Exercise-induced cytokines alleviate arthritis symptoms

Introduction

The prevalence of lifestyle-related diseases in Japan has been increasing as the society ages, leading to increased health consciousness among the public. Among lifestyle diseases, obesity requires attention as it can cause chronic inflammatory states and increase the risk of cardiovascular disease. Establishing an exercise habit is an essential part of health, and so proactive incorporation of exercise into the daily routine is highly recommended. Stimulation from exercise promotes the secretion of a variety of substances, many of which act on various organs to help maintain health. Among these substances are cytokines, which are immune substances produced and secreted by leukocytes. Cytokines secreted by skeletal muscles in response to exercise are called myokines. Cytokines have a variety of effects. For instance in rats, IL-15 has been reported to increase the weight of the spleen, which participates in immunity, and reduces white adipocytes, which can cause hypertension. IL-6 promotes the breakdown of neutral fats and glycogen in the liver. Therefore, cytokines are considered to be extremely effective in maintaining health. However, not all cytokines have positive effects on the body. Cytokines are classified into those with anti-inflammatory effects, like IL-6, IL-8, and IL-1ra, and those with inflammatory effects, like IL-1α, IL-1β, and TNF-α. Normally, there exists a balance between inflammatory and anti-inflammatory cytokines in the body. If this balance is disrupted, inflammatory cytokines become predominant leading to chronic inflammatory states. For instance, in obesity, this can increase the risk of heart disease. Arthritis is one such disease where inflammatory cytokines play a major role. Predominance of inflammatory cytokines in arthritis promotes joint destruction. In this case, over-expression of inflammatory cytokines has a negative effect on the
Currently, there are around 700,000 rheumatoid arthritis patients in Japan, making up about 0.5% to 1% of the population, or one in every 100 to 200 people. Rheumatoid arthritis is the most prevalent autoimmune disease and approaching the figure of roughly 810,000 patients with ischemic heart disease. Therefore, there is a need to be proactive about preventing and treating rheumatoid arthritis. However, the current medicinal strategies lack the means of preventing or curing rheumatoid arthritis. The goals of treatment include early diagnosis and controlling arthritic progression before inflammation spreads. To achieve this, palliative care is administered through pharmaceuticals such as non-steroidal anti-inflammatory drugs and steroids. Gradual elucidation of the onset mechanism of autoimmune diseases and rheumatoid arthritis in recent years has generated new treatment possibilities. Th17 cells produce IL-17, which facilitates the receptor activator of nuclear factor-κB ligand (RANKL) expression on synovial fibroblasts. Th17 cells also promote local inflammation, leading to the production of inflammatory cytokines like TNF-α and IL-1, which can induce RANKL. All this increases osteoclast differentiation resulting in excessive bone destruction.

While this is only a part of the rheumatoid arthritis mechanism, this understanding has created the possibility of complete cure through what is called “anti-cytokine therapy”. In anti-cytokine therapy, drugs are administered to inhibit the secretion of TNF-α, IL-1, and other cytokines secreted via synovial macrophage activity. This suppresses signal transduction from synovial fibroblasts and osteoclast precursors, which inhibits osteoclast differentiation. The Japan College of Rheumatology considers anti-cytokine therapy using TNF inhibitors to be the best choice for improving clinical symptoms of rheumatoid arthritis, controlling the progression of joint destruction, and improving physical function. However, since side effects and serious adverse events are possible with this treatment, it cannot be used in all affected patients and the patients and conditions eligible to use this drug are limited.

To address this problem, we hypothesized that it may be possible to control the production and secretion of inflammatory cytokines using the natural healing ability...
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of the body. Control of inflammatory cytokines would require secretion of sufficient amounts of anti-inflammatory cytokines. Therefore, we focused on cytokines (myokines) that are produced and secreted by skeletal muscles in response to exercise stimulation. Myokines include IL-6, IL-8, and IL-15. Through humoral regulation of the endocrine system, these substances travel through the blood to affect many tissues and organs of the body. Among them, IL-6 in particular, has been found to have anti-inflammatory effects. In other words,

![Diachronic weight change](image1)

**Fig. 2.** Changes in body weight with age, from 8 weeks to 18 weeks. Mannan administration and exercise stimulation were initiated simultaneously in 8-week-old SKG/Jcl mice. Exercise stimulation was terminated at the age of 18 weeks. The solid line represents the stimulation group and the dotted line indicates the non-stimulation group.

