place of the antibodies and lipopolysaccharide. Five, 7, and 14 days after antibody injection, the mice were deeply anaesthetized with intraperitoneal sodium pentobarbital (60 mg/kg), and the portions of the lumbar second (L2) to lumbar fifth (L5) dorsal spinal

cord and the bilateral L2–L5 DRGs were immediately dissected out. Each sample was stored at -80°C until use for RNA extraction.

Assessment of Joint Swelling

The severity of joint swelling as an index of arthritis was graded on a 0 to 3 scale as previously described³⁸: 0, normal; 1, swelling of the ankle or wrist, or limited to digits; 2, swelling of the entire paw; and 3, maximal swelling. Each limb was independently graded, and the values were added together to give a maximum arthritis score of 12 for each animal. The arthritis scores were evaluated on day 0 before antibody injection and on days 5, 7, 10, and 14 after antibody injection.

Behavioral Test

Mechanical allodynia was examined on day 0 before antibody injection and on days 5, 7, 10, and 14 after antibody injection. Paw withdrawal in response to mechanical stimuli was measured with a set of von Frey filaments with bending forces ranging from 0.07 to 3.57 g. Each mouse was placed on a metallic mesh floor covered with a plastic box, and a von Frey monofilament was applied to the plantar surface of the right hind paws from underneath. The weakest force inducing paw withdrawal at least twice in 3 trials was referred to as the paw withdrawal threshold.

Administration of Indomethacin and the NK₁ Antagonist WIN 51708

To examine how NK₁ receptors are involved in mechanical allodynia and joint swelling and in tachykinin mRNA expression levels in the RA model, we intraperitoneally administered WIN 51708, an NK₁ receptor antagonist, and indomethacin, a nonsteroidal anti-inflammatory drug (NSAID) commonly used for the treatment of RA^{39,40}, as a positive control. Indomethacin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved with sonication in Tween-80 and then diluted to 1% with saline. The NK₁ receptor antagonist WIN 51708 (Sigma-Aldrich) was dissolved in a solution of 20% dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd., Osaka), 40% propylene glycol (Wako), and 40% saline.

Indomethacin (3 mg/kg) and WIN 51708 (20 mg/kg) were intraperitoneally administered once daily on days 0 to 7 after antibody injection.

Quantitative Polymerase Chain Reaction

Quantitative analyses of TAC1 and TAC4 mRNA levels were performed as previously described⁴¹. Total RNA was extracted from the mouse samples with the extraction reagent RNAiso (Takara Bio Inc., Otsu) and purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The bilateral L2–L5 DRGs were processed collectively. To avoid contamination by genomic DNA, the extracted RNA was treated with 10 μL of DNase (0.125 U/ μL) at 37°C for 30 minutes on the column of the RNeasy Mini Kit. First-strand complementary (c) DNAs were synthesized with 0.5 μg of total RNA with Oligo (dT) primer, dNTP mix, and Superscript II Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). For polymerase chain reaction (PCR) amplification, 1 μL of the first-strand cDNAs was added to 19 μL of a reaction mixture containing TaqMan Gene Expression Master Mix (Life Technologies) and 900 nM of a primer pair and 250 nM of TaqMan probe and amplified with the StepOnePlus real-time PCR system (Life Technologies). The PCR primers specific for the mouse TAC1 gene encoding SP and neurokinin A and mouse TAC4 gene encoding HK-1 were designed on the basis of cDNA sequences deposited in GenBank (NM009311 and AF235035, respectively) with Primer Express version 2.0 (Life Technologies). The respective forward primers, reverse primers, and probes were as follows: 5'-TGA CCAGATCAAGGAGGCAAT-3', 5'-TGGGTCTTCGG GCGATT-3', and 5'-CTTTGAGCATCTTCTGCAG-3' for TAC1; and 5'-CTCCAGAGTCGAGAGGAAGATG AG-3', 5'-AGAGATGGGTGGCAGAGTGCCTA-3', and 5'-TCCCCTAACCACCAGCAA-3' for TAC4. For quantification, the cDNA sequences of TAC1 and TAC4 were inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA) and the pBluescript II SK(+) vector (Agilent Technologies, Santa Clara, CA, USA), respectively. The plasmids were then serially diluted to concentrations of 1.0×10^3 to 1.0×10^7 and 1.0×10^1 to 1.0×10^5 molecules/reaction tube for use

