ORIGINAL

Associations of serum leptin levels with intra-articular inflammatory cytokine levels in acute arthritic and nonarthritic knees of mice

Koji Aso, Shogo Takaya, Yusuke Kasai, Masashi Izumi, and Masahiko Ikeuchi

Department of Orthopedic Surgery, Kochi Medical School, Kochi University, Nankoku, Japan

Abstract : Background : The roles of serum leptin in knee joint inflammation are unclear. The objective of this study was to identify any associations of serum leptin level with intra-articular inflammatory cytokine levels in acute arthritic and nonarthritic knees of mice. Methods : Acute arthritis was induced by intra-articular injection of 2% carrageenan. Three groups (leptin-deficient ob/ob, wild-type (WT) and high-fat diet (HFD)-fed WT) were made. Serum leptin and inflammatory cytokines in the infrapatellar fat pad and synovium were measured before and 24 hr after injection. Affected knee joints were excised for histology 24 hr after injection. Results : The HFD-WT group had significantly higher serum leptin than the ob/ob and WT groups before and after carrageenan injection. The HFD-WT group had significantly higher IL-1β and IL-6 in the infrapatellar fat pad and synovium than ob/ob and WT before injection but significantly lower IL-1β, IL-6 and TNF-α than the ob/ob group at 24 hr. Conclusions : Hyperleptinemia induced by a HFD is involved in low-grade intra-articular inflammation in nonarthritic knee joints. In contrast, leptin deficiency causes excessive intra-articular inflammation in carrageenan-induced acute arthritis. Leptin alleviates acute arthritis, while chronic hyperleptinemia is involved in low-grade inflammation in normal knee joints. J. Med. Invest. 70 : 54-59, February, 2023

Keywords **:** *Leptin, Acute arthritis, Knee joint*

INTRODUCTION

Leptin is an adipokine that is produced mainly in white adipose tissue, and it regulates food intake and energy expenditure at the hypothalamic level (1). Circulating leptin levels are correlated with the degree of obesity and act as a signal for the hypothalamus to inhibit food intake and to stimulate energy expenditure (1). Leptin has a critical role not only in the regulation of metabolic processes but also in the control of immune homeostasis and inflammation (2, 3). Elevated levels of circulating leptin in obese patients contribute significantly to a low-grade inflammatory state (4). In patients with chronic inflammatory arthritic diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA), serum leptin levels are higher than those in healthy controls (5, 6). For these reasons, leptin has been regarded as a proinflammatory cytokine. However, a recent animal study reported that hyperleptinemia suppressed the IL-6 response and the progression of joint inflammation in a mouse model of collagen antibody-induced arthritis, which suggests that leptin works as an anti-inflammatory cytokine (7, 8). At the present moment, the potential roles of serum leptin in inflammation are unclear, and no study has reported the effects of leptin deficiency and hyperleptinemia on the inflammatory response in acute arthritis.

Carrageenan-induced arthritis is a commonly used model in multiple different animal types, including rodents, to mimic acute inflammatory arthritis. Intra-articular injection of 2% carrageenan into rat knee joints produces synovitis and pain behavior (9). The objective of this study was to identify any associations of serum leptin level with intra-articular inflammatory cytokine levels in acute arthritic and nonarthritic knees of mice. Specifically, we evaluated the association of serum leptin level with interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α in the infrapatellar fat pad and synovium. Additionally, knee joint histology and pain-related behaviors were evaluated. We hypothesized that leptin suppressed the intra-articular inflammatory cytokine levels in acute arthritic knees of mice.

