# Rheumatic Arthritis-induced Alteration of Morphology and Function in Muscles

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## ABSTRACT

**Clinical arthritis is typically divided into rheumatoid arthritis (RA) and osteoarthritis (OA). Arthritis-induced muscle weakness is a major problem in aged people, leading to a disturbance of balance during the gait cycle and frequent falls. The purposes of the present study were to confirm fiber type-dependent expression of muscle atrophy markers induced by arthritis and to identify the relationship between clinical signs and expression of muscle atrophy markers. Mice were divided into four experimental groups as follows: (1) negative control (normal), (2) positive control (CFA+acetic acid), (3) RA group (CFA+acetic acid+type Ⅱ collagen), and (4) aging-induced OA group. DBA/1J mice (8 weeks of age) were injected with collagen (50 μg/kg), and physiological (body weight) and pathological (arthritis score and paw thickness) parameters were measured once per week. The gastrocnemius muscle from animals in each group was removed, and the expression of muscle atrophy markers (MAFbx and MuRF1) and myosin heavy chain isoforms were analyzed by reverse transcription-polymerase chain reaction. No significant change in body weight occurred between control groups and collagen-induced RA mice at week 10. However, bovine type Ⅱ collagen induced a dramatic increase in clinical score or paw thickness at week 10 ($p<0.01$). Concomitantly, the expression of the muscle atrophy marker MAFbx was upregulated in the RA and OA groups ($p<0.01$). A dramatic reduction in myosin heavy chain (MHC)-Iβ was seen in the gastrocnemius muscles from RA and OA mice, while only a slight decrease in MHC-Ⅱb was seen. These results suggest that muscle atrophy gene expression occurred in a fiber type-specific manner in both RA- and OA-induced mice. The present study suggests evidence regarding why different therapeutic interventions are required between RA and OA.**

**(Key words : Rheumatoid arthritis, Collagen, Muscle atrophy, Therapeutic intervention)**

## INTRODUCTION

Arthritis has long been clinically categorized as osteoarthritis (OA) and rheumatoid arthritis (RA). OA frequently occurs in the adult population, and the articular cartilage is the primary target for disease development. OA is caused by a complex system involving interactions between mechanical, biochemical, and molecular feedback loops. Joint tissue destruction eventually occurs secondary to the failure of cells to maintain homeostasis between matrix synthesis and degradation (Martel-Pelletier and Pelletier, 2010). When catabolism exceeds anabolism, progressive bone absorption occurs. Clinical symptoms include pain, loss of motion, and muscle weakness. These cause secondary impairments such as imbalance, frequent falls, and an abnormal gait.

RA is a prototypical inflammatory joint disease defined as inflammation of the synovium. "Pannus" forms secondary to infiltration of inflammatory cells into the joint space and overgrowth of synoviocytes that cover the surfaces of articular cartilage and bone. This pathological mass produces degradative factors that destroy the joint structure (Walsh and Gravallese, 2010). Most symptoms of RA are similar to OA; however, there are also differences. Joint pain and stiffness occur in the morning rather than at night. Circulating cyto-

* This research was supported by grants from BioGreen 21 Program (Code #20110301-061-542-03-00 to Y. Hong), Rural Development Administration, and from KRIBB Research Initiative Program (KGM0321112), Republic of Korea.

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kines, such as interleukin-4 (IL-4) and tumor necrosis factor α (TNF α), reportedly enhanced pain reactivity in RA patients (Sommer and Kress, 2004). Moreover, joint pain has been considered a major cause of abnormal gait in RA patients (van der Leeden *et al.*, 2007; van der Leeden *et al.*, 2006). Recent studies reported that the gait pattern in arthritis-induced animals was characterized by a significant reduction in the distance between the left and right paws, increased guarding scores, and imbalance. These problems result in great loss of muscle strength and frequent falls in elderly people (Boettger *et al.*, 2009).

Generally, skeletal muscle fibers are classified as type I or II (Wang *et al.*, 2004). The characteristics of each type are markedly different. Muscles with prominent type I fibers (e.g., soleus) are oxidative, aerobic, slow muscles because of their high numbers of mitochondria. In contrast, muscles with prominent type II fibers (e.g., gastrocnemius) are glycolytic, anaerobic, fast muscles, due a relatively smaller number of mitochondria in cells. Type II fibers consists of three subtypes: IIa, IIx, and IIb. The latter relies mainly on glycolysis as an energy source, because its mitochondria content is the lowest among all fiber types (Berchtold *et al.*, 2000). In addition to the metabolic pathway, fiber type-dependent differences in susceptibility to atrophy were studied.