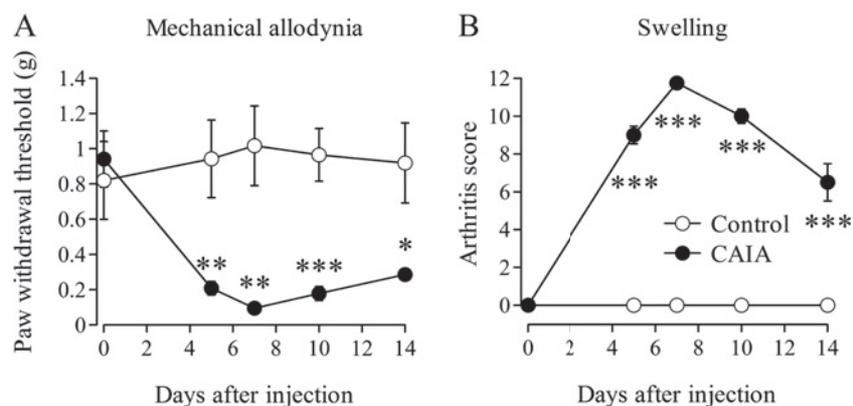


Fig. 1 Mechanical allodynia (A) and joint swelling (B) in CAIA mice. The withdrawal thresholds of the right hindpaw in response to mechanical stimuli and the severity of joint swelling were measured in control mice and CAIA mice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared by means of unpaired t -tests with the values for control mice on the corresponding days ($n=8$).

as standards for TAC1 and TAC4 amplifications, respectively. All PCR amplifications using the standards and samples were performed in triplicate at 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The number of cDNA copies was calculated using a standard curve obtained from the set of control plasmids in each assay.

Statistical Analysis

Values are expressed as means \pm SEM. Unpaired t -tests were used to compare threshold values, arthritis scores, and expression levels of TAC1 and TAC4 mRNAs between the CAIA mice and control mice. Differences in the threshold values and arthritis scores among the CAIA mice that received no treatment, indomethacin treatment, or WIN 51708 treatment were analyzed with one-way analysis of variance (ANOVA), followed by individual *post hoc* multiple comparison tests (Tukey-Kramer's tests). Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Time Course of CAIA

Before antibody injection on day 0, the paw withdrawal thresholds in response to mechanical stimuli were 0.82 ± 0.22 g and 0.94 ± 0.16 g for mice assigned to the control group and CAIA group,

respectively ($n=8$; **Fig. 1A**). After antibody injection, the paw withdrawal threshold was significantly decreased on day 5 (0.21 ± 0.04 g for CAIA vs. 0.94 ± 0.22 g for control, $P < 0.01$; $n=8$; **Fig. 1A**) and reached the lowest value on day 7 (0.10 ± 0.01 g; $n=8$; **Fig. 1A**). The decreased the paw withdrawal threshold gradually began to increase over the following 7 days ($n=8$; **Fig. 1A**).

Before antibody injection, no joint swelling was observed in any of the mice ($n=8$; **Fig. 1B**). The arthritis score was significantly increased on day 5 after antibody injection compared with vehicle injection (9.00 ± 0.46 for CAIA vs. 0.00 ± 0.00 for control, $P < 0.001$; $n=8$; **Fig. 1B**). The score reached its peak value on day 7 (11.8 ± 0.16 ; $n=8$; **Fig. 1B**) and gradually declined over the following 7 days. The control mice did not show any changes in arthritis score ($n=8$; **Fig. 1B**).

TAC1 and TAC4 mRNA Expression Levels in DRGs in CAIA

In intact mice, the numbers of TAC1 (SP-encoding gene) and TAC4 (HK-1-encoding gene) mRNA molecules in the L2-L5 DRGs were $3.37 \times 10^7 \pm 2.20 \times 10^6$ and $1.05 \times 10^4 \pm 4.37 \times 10^2$ molecules/ μ g total RNA, respectively ($n=9$). On day 7 after antibody injection, when the arthritis was fully developed, the expression level of TAC4 mRNA in the DRGs was significantly decreased compared with that in