![Arthritis coefficient](image2)

**Fig. 3.** Chronological changes in arthritis coefficient. The graph shows changes in the arthritis coefficient until termination of exercise stimulation and collection of muscle samples at the age of 18 weeks. The solid line represents the stimulation group and the dotted line shows the non-stimulation group.
we hypothesized that exercise could serve as a kind of self-induced anti-cytokine therapy. Therefore, in the present study we administered exercise stimulation to a mouse model of spontaneous arthritis to for self-induction of myokines such as IL-6 and IL-10 from the skeletal tissue. We examined whether these myokines suppressed the secretion of TNF-α and other inflammatory cytokines, and investigated effects, such as delayed onset of arthritis and alleviation of symptoms.

Materials and methods

1) Animal model

Twelve 7-week-old male SKG/Jc1 mice (CLAIR Japan Inc.) were used. Autoimmune arthritis with a strong immunopathological resemblance to rheumatoid arthritis occurs spontaneously in these mice. The animals were kept in groups in bedded cages (3 per cage) and were used for the experiment after 1 week of acclimatization. This experiment followed the animal experimentation guideline of the Fujita health University (approval No. H0771). Arthritis was induced with 20 mg of mannan (100 mg/ml) (Sigma-Aldrich Corporation) and the control group received the same amount of it solution via intraperitoneal administration.

2) Method of exercise stimulation

The animals were divided into an experimental group and a control group of 6 animals each. The experimental group was subjected to exercise stimulation for 30 minutes per day after mannan administration for 6 days in a row followed by 1 day of rest, for 10 consecutive weeks (stimulation group). In the control group, the animals were kept normally after mannan administration without any exercise stimulation (non-stimulation group). Apart from exercise stimulation, both groups were maintained under the same conditions.

3) Exercise stimulation device

Exercise stimulation was administered using devices that we developed. One device was a horizontal-rotation device that administered uniform 360° horizontal shaking stimulation 150 times per minute (NX-25D Nissin Scientific Corporation, Japan). The other device was a vibrator that administered 0.5 mm tornado-type vibra-

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**Edema and erythema of multiple fingers**

![Image](image.png)

**Fig. 4.** An edema and erythema of multiple fingers. The images show foot bottom and foot fingers of SKG/Jcl arthritis model mice. (a) show no signs of arthritis and (b) show an edema and erythema of multiple fingers. Black arrowhead show these.

**Table 1.** Limbs were assigned a arthritis score as follows:

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no signs of arthritis</td>
</tr>
<tr>
<td>1</td>
<td>edema and erythema of one finger or multiple fingers</td>
</tr>
<tr>
<td>2</td>
<td>edema and erythema of all fingers</td>
</tr>
<tr>
<td>3</td>
<td>ankylosis of the finger</td>
</tr>
</tbody>
</table>

The total of each score was arthritic score (normal : 0 The most serious state : 12)
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4) Evaluation of arthritis symptoms

Body weight was measured once every week. Arthritis coefficient was calculated using a modified version of a method previously described by Kokkola et al.\(^{10-12}\) and was assessed once every week (Table 1). The symptom of the arthritis was observed of edema and erythema of multiple fingers (Figure 4).

5) Tissue analysis

Tissue samples of knee joints were collected from mice at 3 hours after the final exercise stimulation. The animals were anesthetised with Somnopentyl. After thoracotomy and PBS perfusion, perfusion fixation was performed using 10% neutral buffered formalin. Next, the knee joints were collected and fixed for 48 hours with 10% neutral buffered formalin. Using standard methods, the samples were then decalcified for 48 hours with 10% EDTA-2Na (pH 7.4), dehydrated for 48 hours with an automatic embedding device, defatted, and embedded in paraffin. Section of 8 µm thickness were stained with Safranin O, which specifically stains the cartilage.