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Address correspondence and reprint requests to Koji Aso, MD PhD, Department of Orthopedic Surgery, Kochi Medical School, Kochi University, 185-1 Oko-cho Kohasu, Nankoku, JAPAN, 783-8505 and Fax : +81-88-880-2388. E-mail : koji.aso@gmail.com

MATERIALS AND METHODS

Mice and arthritis induction

Male 7-week-old wild-type (WT) mice (C57BL/6J Ham Slc-ob/ob +/+) and leptin-deficient ob/ob mice (C57BL/6J Ham Slc-ob/ob) were purchased from Japan SLC and were housed in the Kochi Medical School Vivarium. Mice were kept on a 14-hour light/10-hour dark cycle with unlimited access to food and water for the duration of this study. Three groups (leptin-deficient ob/ob, WT and HFD (high-fat diet)-WT groups) were made. The ob/ob and WT groups were fed a control diet (12% kcal from fat ; CA-1, CLEA Japan), while the HFD-WT group was fed a high-fat diet (60% kcal from fat ; HFD-60, Oriental Yeast). To induce acute arthritis, mice were injected with 20 μl of 2% carrageenan using a 27 G needle with a Hamilton syringe inserted through the patellar ligament into the intra-articular space of the right knee under anesthesia with 2–3% isoflurane.

Experimental protocol

After giving the ob/ob and WT groups the control diet and the HFD-WT group the high-fat diet for 6 weeks, all mice were injected with 20 μl of 2% carrageenan. Serum leptin levels were evaluated before injection in all groups and 24 hr after injection in the WT and HFD-WT groups (n=6 per group). After evaluation at 24 hr, mice were euthanized, and the affected-side infrapatellar fat pads and synovium were resected for inflammatory

cytokine measurement (n=12 in each group before injection and n=15 in each group 24 hr after injection). The affected knee joints were excised for histology (n=5 in each group). Pain-related behavior tests were performed before and 3 hr and 24 hr after injection. All experiments were approved by the Animal Care and Use Committee of authors' institutions (Approval number ; L00069).

Measurement of leptin and inflammatory cytokine

Serum levels of leptin were measured with a commercially available ELISA kit (Morinaga Institute of Biological Science, Kanagawa, Japan) according to the manufacturer's instructions. The blood samples were taken by cardiac puncture.

For quantitative analysis of IL-1β, IL-6 and TNF-α, total RNA from the affected-side infrapatellar fat pad and synovium was extracted with RNAiso Plus (Takara Bio, Kusatsu, Japan) and Pure Link RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was then synthesized by using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative real-time PCR was performed by using designed primer pairs (Perfect Real Time Support System ; Takara Bio, Kusatsu, Japan) in Supplementary Table 1. SYBR Premix Ex Taq II was used for amplification according to the manufacturer's instructions in a Step One Plus Real Time PCR system (Applied Biosystems Japan, Tokyo, Japan). These samples were assessed by combining 3 pieces of fat pad into one sample because the infrapatellar fat pad and synovium of mice are too small to assess with one piece. mRNA levels were determined relative to those of HPRT.

Histological synovitis evaluation of knee joint

Right knee joints were excised and fixed in 10% formaldehyde, decalcified by 13% formic acid in phosphate-buffered water for 7 days and embedded in paraffin. Five-micrometer-thick sagittal sections of medial weight-bearing regions were cut and stained with hematoxylin and eosin (H&E). The severity of synovial inflammation was evaluated according to the synovitis scoring system for synovial hyperplasia (from 0 = the synovial lining cells form one layer to 3 = more than 5 layers) and inflammatory cell infiltration in the synovial stroma (from 0 = normal to 3 = greatly increased cellularity with possible pannus formation and granulomas) (10). The synovitis score was the sum of the synovial hyperplasia and inflammatory cell infiltration scores (maximum score 6).

Pain-related behaviors tests

The mechanical sensitivity of the paw was examined using von Frey filaments (11). Mice were placed inside a Plexiglas cage on an elevated mesh steel platform and acclimated. Von Frey filaments of varying bending forces (2.0, 1.4, 1.0, 0.6, 0.4, 0.16, 0.07, 0.04, 0.02, 0.008 × g) were applied to the plantar surface of the affected side paw. The minimum bending force to induce leg withdrawal was recorded three times. The median of these values was recorded as the mechanical threshold of the paw.