Skeletal muscle atrophy can be described as the wasting of muscle mass because of injury, immobilization, or disease. As muscle atrophy progresses, molecular markers that degrade muscle proteins are activated. F-box protein 32 (MAFbx) and muscle-specific ring finger protein 1 (MuRF1) are known as ubiquitin E3 ligases. Interestingly, MuRF1 associates with myofibril components, including titin, and acts as a cytoskeletal adaptor and signaling molecule (McElhinny *et al.*, 2004).

Clinically, researchers have divided muscle atrophy into two categories, cachexia and sarcopenia. Cachexia describes muscle wasting resulting from certain diseases, such as myopathy, neuropathy, and cancer. In contrast, sarcopenia is defined as an age-related reduction in muscle mass. Recent studies have suggested that cytokines are secreted from arthritis-responsive immune cells, thereby inducing an inflammatory myopathy.

Based on previous studies, the objectives of present study were to confirm the fiber-dependent expression of muscle atrophy markers after RA induction and to identify any relationship between clinical signs and the expression of muscle atrophy markers.

## MATERIALS AND METHODS

### Animals

The Ethical Committee for Animal Care and Use at Inje University approved all of the animal procedures performed. Male DBA1/J mice weighing 18~20 g (6 weeks of age) were purchased from Orient (Korea) and used for the RA animal model. Aged C57BL/6 mice (>2 years of age) were used for the OA animal model. Mice were housed at 23.1±1℃ in a 12/12-h light/dark cycle (lights on: 06:00) and allowed free access to food and water.

### Induction of RA

Collagen-induced RA was performed as described previously. Eight-week-old male DBA/1J mice were initially immunized with bovine type II collagen (Chondrex) mixed with complete Freund's adjuvant (CFA, Sigma) containing 1 mg/mL of heat-killed *Mycobacterium tuberculosis*. On day 21 from the first immunization, a boosting injection was administered with a collagen mixture containing incomplete Freund's adjuvant (Fig. 1).

### Assessment of Arthritis

Each paw was measured using calipers once every week. The clinical scores, indicators of disease onset, were determined and are shown in Table 1. The sum of the scores for all paws from each mouse was used as the total clinical score.

### Tissue Collection

Collagen-induced RA animals were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) and sacrificed at 5 weeks from the initial immunization. Gastrocnemius muscles from animals in each group were obtained at this time.

**Table 1. Scoring system for subjective evaluation of arthritis severity (Malfait *et al.*, 2001).**

| Severity score | Degree of inflammation |
|---|---|
| 0 | No evidence of erythema and swelling |
| 0.5 | Erythema and mild swelling confined to the tarsals or ankle joint |
| 1 | Erythema and mild swelling extending from the ankle to the tarsals |
| 1.5 | Erythema and moderate swelling extending from the ankle to metatarsal joints |
| 2 | Erythema and severe swelling encompass the ankle, foot & digits, or ankylosis of the limb |

**Table 2. Sequence-specific primers used for RT-PCR**

| Gene | Primer sequence (5'-3') | Size (bp) | GeneBank accession No. |
|---|---|---|---|
| MuRF1 | F: AGG TGA AGG AGG AAC TGA G<br>R: AAC TGC TCT CGG TAC TGG | 86 | NM_080903 |
| MAFbx | F: GCA AAA CAT AAG ACT CAT ACG<br>R: GTA GAG TGG TCT CCA TTC G | 83 | NM_133521 |
| MHC1 β | F: ACA GAG GAA GAC AGG AAG AAC CTA C<br>R: GGG CTT CAC AGG CAT CCT TAG | 288 | K01463 |
| MHC2b | F: AGC CTG CCT CCT TCT TCA TCT GG<br>R: CAC GGT TGC TTT CAC ATA GGA CTC | 229 | DQ872906 |
| Gapdh | F: GTA TGA CTC CAC TCA CGG CAA A<br>R: GGT CTC GCC TCC TGG AGG ATG | 100 | BC094037 |

### Analysis of Gene Expression

Gastrocnemius muscles were homogenized with 1 mL TRI reagent to prepare total RNA. The concentration of total RNA was determined using a spectrophotometer (Optizen 2120UV). Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a Px2 Thermal Cycler HBPX2220 (Thermo Electron Corporation) according to the manufacturer's instructions. The cDNA amplification was carried out as follows: denaturation step, 95℃ for 30 s, annealing step, primer-specific temperature for 1 min; and extension step, 72 ℃ for 1 min. The specific primers used for the PCR reaction are shown in Table 2. PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The expression densities of the amplified bands were quantified with the ImageJ software program (ver. 1.6).