6) Protein expression analysis

Protein analysis samples were collected from mice at 3 hours after the final exercise stimulation. The animals were anesthetised with Somnopentyl. After thoracotomy and PBS perfusion, the following four muscles were collected: quadriceps, hamstring, triceps surae, and tibialis anterior. These muscles were rapidly frozen using dry ice and were stored temporarily at -80°C. The samples were processed by removing the muscles from frozen storage and crushing them with a mortar and pestle under liquid nitrogen. The samples were then suspended in T-PER (Thermo Scientific PB196592) containing a protease inhibitor (Complete protease inhibitor cocktail, Roche Applied Science 10190300). These were then subjected to centrifugation and the supernatant was collected. After adjusting the concentration to 1 µg/µl, the samples were used for the expression analysis. Cytokine measurements were performed using the Mouse IL-6

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Knee joint: safranin O staining

Fig. 5. Safranin O-stained knee joints. The images show Safranin O-stained sagittal sections of knees of SKG/Jcl arthritis model mice. (a) and (b) show low-power fields with 100 µm scale bars. (c) and (d) show high-power fields with 50 µm scale bars. (a) and (c) are from mice that underwent exercise stimulation (+). (b) and (d) are from mice that did not have exercise stimulation (−). The symbols in the images are T: tibia, F: femur, AC: articular cartilage, black arrowhead: fibroblast, and white arrowhead: chondrocyte.
Quantikine ELISA kit (R&D SYSTEMS 312184), TNF-α Mouse ELISA kit (Abcam GR195763-1), IL-10 Mouse ELISA kit (Abcam GR155247-5), and IL-15 Mouse ELISA kit (Abcam GR128196-1).

We performed the statistical method to compare with the control group using the t-test method. All the statistical method of Fig.5 depends on this method. Statistical analyses were conducted with SPSS Statistics 22.0 (IBM Japan, Ltd.) software. For all analysis, the level of significance was set at $p < 0.05$.

**Results**

Body weight measurements taken every week showed that weight increased along with age in both the stimulation and non-stimulation groups (Figure 2). The weekly arthritis coefficient assessments showed that initial symptoms of arthritis appeared after 1 week of mannan administration in the non-stimulation group (at 9 weeks of age). In the stimulation group, initial arthritis symptoms began appearing 4 weeks later than the non-stimulation group (at 14 weeks of age). The score of the non-stimulation group increased markedly starting at the age of 12 weeks, reaching a maximum of 3. In contrast, the score of the stimulation group increased starting at the age of 14 weeks, but only reached a maximum of 1.5 (Figure 3).

Safranin O-stained knee tissue samples showed thicker knee cartilage in the stimulation group than in the non-stimulation group. These areas also showed multiple chondrocyte clusters (Figure 5). IL-6, IL-10, IL-15, and TNF-α were measured using ELISA to evaluate cytokine expression in the skeletal muscle (Figure 6). Higher levels of IL-6 and IL-10 were observed in the stimulation group than in the non-stimulation group. IL-15 levels were significantly higher in the stimulation group ($p < 0.01$) and TNF-α ($p < 0.05$).

![Fig. 6. Muscle protein expression. The graphs show ELISA results for (a) IL-6, (b) IL-15, (c) IL-10, and (d) TNF-α. The black bars represent the stimulation group (+) and the white bars represent the non-stimulation group (-). Significant differences were observed for IL-15 ($p < 0.01$) and TNF-α ($p < 0.05$).](image-url)
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0.01), while TNF-α levels were significantly higher in the non-stimulation group ($p < 0.05$).