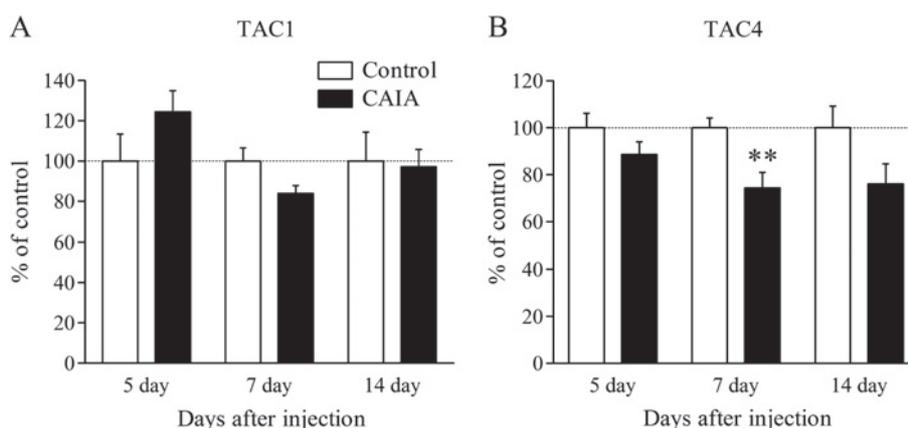


Fig. 2 Time courses of TAC1 (A) and TAC4 (B) mRNA expression levels after antibody injection in CAIA mice. The expression levels of TAC1 and TAC4 mRNAs were examined in the L2–L5 DRGs of control mice and CAIA mice. The values are expressed as percentages of values in control mice. ** $P < 0.01$ compared by means of unpaired t -tests with the values for control mice on the corresponding days ($n = 6-9$).

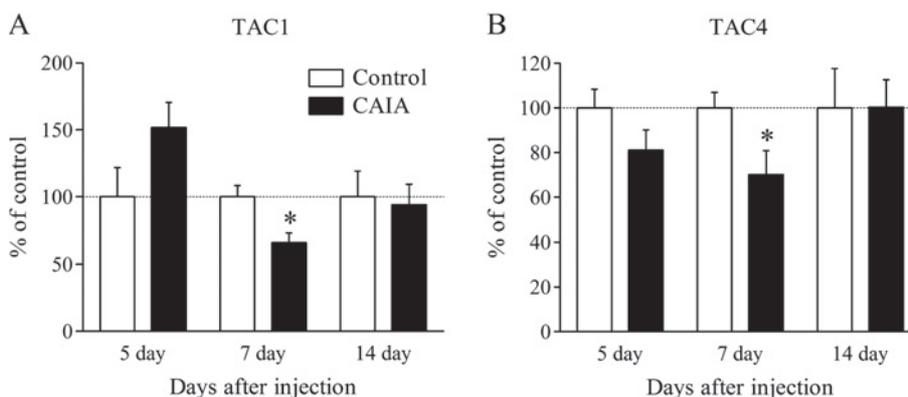


Fig. 3 Time courses of TAC1 (A) and TAC4 (B) mRNA expressions after antibody injection in CAIA mice. The expression levels of TAC1 and TAC4 mRNAs were examined in the L2–L5 dorsal spinal cord of control and CAIA mice. The values are expressed as percentages of the values in control mice. * $P < 0.05$ compared with the values for control mice at the corresponding days by means of unpaired t -tests ($n = 6-9$).

control mice ($74.41\% \pm 6.57\%$ expressed as a percentage of the value for control mice; $P < 0.01$; $n = 6-9$; **Fig. 2B**). On the other hand, on days 5 and 14 after antibody injection, when the arthritis was developing and dissipating, respectively, the expression levels of TAC4 mRNA were unchanged ($n = 7-8$; **Fig. 2B**). In contrast, the expression levels of TAC1 mRNA remained unchanged in the DRGs throughout the course of the arthritis (**Fig. 2A**).

TAC1 and TAC4 mRNA Expression Levels in the Dorsal Spinal Cord in CAIA

In the intact mice, the numbers of TAC1 and TAC4 mRNA molecules in the dorsal spinal cord were $1.36 \times 10^7 \pm 1.15 \times 10^6$ and $1.07 \times 10^4 \pm 7.47 \times 10^2$ molecules/ μg total RNA, respectively ($n = 9$). In the CAIA mice, the expression levels of TAC1 and TAC4 mRNA were not significantly changed in the dorsal spinal cord on day 5, when the arthritis was developing ($151.81\% \pm 18.68\%$ for TAC1 and $81.04\% \pm 9.03\%$ for TAC4 expressed as percentages of the values for control mice; $n = 7-8$; **Fig. 3**). Subsequently,