### Statistical Analysis

Data were collected from repeated experiments and are presented as means±SEM. One-way ANOVA and Student's *t*-test were used for statistical analyses. Differences were considered significant at $p<0.05$. All analyses were performed using the SPSS software (ver. 18.0).

## RESULTS

### Changes in Body Weight in Collagen-Induced RA Animals

Body weights of type II collagen-induced RA mice were measured once every week. Compared with control groups, there was a significant increase in body weight in the collagen-treated group at week 1 after primary immunization ($p<0.05$). Body weights of the mice increased continuously until week 10; however, no statistically significant difference was seen at week 10 among the groups (data not shown).

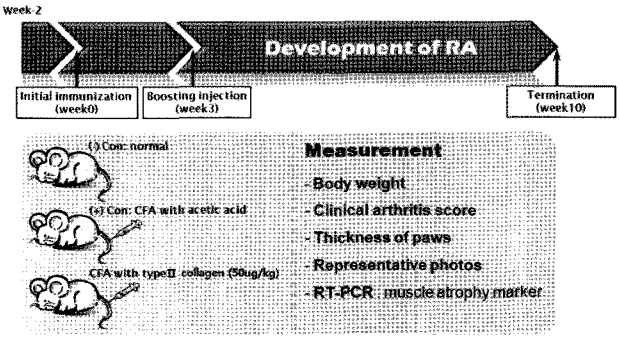



**Fig. 1. Schematic diagram of the experimental design.**

### Disease Development from Initial Immunization

### Clinical Score

The clinical arthritis score, an indicative index of disease onset, was determined by the sum of the values of either swelling or reddening in each animal (data not shown). There was no significant difference among the groups at week 0. A dramatic increase in the score was induced by collagen at week 4 after the initial immunization ($p<0.01$). The positive control group showed a significant reduction at week 7 ($p<0.01$); it was subsequently retained close to the baseline. However, immunization with collagen resulted in a continuous increase until week 10 ($p<0.01$). A statistically significant difference was evident between control groups (positive and/or negative control) and the collagen-induced RA group at weeks 4, 7, and 10 ($p<0.01$).

### Representative Photographs and Paw Thickness

Representative photographs show that immunization with type II collagen induced swelling and redness at weeks 7 and 10 (Fig. 2). However, no change was found in the positive or negative control groups. Com-

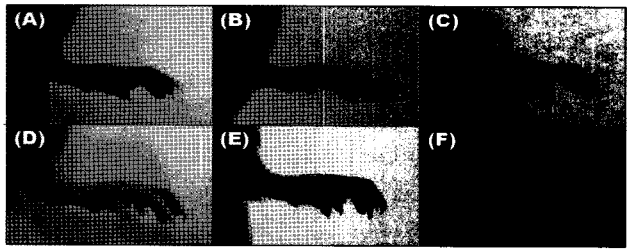



**Fig. 2. Representative photographs of paws with and without RA.** (A-C) Photos at week 7; (D-E) photos at week 10. The paws of (A, D) negative controls, (B, E) positive controls, and (C, F) type II collagen-induced RA mice are shown.

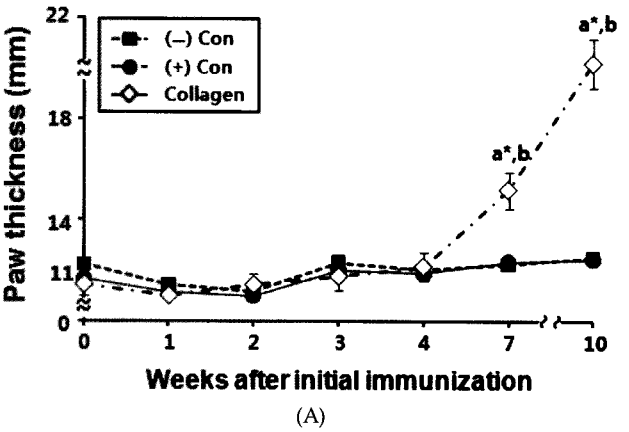



**Fig. 3. Immunization-induced alteration of paw volumes.** Changes in the thickness of (A) forelimb and (B) hindlimb are shown. [a] $p<0.05$ and [a*] $p<0.01$, normal/acetic acid vs. type II collagen at the same time; [b] $p<0.01$, immediately previous time vs. indicated time.

pared with the control groups, a significant increase in the thickness of all paws was induced by collagen at weeks 7 and 10 ($p<0.01$; Fig. 3A, 3B). The forelimbs were thicker in control mice than in collagen-induced RA mice at weeks 7 and 10 ($p<0.01$). Furthermore, thicknesses of the hind limbs were significantly increased at weeks 2 and 3, resulting in a dramatic increase at week 10 ($p<0.01$).