**Discussion**

Rheumatoid arthritis is a disease that involves synovial inflammation and osteoclast activation, leading to bone resorption. In the present study, we administered exercise stimulation in the form of double mechanical stress that combined shaking and vibration in a mouse model of arthritis. We then compared these animals to ones that did not receive any stimulation. The results showed thicker cartilage in the stimulation group compared than that in the non-stimulation group, which we surmise was due to vigorous cellular activity. This observation suggests that exercise stimulation suppressed osteoclast activity and mitigated bone resorption. Increasing bone density is considered to require direct mechanical stimulation from contracting muscles that attach to bones via tendons 13), suggesting that muscle contraction activates bone remodelling. Further, we observed a delay in the onset of arthritis and slower progression of symptoms in the stimulation group compared with the non-stimulation group. These results suggest that stimulation via double mechanical stress had positive effects on bones and cartilage in arthritic.

Analysis of muscle proteins after the final exercise stimulation, confirmed the expression of anti-inflammatory cytokines IL-6 and IL-105–7, 14) and the inflammatory cytokines, IL-15 and TNF-α. In addition to the myokines, IL-6 and IL-15, we observed the expression of IL-10 and TNF-α in the muscles, which has never been reported and suggests that these are a type of myokine. It is thought that IL-6 induces IL-1015) whereas IL-15 induces TNF-α16–17). Greater expression of both IL-6 and IL-15 were observed in the stimulation group compared that in the non-stimulation group, the difference for IL-15 being significant. Similarly, we expected that the stimulation group would show more secretion of IL-10 and TNF-α than the non-stimulation group, but in contrast TNF-α secretion was reduced in the stimulation group ($p < 0.05$). Moreover, expression of the anti-inflammatory cytokines, IL-6 and IL-10 was greater in the stimulation group than in the non-stimulation group. This indicates that IL-6 or IL-10 secreted in response to exercise stimulation inhibited TNF-α secretion, or that there is a mechanism by which IL-15 inhibits TNF-α. It has been reported that IL-6 and IL-10 suppress TNF-α secretion18–19), so it is logical that IL-6 and IL-10 induced by exercise stimulation might inhibit TNF-α secretion. The mechanism of increased IL-15 expression was not clarified in the present study, but we presume that some mechanism to suppress the expression of inflammatory cytokines exists.

One aspect of the onset mechanism of rheumatoid arthritis involves TNF-α secreted by macrophages in the synovial membrane that directly participate in the bone resorption process20). This occurs because this substance can strongly induce RANKL expression in synovial fibroblasts and osteoblasts. TNF-α is an important target molecule for drugs to treat rheumatoid arthritis and is an important factor in the onset mechanism of this disease. In the present study, we surmise that the myokines, IL-6 and IL-10 from the endocrine system travelled through blood to distal joints and inhibited the expression of TNF-α secreted by macrophages in the synovial membrane, resulting in the retardation of arthritic symptoms. However, the onset and progression of rheumatoid arthritis occurs through a complex pathway, not all of which has been elucidated. Therefore, it would be difficult to suppress all the onset mechanisms that are thought to exist. The present study showed that while continuous exercise stimulation administered prior to onset cannot prevent disease, it could delay onset and slow the progression of arthritic symptoms.

SKG/Jcl, the arthritic model mouse used in the present study, spontaneously exhibits autoimmune arthritis due to a Zap-70 mutation8). In cases of arthritis thought to involve genetic factors, continuous administration of exercise stimulation prior to its onset could help inhibit and prevent inflammation.

We did not examine different conditions such as exercise intensity or timing of myokine appearance after stimulation, in detail. Therefore, we did not demonstrate the intensity or frequency of exercise that is needed to effectively delay arthritis onset or mitigate its symptoms. Further, cytokines induced by exercise stimulation travel throughout the body through circulation. Thus, myokines may affect other organs besides joints, which is another factor that we did not examine. In the future, detailed studies on exercise methods, intensity, and frequency need to be conducted considering clinical applications for humans. Moreover, it is now possible to investigate the congenital factors such as arthritis risk genes and MHC. Based on these results, exercise stimulation as a form of self-induced myokine therapy could be started in people who may be at risk of arthritis at an early stage. We hope this study can provide the foundation to create new methods of preventing and treating arthritis.

**Acknowledgement**

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