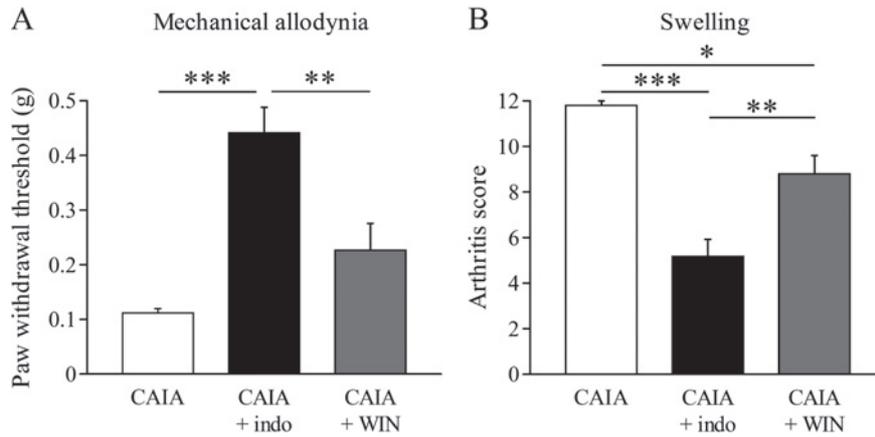


Fig. 4 Effects of indomethacin and WIN 51708 on the mechanical allodynia (A) and joint swelling (B) in CAIA mice. Indomethacin and WIN 51708 were intraperitoneally administered once daily on days 0 to 7. The thresholds of paw withdrawal for the right hindpaw in response to mechanical stimuli and the severity of joint swelling were measured in CAIA mice and CAIA mice that had received injections of indomethacin (indo) or WIN 51708 (WIN) on day 7. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by means of one-way ANOVA, followed by the Tukey-Kramer's test ($n = 6-7$).

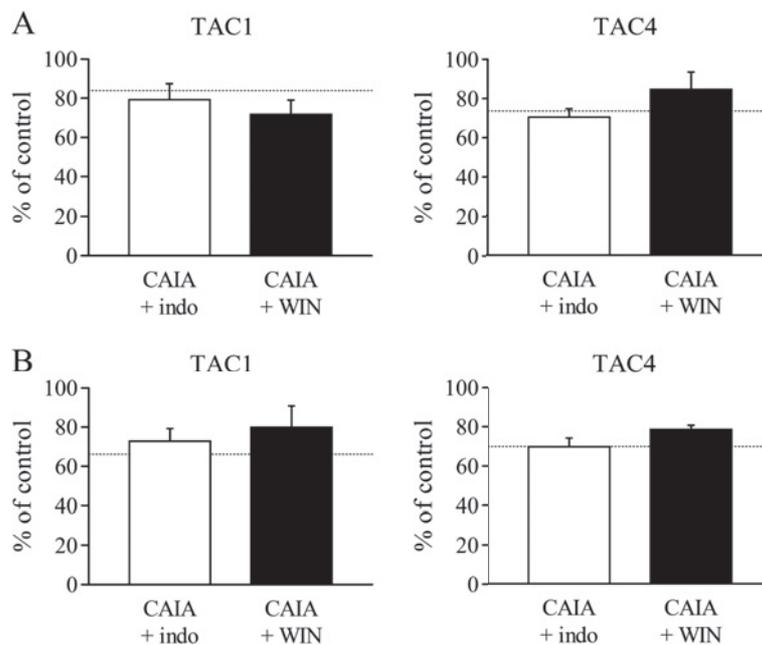


Fig. 5 Effects of indomethacin and WIN 51708 on TAC1 and TAC4 mRNA expression levels in CAIA mice. Indomethacin and WIN 51708 were intraperitoneally administered once daily on days 0 to 7. The expression levels of TAC1 and TAC4 mRNAs were examined in the L2-L5 DRGs (A) and dorsal spinal cord (B) of CAIA mice that received injections of indomethacin (indo) or WIN 51708 (WIN) on day 7. The values are expressed as percentages of the values for control mice ($n = 6-9$). The dashed lines indicate the average values for CAIA mice.

the expression levels of TAC1 and TAC4 mRNAs were significantly decreased in the dorsal spinal cord on day 7 in association with the peak of arthritis, compared with the values for control mice ($66.12\% \pm 7.43\%$ for TAC1 and $70.12\% \pm 10.69\%$ for TAC4 expressed as percentages of the values for control mice; $P < 0.05$; $n = 6-9$; **Fig. 3**). The decreased expression levels of TAC1 and TAC4 mRNAs had returned to near the baseline levels on day 14 with the reduction in allodynia and joint swelling.

Effects of Indomethacin and WIN 51708 on Mechanical Allodynia and Joint Swelling

On day 7 after antibody injection, mechanical allodynia was significantly inhibited by indomethacin treatment (0.44 ± 0.05 g for CAIA plus indomethacin injection vs. 0.11 ± 0.01 g for CAIA, $P < 0.001$; $n = 6-7$; **Fig. 4A**). Joint swelling was also decreased by indomethacin treatment (4.75 ± 0.53 for CAIA plus indomethacin injection vs. 11.75 ± 0.16 for CAIA, $P < 0.001$; $n = 6-7$; **Fig. 4B**). In contrast, the mechanical allodynia was not significantly inhibited by injection of WIN 51708 (0.23 ± 0.05 g for WIN 51708 injection; $n = 6$; **Fig. 4A**), whereas the joint swelling was significantly decreased on day 7 in CAIA mice (8.80 ± 0.80 , $P < 0.05$; $n = 6$; **Fig. 4B**).