### Altered Expression of Muscle-Specific Atrophy Markers in RA-Induced Mice

To analyze the severity of muscle atrophy at the molecular level, we performed RT-PCR using primers for atrogin (MuRF1/MAFbx). No significant alteration was seen in the expression of MuRF1 among the groups (Fig. 4A). However, the expression of MAFbx changed significantly in a time-dependent manner. At week 1 from the initial immunization, both the positive control and RA-induced groups showed increased expression of MAFbx mRNA ($p<0.01$). MAFbx was transcribed continuously to week 4 in the positive control and collagen-treated groups, while a dramatic reduction was seen at week 10 (Fig. 4B). MAFbx expression was increased significantly at week 4 compared with week 1 ($p<0.01$).

The expression of myosin heavy chain (MHC) iso-

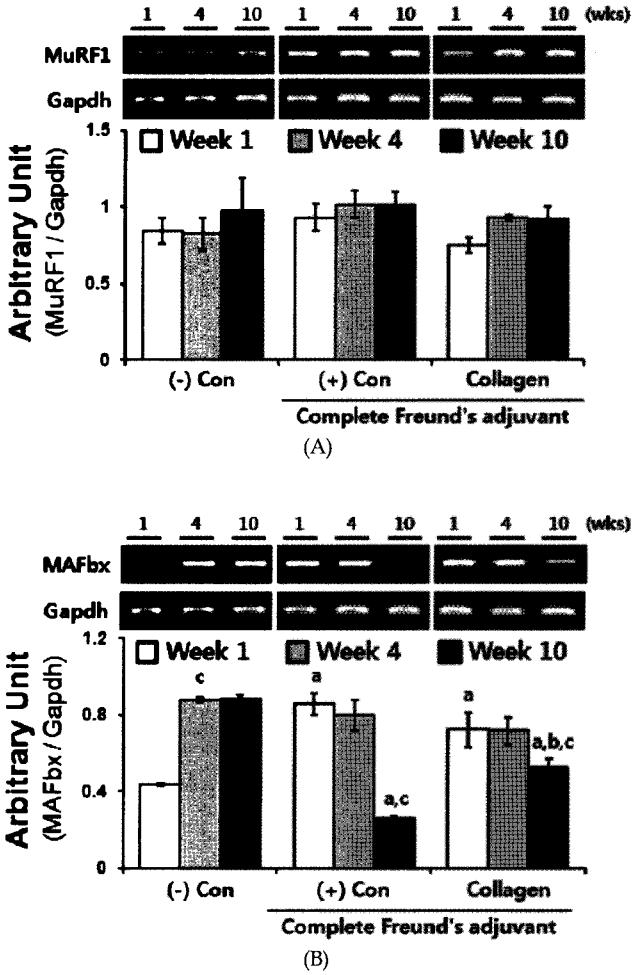
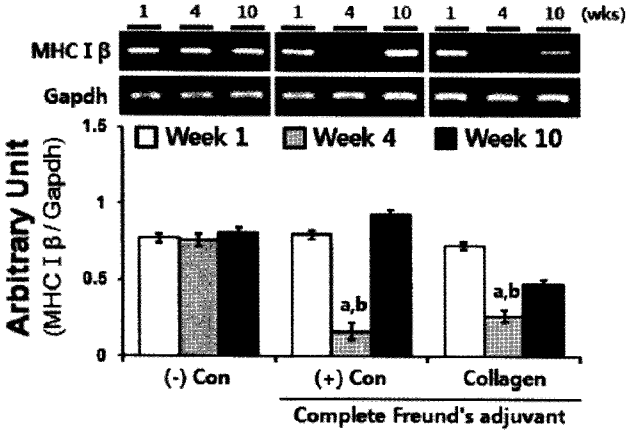
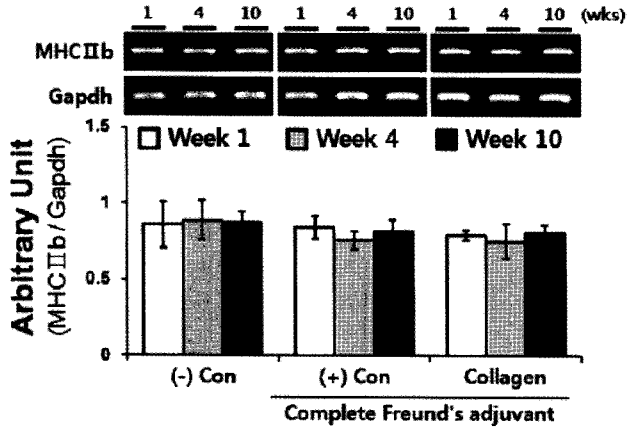