Spontaneous nociceptive behavior was examined using the foot stance score (12). Mice were placed in individual chambers and recorded on a digital camera for 5 min. The recordings were scored using a modified rating scale previously described for rats : 0 = no visible impairment of gait or stance, foot firmly placed flat on the surface with normal spread of toes ; 1 = moderate impairment of stance, foot placed on the ground but with toes tightly contracted together ; 2 = severe impairment of gait and stance, foot either entirely off the ground or only the lateral part of the foot very lightly touching the ground, toes tightly pulled together.

Statistical analysis

Statistical analysis was carried out using IBM SPSS statistics version 26 (IBM, Armonk, NY, USA). All data in each group show a normal distribution, therefore two-way ANOVA tests were performed to compare body weight and pain-related behaviors, and one-way ANOVA tests were performed to compare serum leptin and inflammatory cytokine mRNA levels between the ob/ob, WT and HFD-WT groups. Tukey's honestly significant difference test was used for comparison when ANOVA detected significant differences. Pearson's r was used for Correlation analysis between serum leptin and cytokines. A p-value of <0.05 was considered statistically significant.

RESULTS

Body weight

Before carrageenan injection, the ob/ob group had a significantly heavier weight than the WT and HFD-WT groups, and the HFD-WT group had a significantly heavier weight than the WT group. The mean body weights (95% confidence interval (CI)) (g) of the ob/ob, WT and HFD-WT groups were 48.8 (44.0–53.4), 28.0 (26.8–29.1) and 35.2 (32.9–37.5), respectively.

Leptin and inflammatory cytokine

Serum leptin was mostly not detected in the ob/ob group before carrageenan injection (Figure 1). Serum leptin level in the HFD-WT group was significantly higher than that in the WT group before carrageenan injection, and the levels were not changed by carrageenan injection (Figure 1). The HFD-WT group had significantly higher gene expression of IL-1β and IL-6 in the infrapatellar fat pad and synovium than the ob/ob and WT groups before carrageenan injection. The expression of IL-1β and IL-6 were significantly positive associated with serum leptin (IL-1β ; Pearson's r = 0.82, p = 0.001, IL-6 ; Pearson's r = 0.82, p = 0.002). However, the HFD-WT group had significantly lower IL-1β, IL-6 and TNF-α than the ob/ob group at 24

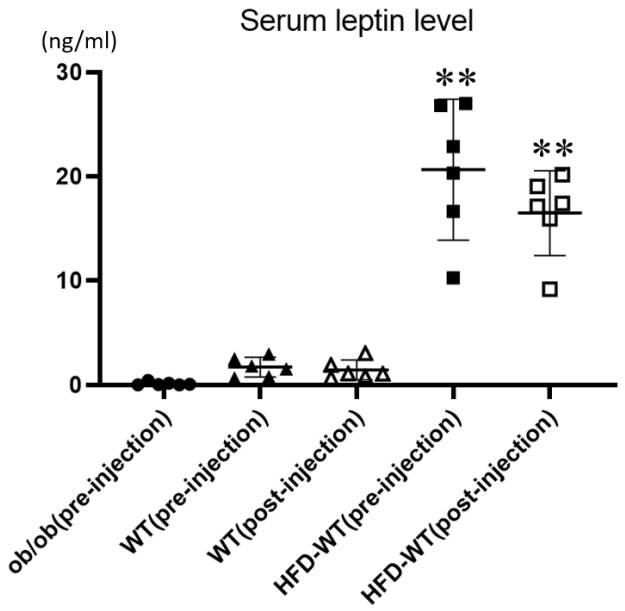

Figure 1. Serum leptin level before and after carrageenan injection
Lines represent means and 95% CIs.
**p<0.01 vs. ob/ob (pre-injection), WT (pre-injection) and WT (post-injection). n = 6 in each group.
WT ; wild-type, HFD ; high-fat diet

hr after injection. Furthermore, the WT group had significantly lower IL-1β and IL-6 levels than the ob/ob group at 24 hr after injection (Figure 2). The expression of IL-1β were significantly negative associated with serum leptin (IL-1β ; Pearson's r = -0.71, p = 0.01)

Histological synovitis evaluation of knee joint

Intra-articular carrageenan injection induced of the synovial lining cell layer in all groups and increased density of the cells in the synovial stroma, especially in the ob/ob group compared with the HFD-WT group. However, there were no significant differences in synovitis score between the three groups (Figure 3).