Effects of Indomethacin and WIN 51708 on Tachykinin mRNA Expression Levels

On day 7 after antibody injection, neither indomethacin nor WIN 51708 affected the expression levels of TAC1 and TAC4 mRNAs in the DRGs and dorsal spinal cord ($n = 6-9$; **Fig. 5**).

Discussion

In the present study, we first showed that TAC1 and TAC4 mRNA expression levels were decreased in the DRGs and spinal cord at the peak of the inflammatory symptoms in CAIA. These expression changes in tachykinin mRNAs are in contrast to those observed in a neuropathic pain model in our previous study³⁰, in which TAC1 mRNA expression was increased in the DRGs at the initial phase of pain development while TAC4 mRNA expression was increased in the spinal cord. Marchand et al.⁴² have also reported increased expression levels of SP

peptide and its mRNA (TAC1) in the DRGs in the early phase after nerve injury. In adjuvant-induced arthritis models, the expression changes of SP in the DRGs are controversial. In one report, TAC1 mRNA expression was decreased on day 6⁴³, whereas other reports described increases in SP protein and TAC1 mRNA with various time courses⁴⁴⁻⁴⁶. It is thought that SP released from the central axon terminals of DRG neurons in the spinal cord binds to NK₁ receptors expressed in nociceptive projection neurons and sensitizes these neurons to contribute to hyperalgesia^{47,48}. HK-1 can also bind to NK₁ receptors with a similar potency to SP^{19,20} and can induce nociceptive behavior^{28,29}. Therefore, the extent and time course of the central sensitization via NK₁ activation may vary among different arthritis models. The decreased expression levels of TAC1 and TAC4 mRNAs in the present study suggest that NK₁ receptor-mediated central sensitization of the nociceptive pathways is less involved in CAIA. Consistently, the NK₁ receptor antagonist WIN 51708 was less effective in relieving the hyperalgesia in the present study. In addition, the changes in the expression of TAC1 and TAC4 mRNAs were unaffected by indomethacin treatment, although indomethacin suppressed the hyperalgesia, possibly through perturbation of prostaglandin pathways. In the present model, the inflammatory cascade is triggered by antibodies binding to complement on the cartilage surfaces of the joints, with subsequent local activation of mononuclear cells, which in turn release proinflammatory cytokines to induce macrophage and neutrophil recruitment in the absence of a primary immune response³¹. These inflammatory symptoms have an early onset, but are self-limited, as observed in the present study. Therefore, the expression levels of the tachykinin mRNAs may reflect the nature of this arthritis model, such as possible concomitant anti-inflammatory responses for the spontaneous recovery from the diseased state.

The NK₁ receptor antagonist WIN 51708, as well as indomethacin, attenuated the joint swelling in the present mouse model of RA. SP has been reported to cause plasma extravasation and vasodilation⁵⁻⁷. SP induces nitric oxide release in rheumatoid

synoviocytes⁴⁹. Joint swelling induced by complete Freund's adjuvant is also suppressed by NK₁ receptor antagonists^{13,14,50}. It has been reported that SP is expressed in immune cells, such as T cells, B cells, and dendritic cells^{8,21,23,24}, which infiltrate joints in RA^{51,52}. Infiltration of lymphocytes is observed in the RA model used in this study⁵³. Synovial fibroblasts obtained from patients with RA also release SP, to a greater degree than those obtained from patients with osteoarthritis⁵⁴. The SP levels of synovial fluid are higher in patients with RA than in patients with osteoarthritis^{16,55}. In addition, HK-1 is expressed mainly in a variety of peripheral immune cells, including B cells, dendritic cells, monocytes, and macrophages^{21,23,56,57}. Because HK-1 activates NK₁ receptors with a similar potency to SP^{19,20}, HK-1 may also contribute to the joint swelling in RA. In the present study, although the implication of the NK₁-mediated central sensitization is less clear, the NK₁ antagonist substantially decreased joint swelling, suggesting that NK₁ is involved in the inflammation in CAIA mice. Although NSAIDs, conventional anti-inflammatory drugs, have been used to treat RA, they have side effects, including gastric ulcer. Because the mechanisms of action of NK₁ antagonists differ completely from those of NSAIDs, the combination of NSAIDs and NK₁ antagonists might be a safer and more effective treatment for RA.

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