The cyclic peptide WP9QY (YCWSQYLCY), which was designed to mimic the most critical tumor necrosis factor (TNF) \( \alpha \) recognition loop on type1 TNF receptor, antagonizes the effects of TNF\( \alpha \). In this study, we investigated the effects of WP9QY peptide on collagen- induced arthritis (CIA) mice to evaluate its effects on inflammatory bone destruction. DBA/1J mice were injected intradermally at the base of the tail with bovine type II collagen, emulsified in complete Freund’s adjuvant on day 0 and 21. The three sets of WP9QY peptide injections (24 mg/kg \( \times \) 8 times per day) were performed before the onset of paw swelling. Mice were sacrificed at day 38 and thereafter, the arthritis scores as well as radiographical and histological outcomes were assessed. WP9QY peptide inhibited CIA- induced increase in the arthritis score. Furthermore, histomorphometric analysis of the tibial epiphysis region revealed that WP9QY peptide inhibited the increase of synovial pannus infiltration and the decrease of bone volume, which were induced by the CIA. The WP9QY treatment prevented the inflammation as well as bone destruction of the joints in the CIA mice, suggesting that the administration of WP9QY peptide might be useful for developing a drug to prevent inflammatory bone destruction.

Key words: WP9QY peptide, TNF\( \alpha \), antagonist, inflammation, bone resorption

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that leads to chronic inflammation of multiple joints and subsequent destruction of cartilage and bone\(^{1,2}\). Proinflammatory cytokines such as tumor necrosis factor \( \alpha \) (TNF\( \alpha \)), interleukin-1 (IL-1), and interleukin-6 (IL-6) play a crucial role in the pathogenesis of RA\(^3\). Especially, TNF\( \alpha \) occupies a pivotal position in a cytokine cascade that regulates the production of inflammatory mediators such as IL-1, IL-6 in the synovial membrane culture system\(^4,5\).

Collagen- induced arthritis (CIA) is a murine model of human RA which is currently used for its analysis. In
the CIA mice, the etiology is associated with local overproduction of TNF-α. The TNF-α mRNA level was upregulated before the onset of the swelling of the paws. Immunohistochemical observations identified TNF-α protein in the pannus at all stages of the disease. Thus, TNF-α seems to be an important cytokine in the pathogenesis of RA inducing the chronic inflammatory state and several TNF-α-blocking therapies ameliorated the development of inflammation and disease activity in RA.

The WP9QY peptide (Tyr^1^- Cys^2^- Trp^3^- Ser^4^- Glu^5^- Tyr^6^- Leu^7^- Cys^8^- Tyr^9^) has a disulfide bond between Cys^2^ and Cys^8^ and composes a cyclic peptide. It is designed to mimic the loop 1 of domain 3 on type 1 TNF receptor (TNFR(I)), which is the most critical site for TNF-α recognition. Twenty five μM of WP9QY peptide inhibits the TNF-α binding to TNFR(I) as a same extent as 1 nM of soluble TNF- receptor in a competitive radioreceptor assay. WP9QY peptide protected dose-dependently from TNF-α induced cells death in vitro. Recently Suzuki et al. reported that WP9QY peptide dose-dependently inhibits inflammatory bone resorption induced by Porphyromonas gingivalis infection in mice, suggesting that WP9QY peptide works as a TNF-α antagonist, in vivo. Furthermore, WP9QY peptide also prevented the bone loss in ovariectomized (OVX) mice, where TNF-α is involved in the bone resorption. These results suggested that WP9QY peptide has an inhibitory effect on TNF-α-mediated bone resorption in vivo.

In this report, the effects of WP9QY peptide on the CIA mice were investigated if this peptide has beneficial effects on inflammatory bone destruction. We found that the WP9QY peptide inhibits both inflammation and bone destruction in the CIA mice.

### Materials and Methods

#### Materials

The WP9QY peptide was synthesized and its activity against TNF-α/TNFRI(I) interaction was assayed as described elsewhere. Bovine type II collagen was obtained from the Collagen Research Center (Tokyo, Japan) and complete Freund’s adjuvant from Difco (Detroit, MI). Specific pathogen-free 6 week-old male DBA/1J mice were purchased from Japan Charles River Breeding Laboratories (Kanagawa, Japan). Prior to the experiment, all mice were allowed to have ad libitum the food (Oriental Yeast Co., Ltd., Tokyo, Japan) and water for 7 days for acclimatization. They were maintained in a 12-hour light and dark cycle. The experimental procedures were reviewed and approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University.