Pain-related behavior tests

Intra-articular carrageenan injection decreased the mechanical threshold in the right hind paw and induced spontaneous nociceptor activity in all groups. There were no significant differences between the three groups in the von Frey test or stance score (Figure 4).

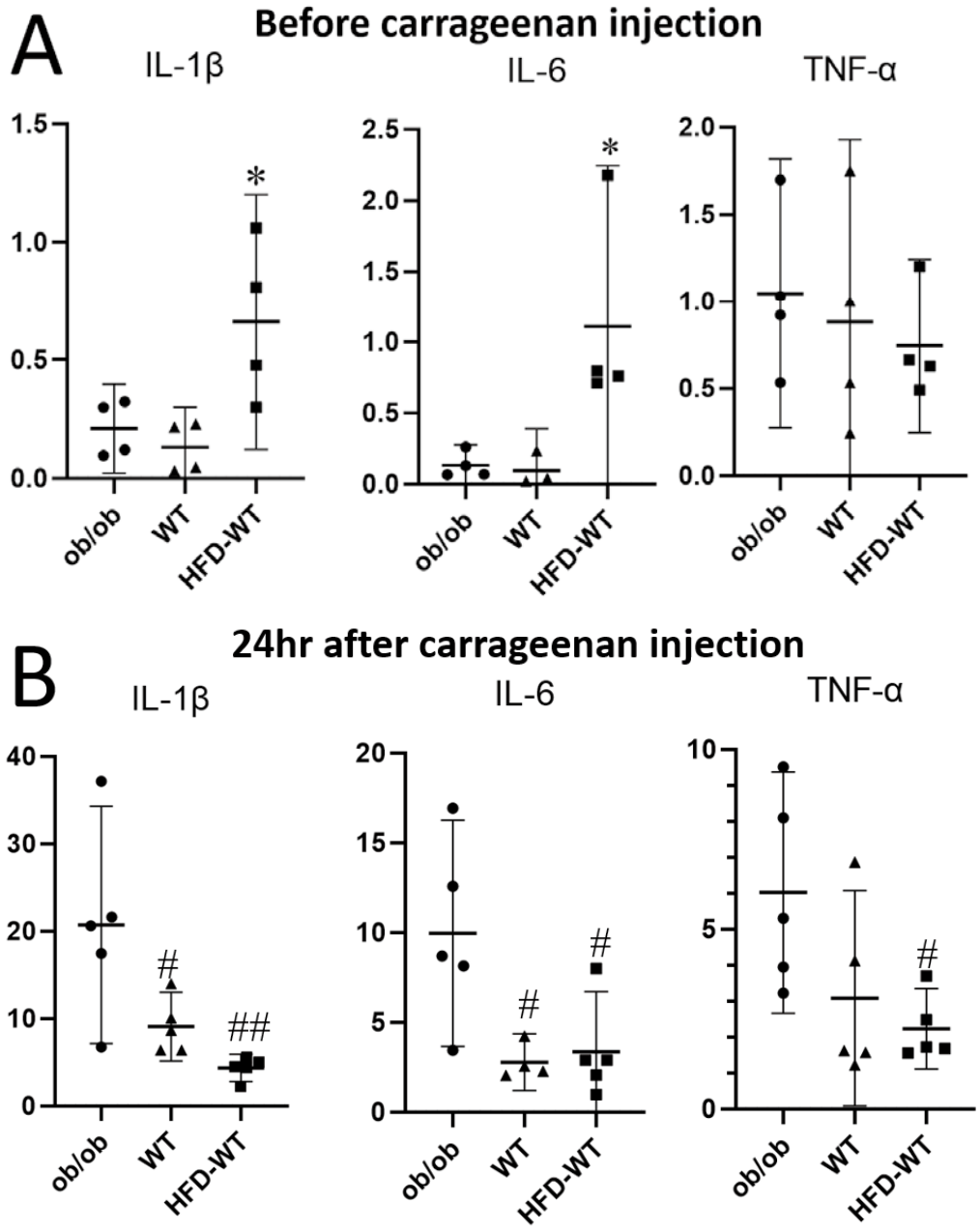

Figure 2. Inflammatory cytokine mRNAs in the infrapatellar fat pad and synovium before and 24 hr after injection
Lines represent means and 95% CIs. n = 15 in each group
One dot indicates the result of 3 mice.
*p<0.05 vs. ob/ob and WT. #p<0.05 vs. ob/ob. ##p<0.01 vs. ob/ob.
WT ; wild-type, HFD ; high-fat diet

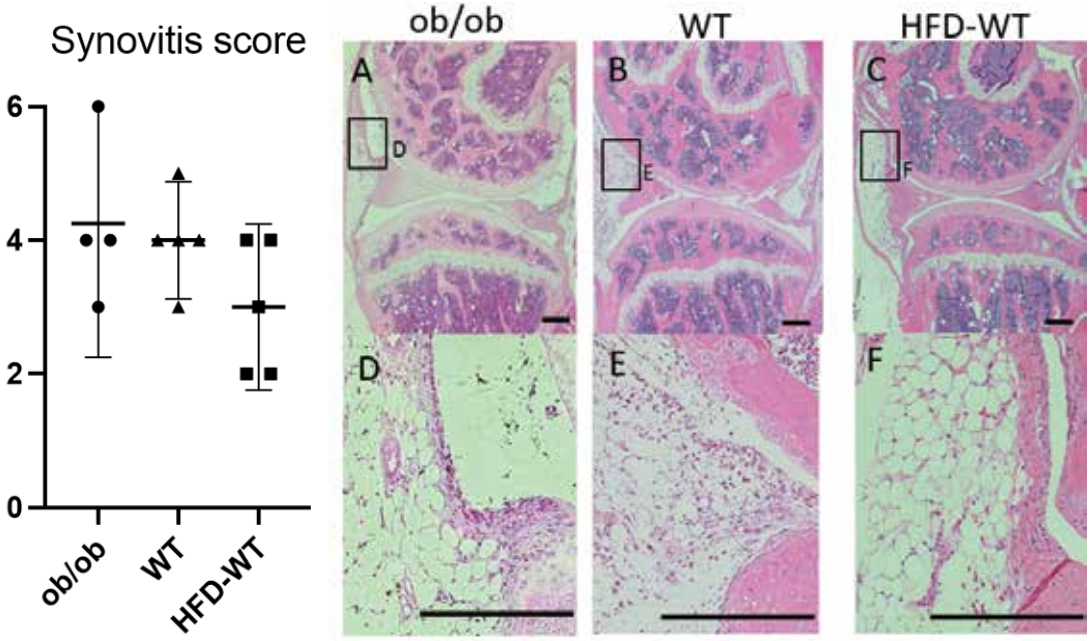

Figure 3. Histological synovitis evaluation of knee joints
Photographs show enlargement of the synovial lining cell layer in all groups and increased density of the cells in the synovial stroma, especially in the ob/ob group compared with the HFD-WT group. However, there were no significant differences in synovitis score between the three groups. Histological phot (D) shows synovitis score 4 (synovial hyperplasia is 3 points and inflammatory cell infiltration is 1 point). Histological phot (E) shows synovitis score 4 (synovial hyperplasia is 2 points and inflammatory cell infiltration is 2 points). Histological phot (F) shows synovitis score 2 (synovial hyperplasia is 1 points and inflammatory cell infiltration is 1 point).
Sections of medial weight-bearing regions were stained with hematoxylin and eosin (H&E).
Lines represent means and 95% CIs. n = 5 in each group
WT ; wild-type, HFD ; high-fat diet
Scale bars = 200 μm