#### Induction of CIA

The induction and assessment of CIA were performed as previously described. Briefly, 200 μg bovine Type II Collagen in 0.05 M acetic acid emulsified in complete Freund’s adjuvant was intradermally injected at the base of the tail to the male DBA/1J mice (7 week-old). Twenty-one days after the primary immunization, the mice were boosted in the same way.

#### Treatment with WP9QY peptide

The WP9QY peptide was dissolved in NaOH, neutralized in NaH₂PO₄, and buffered in PBS. The three sets of vehicle (the mixture of NaOH, NaH₂PO₄, PBS) and WP9QY peptide (24 mg/kg×8 times per day) injections (70 μl/mouse) were performed subcutaneously, before the first appearance of clinical signs of disease (at day 22, 24, and 26). In our previous study, this dose was proven to have an inhibitory effect on bone resorption induced by a low calcium diet in mice (data not shown). The mice were divided into three groups; 1) non-immunized mice received vehicle (normal-vehicle mice; n = 6), 2) immunized mice receiving vehicle (CIA-vehicle mice; n = 6), and 3) immunized mice receiving WP9QY peptide (CIA-WP9QY mice; n = 6).

#### Assessment of arthritis

The arthritis score of the mice was examined daily, from the day of the second immunization (day 21). Initial observation of erythema and/or swelling was considered to be the onset of arthritis. Arthritis score were given as 0 to 3 to the four paws as previously described. The criteria for the grading were as follows: 0 = normal; 1 = slight swelling and/or erythema; 2 = pronounced edematous swelling; and 3 = deformed paw or joint, with ankylosis, joint rigidity.

#### Radiological assessment of arthritis

At the end of the experiment (day 38), mice were sacrificed under ether anesthesia, and the hind paws were collected and fixed in 4% cacodylate-buffered glutaraldehyde-formalin fixative (pH 7.4) for 2 days, washed with water for a day and later used for radiological analysis. Three-dimensional reconstruction images and coronal images of ankle joints and knee joints were obtained with a micro focus X-ray computed...
tomography (Micro-CT; SKYSCAN 1072, Aartselaar, Belgium).

**Histomorphometric evaluation of arthritis**

Collected hind paws were embedded in GMA resin (JB4, Polyscience, Warrington, PA). Standard decalcified sections (1.5 μm) were prepared and stained with hematoxylin and eosin (H&E). In order to quantitatively assess the arthritis, we analyzed the infiltration rate of the synovial pannus of the articular cartilage surface of the proximal end of tibia, using H&E stained undecalcified sections. The calculation formula is as follows: the infiltration rate of pannus (%) = (the length of the synovial pannus facing the proximal end of tibia) / (the total length of the proximal end of tibia) X 100. To evaluate bone destruction of the knee joints, approximately 0.38 mm² area of the epiphysis, starting 0.5 mm proximal from the growth plate-epiphyseal junction, was determined in order to exclude the cortical bone facing the epiphyseal growth plate using undecalcified sections with toluidine blue staining. The bone volume (BV/TV) in the epiphysis of proximal tibia was measured using an image analyzing system (KS400, Carl Zeiss, Jena, Germany) as previously described.

**Bone histomorphometry of tibial metaphysis**

To perform bone histomorphometry in the tibial metaphysis, sagittal sections of proximal tibia were stained with both tartrate resistant acid phosphatase (TRAP) and toluidine blue, and observed under a light microscope with a 20× objective. The measurements were performed in the secondary spongiosa starting 0.3 mm distal from the growth plate to exclude the primary spongiosa as previously described. A standard histomorphometric analysis was performed using the image analyzing system as described above. TRAP-positive cells that formed resorption lacunae at the surface of the trabeculae and contained two or more nuclei were designated as osteoclasts.

**Measurements of serum anti-Type II collagen (anti-CII) antibodies**

At the end of the experiment (day 38), serum samples were obtained and the levels of the serum anti-CII antibody were assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Chondrex, Inc. NE Redmond, WA, USA).

**Statistical analysis**

The analysis of arthritis scores was performed by Mann-Whitney U-test. The other data were first analyzed by ANOVA. When a significant F ratio (P < 0.05) was identified, groups were compared using Fisher’s protected least significant difference post hoc test. Tests were carried out using Apple software, Stat View 4.1 (Abacus Concepts, Inc., Cupertino, CA, USA). P values less than 0.05 were considered significant.

**Results**

WP9QY peptide inhibited the disease activity in the CIA mice

To identify the effects of WP9QY peptide on inflammation in CIA model, we assessed the development of inflammation using arthritis score. The onset of arthritis was not delayed by WP9QY treatment compared to that in CIA-vehicle mice, appearing on day 26, but the severity of the disease was less. A significant reduction of the arthritis score appeared from day 28. On day 38, it was still observed in the CIA-vehicle mice when compared to the CIA-WP9QY mice (Figure 1), indicating that the WP9QY peptide had an inhibitory effect on the disease activity.