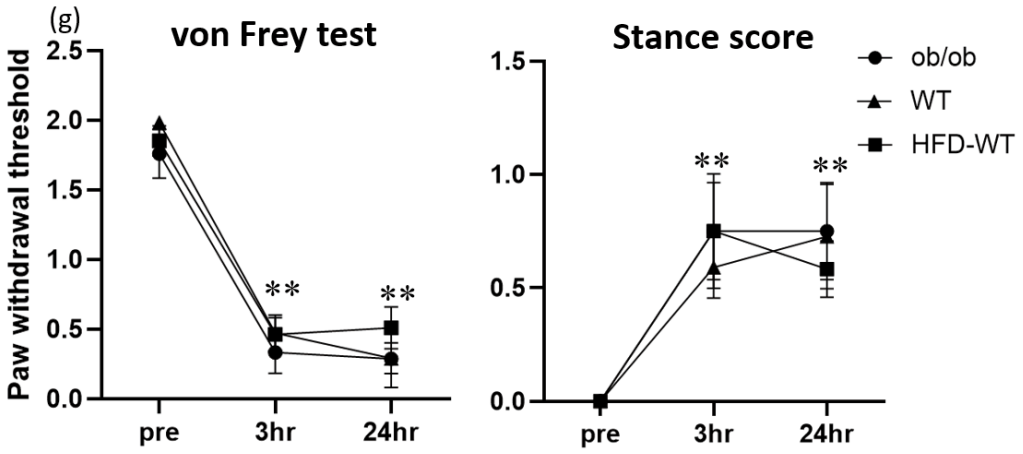

Figure 4. Pain-related behaviors.
Lines represent means and 95% CIs.
**p<0.01 vs. pre in all groups. n = 12 in each group.
WT ; wild-type, HFD ; high-fat diet

DISCUSSION

This study is the first to find that leptin deficiency is associated with the overexpression of inflammatory cytokines in the infrapatellar fat pad and synovium in carrageenan-induced acute arthritis. In contrast, hyperleptinemia induced by a high-fat diet is associated with an increase in inflammatory cytokines in the nonarthritic knee joint of obese mice. These results suggest that leptin works to alleviate intra-articular inflammatory cytokine in acute arthritis, which is consistent with our hypothesis, but chronic hyperleptinemia is involved in low-grade inflammation in nonarthritic knee joints. Appropriate management of serum leptin levels may be important for the prevention of excessive intra-articular inflammation.

Leptin has been shown to be involved in inflammation as a proinflammatory adipokine. Hyperleptinemia is associated with low-grade systemic inflammation in obese people, and serum levels of leptin show a positive correlation with proinflammatory cytokines and a negative correlation with anti-inflammatory cytokines (13). Leptin can modulate T-cell immune responses and increase inflammation by enhancing the production of proinflammatory cytokines (14, 15). We also confirmed that increased serum leptin induced by a high-fat diet was associated with increased IL-1β and IL-6 in the infrapatellar fat pad and synovium in the nonarthritic knees of obese mice.

This study is also the first to show that leptin deficiency caused overexpression of inflammatory cytokines in the infrapatellar fat pad and synovium compared with what was seen in WT and HFD-WT mice with carrageenan-induced acute arthritis. A previous study reported that septic arthritis caused decreased leptin levels during infection and that the administration of leptin reduced the severity of septic arthritis and the inflammatory response, as measured by serum IL-6 levels in mice (16). The study suggested that the decreased production of IL-6 in response to exogenous leptin is a possible pathway for leptin's anti-inflammatory effect (16). In zymosan-induced arthritis, leptin-deficient mice had delayed resolution of acute inflammation (17). In our results, there were no significant differences in inflammatory cytokine responses caused by carrageenan injection between WT mice with normal leptin levels and HFD-WT mice with high leptin levels. However, a previous study using leptin transgenic mice demonstrated that hyperleptinemia suppressed the IL-6 responses and the progression of joint inflammation caused by collagen antibody compared with control mice with normal leptin levels (8). A previous study showed that high-concentration leptin significantly suppressed IL-6 expression in THP-1 cells, a human monocytic cell line, and suggested that leptin may play an anti-inflammatory role in hyperleptinemia. It is unclear whether hyperleptinemia is needed for suppression of the inflammatory response, but our results suggest that leptin deficiency causes an excessive intra-articular inflammatory response in acute arthritis. Further studies are needed to elucidate the optimal leptin concentration for controlling inflammation in joints.