The serum levels of anti-CII antibody were significantly increased in the CIA mice, but the increase of...
anti-CII antibody was not reduced by anti-TNF treatment. In accordance with this report, the high level of anti-CII antibody was observed in the CIA-vehicle mice. The serum levels did not change significantly between the CIA-vehicle mice and CIA-WP9QY mice, though the WP9QY peptide lowered the CIA-induced increase in the serum levels (data not shown).

The inhibitory effects of WP9QY peptide on the invasion of inflammatory cells and pannus formation in the knee joints of CIA mice

To confirm the inhibitory effect of WP9QY peptide on the activity of CIA, a histological assessment of the knee joints was performed. The histological findings in the CIA-vehicle mice revealed the abnormalities in invasion of inflammatory cells and bone erosion (Figure 2B), but those in the CIA-WP9QY mice showed markedly reduced invasion of inflammatory cells and absence of bone erosion (Figure 2C). Proliferative synovitis composed of abundant fibroblasts, hypertrophic synoviocytes, and infiltration of

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Figure 2. Histological observations of knee joints in CIA mice. Knee joint in CIA-vehicle mice (B) showed pannus formation (arrows) and bone erosion compared to normal-vehicle mice (A). Knee joint in CIA-WP9QY mice (C) showed reduction of infiltrating cells and inhibition of bone erosion. The region within the black rectangle of panel (B) represents the entire field of panel (D). The region within the black rectangle shows the invasion of pannus in the tibial epiphysis. Hematoxylin and eosin staining. Bar; 100 μm.
inflammatory cells composed of neutrophils, macrophages, lymphocytes were observed at the proximal end of tibia in CIA-vehicle mice (Figure 2D). In order to quantitatively evaluate the pannus formation, we calculated the infiltration rate of synovial pannus on the proximal end of tibial epiphysis. This histomorphometric assessment revealed that WP9QY peptide significantly inhibited CIA-induced pannus formation and infiltration (Figure 3). These results suggest that WP9QY peptide inhibited the inflammatory destruction of the knee joints in CIA mice.

**WP9QY peptide prevents bone destruction**

Three-dimensional reconstruction images from the Micro-CT scanning data (Figure 4A-C) illustrated bone erosion on the articular surfaces of the ankle joints in CIA-vehicle mice (Figure 4B) compared to normal-vehicle mice (Figure 4A), while almost no erosion was detected in the CIA-WP9QY mice (Figure 4C). Three-dimensional reconstruction images from the Micro-CT scanning data revealed the protective effects of WP9QY peptide against bone destruction in CIA mice. Similarly, X-ray radiography showed severe bone destruction of the ankle joints in the CIA mice while WP9QY treatment prevented this bone destruction in the CIA mice (data not shown).

Three-dimensional reconstruction images from the Micro-CT scanning data (Figure 5A-C) illustrated bone erosion on the articular surfaces of the knee joints in CIA-vehicle mice (Figure 5B) compared to normal-vehicle mice (Figure 5A), while almost no erosion was detected in CIA-WP9QY mice (Figure 5C). Coronal images of the knee joints by Micro-CT showed that the decrease of trabecular bone of femur and tibia in CIA-vehicle mice (Figure 5E) compared to normal-vehicle mice (Figure 5D), while the WP9QY peptide prevented the CIA-induced decrease of trabecular bone (Figure 5F).

To further investigate the effects of WP9QY peptide on bone erosion accompanied with CIA at the knee joints, histological and histomorphometric assessments were performed in the epiphysis of proximal tibia as described in the Materials and Methods. The histomorphometric measurement showed that bone volume...
of the tibial epiphysis (BV/TV at epiphysis) in CIA- vehicle mice was significantly reduced compared to that of normal- vehicle mice, and this decrease in the bone volume was significantly inhibited by WP9QY treatment (Figure 6). These results suggest that WP9QY peptide prevented bone destruction in the knee joints in CIA mice.

Bone morphometry in the tibial metaphysis
Since it was reported that the induction of arthritis causes bone loss in other parts of the bones as well as the joint destruction\(^1\), a histomorphometric analysis was performed in the tibial metaphysis. The bone volume (BV/TV) was significantly decreased in CIA-vehicle mice compared to normal- vehicle mice, and the decreased BV/TV was significantly blocked in CIA-WP9QY mice (Figure 7A). The number of osteoclasts (N. Oc/BS) was significantly increased in CIA- vehicle mice compared to normal- vehicle mice. This increase in N. Oc/BS was significantly reduced in CIA- WP9QY mice (Figure 7B). This result indicates that WP9QY peptide could inhibit the cancellous bone in the metaphysis of the tibia as well as bone erosion of the articular surface of the knee joints.
The present study demonstrates that WP9QY peptide inhibits both inflammation and bone destruction in collagen-induced arthritis (CIA) mice, a chronic autoimmune model of human RA.

TNF\(\alpha\), IL-1 and IL-6 are the key mediators in the perpetuation of synovitis and bone destruction and are upregulated in the RA synovium and synovial fluid\(^3\). In fact, blockage of these proinflammatory cytokines ameliorated the inflammation of joints in the CIA mice\(^22,24\). Moreover, anti-TNF\(\alpha\) treatment such as neutralizing antibody (Infliximab) or a soluble TNFR:Fc fusion protein (Etanercept) reduced the inflammation in patients with persistently active RA\(^9,10\).

These reports indicated that TNF\(\alpha\) is important for the development of inflammation in RA. The present observation is consistent with these reports. It seems that the WP9QY peptide, a TNF\(\alpha\) antagonist, reduces inflammation in the CIA mice by interfering with the TNF\(\alpha\)/TNFR (I) interaction.

The present study has confirmed that osteoclasts play a pivotal role in RA-mediated joint destruction. The proliferation and differentiation process of osteoclasts...
requires cytokines such as macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) expressed on osteoblast, in combination with RANK expressed on osteoclast. In the synovial inflammatory tissue, activated T cells express RANKL and regulate the osteoclast formation which results in the bone destruction of RA. Actived T cells produce interferon (INF)-γ, a strong inhibitor of osteoclast formation and therefore the balance between RANKL and IFN-γ may be skewed in favor of the RANKL expression in RA. Recently, other studies have reported that the proinflammatory cytokine, TNFα has intrinsic osteoclast-induced properties mediated through signaling which is independent of the RANKL/RANK system. Therefore, the inhibitory effects of WP9QY peptide in the CIA-induced bone resorption of knee joints and tibial epiphysis might be mainly due to the interference with TNFα/TNFRI(II) interaction.

However, there is a report that TNFα itself fails to induce differentiation of osteoclasts in the absence of RANKL priming. Moreover, TNFα-treated RANKL-/− mice showed the rare appearance of TRAP and cathepsin K-positive osteoclasts on bone surfaces and failed to induce hypercalcaemia. Since many reports confirmed that the synergy between TNFα and RANKL promotes bone resorption by enhancing the differentiation and the activity, this synergism seems to lead to the severe inflammatory bone destruction in RA. In view of these reports, it is still not clear whether WP9QY peptide inhibited osteoclastogenesis in the CIA mice only by antagonizing TNFα. It could be speculated that WP9QY peptide inhibited inflammatory bone destruction of the joint by blocking TNFα/TNFRI(II) and/or synergism of TNFα and RANKL in the CIA mice. In addition, similarities between the ligand recognition site on TNFR(I) and the cognate receptor RANK, indicate the possibility that WP9QY peptide directly inhibits the RANKL/RANK interaction, thereby decreasing the osteoclast activity in the CIA mice. Since it is reported that higher serum levels of soluble RANKL are present in RA patients than in healthy, the findings that WP9QY peptide also inhibited the systemic reduction of trabecular and cortical bone of proximal tibia, which is not a site for RA, in this study further support the inhibitory effects of the WP9QY peptide on RANKL/RANK interactions.

The molecular size of the WP9QY peptide that we used in this study is 1226, so the WP9QY peptide is expected to overcome the disadvantages of large macromolecules such as antibodies and soluble forms of receptor, which include poor bioavailability, stability, expense for peptide synthesis, and the risk of severe and occasionally life-compromising side effects. Furthermore, WP9QY peptide apparently had no side effects, as no systematic abnormality or no pathological changes were observed in the tissues even under high dose of peptide administration. Moreover, we found that the half-life of the WP9QY peptide is very short, which could be one of the reasons why this peptide could not delay the onset of arthritis as do anti-TNF agents and why multiple injections were necessary during 24 hours in the experiment. Strategies to overcome these disadvantages should be addressed for further use of this peptide.

In conclusion, the present result showed that WP9QY peptide inhibited both inflammation and bone destruction of the joint in CIA mice, possibly by a mechanism that interferes with the interaction between ligand and receptor pairs, TNFα/TNFRI(II) and RANKL/RANK, by the reduction of synergism of TNFα and RANKL. Therefore, the development of improved type of WP9QY peptide, that prevents more efficiently the interaction of the critical loop on the TNF receptor(I) with its binding site on TNFα may be useful for the amelioration of inflammatory bone destruction such as RA and periodontitis.

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