Synovial fluid leptin concentrations are associated with joint pain in patients with hip or knee osteoarthritis (18). A previous animal study suggested the involvement of leptin in the pathogenesis of pain at the spinal level and a possible role in the development of neuropathic pain (19). However, hyperleptinemia did not influence pain behavior in carrageenan-induced acute arthritic mice in this study. A mild effect of hyperleptinemia on pain may have been undetectable because the effect of carrageenan on their pain was too strong.

Serum leptin has been regarded as a good predictor of nutritional status in elderly patients (20). Clinically, our results suggest that leptin deficiency in malnutrition increases susceptibility to excessive inflammatory responses in acute arthritis. The appropriate management of serum leptin levels by body weight and nutritional status control may be important for the prevention of excessive inflammation in acute arthritis. Furthermore, leptin administration may suppress inflammation in acute arthritis in patients with leptin deficiency. The suppressive effects of leptin administration on septic arthritis and sepsis on a background of leptin deficiency have already been reported (16, 21). Our study showed that hyperleptinemia was associated with intra-articular low-grade inflammation. Intra-articular inflammation is involved in the progression of degenerative diseases such as osteoarthritis (OA) (22). Therefore, leptin control for obese patients with hyperleptinemia may be a treatment for OA.

This study has several limitations. First, the influence of metabolic disorders such as diabetes mellitus, which are expected in the ob/ob and HFD-WT groups, was not measured in this study. Second, the effects of body weight differences between groups on our results were not assessed. Body weight in the ob/ob group was significantly heavier than that in the WT and HFD-WT groups. However, inflammatory cytokines in the ob/ob group were similar to those in the WT group in the normal knee. Therefore, we believe that the effects of body weight differences played little or no role in the inflammatory response in this study. Third, in addition to adipose tissue, leptin is produced by cartilage and other joint tissues in humans, and it can be found in synovial fluid (5, 23), but the direct contribution of intra-articular leptin to inflammatory cytokine levels in the infrapatellar fat pad and synovium was unclear in this study. Future studies should assess this. Forth, inflammatory cytokines were assessed only at 24 hr after carrageenan injection in this study. Carrageenan significantly increased inflammatory cytokines in synovial lavage fluid at 3 hr after carrageenan injection into rat knee joints (24) and the cytokines production from macrophages by induced carrageenan were significantly higher at 24 hr in vitro (25). Therefore, we assessed inflammatory cytokines at 24 hr after carrageenan injection in this study. Multiple times of assessment of inflammatory cytokines are needed in the future study.

In conclusion, hyperleptinemia induced by a high-fat diet is involved in low-grade intra-articular inflammation in nonarthritic knee joints. In contrast, leptin deficiency causes excessive intra-articular inflammation in carrageenan-induced acute arthritis. Appropriate management of serum leptin levels may be important for the prevention of excessive intra-articular inflammation.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest associated with this study.

AUTHOR CONTRIBUTIONS

K.A. and S.T. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All author designed the experiments, and S.T. conducted it. K.A. and S.T. analyzed results and wrote the manuscript. M.I. directed this study.

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Supplementary Table 1. Sequences of primers used in this study.

Primer	Sequences [5' – 3']
TNF-α	F : ACTCCAGGCGGTGCCTATGT R : GTGAGGGTCTGGGCCATAGAA
IL-1β	F : TCCAGGATGAGGACATGAGCAC R : GAACGTCACACACCAGCAGGTTA
IL-6	F : CCACTTCACAAGTCGGAGGCTTA R : CCAGTTTGGTAGCATCCATCATTTC
Leptin	F : CCAGGATCAATGACATTTCACACAC R : AGGTCATTGGCTATCTGCAGCAC
HPRT	F : TTGTTGTTGGATATGCCCTTGACTA R : AGGCAGATGGCCACAGGACTA