**Fig. 4. Changes in the expressions of muscle-specific atrophy markers in RA mice.** The expression of (A) MuRF1 and (B) MAFbx is shown. [a] $p<0.01$, negative control vs. positive control/collagen-treated group at the same week; [b] $p<0.01$, positive control vs. collagen-treated group at the same week; [c] $p<0.01$, immediately previous time vs. indicated time.




(A)




(B)

**Fig. 5. Changes in the expressions of MHC isoforms in RA mice.** The expression of (A) MHC-Iβ and (B) MHC-IIb is shown. [a] $p<0.01$ negative control vs. positive control/collagen-treated group at the same week; [b] $p<0.01$, immediately previous time vs. indicated time.

forms was also analyzed to identify characteristics of muscle fiber types within the gastrocnemius. MHC-Iβ, which is distributed primarily in slow-twitch muscles, was dramatically decreased in the positive control and collagen-immunized mice at week 4 ($p<0.01$; Fig. 5A). Only the positive control recovered from a decrease in MHC-Iβ at week 10. However, the expression of MHC-Iβ in RA mice was still lower than that of the positive and negative control groups ($p<0.01$). Compared with the negative control group, MHC-IIb, a well-known marker for fast twitch muscles, was not changed in the positive control group. At week 10, a definite reduction was seen in the collagen-treated group (Fig. 5B).

### Different Expression of MHC Isoforms between RA and OA

Finally, we compared the expression of MHC isofo-

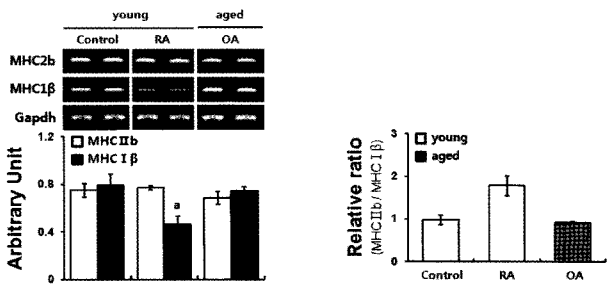



**Fig. 6. Comparison of MHC isoforms between RA- and OA-induced mice.** [a] $p<0.01$ MHC-IIb vs. MHC-Iβ.

rms (Fig. 6). The expression of MH-IIb did not significantly differ between RA and OA, while that of MHC-Iβ was dramatically decreased in collagen-induced RA mice ($p<0.01$). These changes indicated the relative amounts of MHC-IIb within gastrocnemius muscles.

## DISCUSSION

The present study focused on relationships among muscle fiber types, expression of atrophy markers, and clinical signs. We used a mixture emulsifying collagen to induce RA and observed disease development by measuring several parameters: body weight, clinical arthritis score, paw thickness, and expression of atrophy markers.

Various methods have been introduced to induce RA in animals, including zymosan-induced, antigen-induced, and genetic manipulation methods. In particular, advantages of collagen-induced RA models include the sharing of many similarities with human RA (HLA-DR1/HLA-DR4) and usefulness in preclinical development of novel therapeutics (Asquith *et al.*, 2009).

We expected that body weights would be lower in the collagen-induced arthritis group than in the control groups. However, no significant change was seen at week 10. A significant increase in paw thickness was seen at week 7, the timing of which was considered delayed. Previous studies have reported that 100 μg is the most common dose. However, we used 50 μg collagen for immunization; mild symptoms may be caused by the lower dose (Hartog *et al.*, 2009; Vincelette *et al.*, 2007).

In both the acetic acid- and collagen-treated groups, an increase in the clinical arthritis score was seen at week 4. Clinical signs of disease reportedly typically develop on days 21 to 25 and peak at approximately day 35 after the initial immunization (Mauri *et al.*, 1996). In our study, induction of inflammation was similar to previous research. However, the clinical score in the acetic acid-treated group increased and recovered to

**Table 3. Comparison of differences between cachexia and sarcopenia**

| | Cachexia | Sarcopenia |
|---|---|---|
| Main cause | Diseases including RA | Age-related decline of body function |
| Possible mechanisms | - Proinflammatory cytokines<br>- Hypermetabolic state<br>- Starvation | - Neurodegenerative disease<br>- Disability/ inactivity<br>- Age related hormones |
| Characteristics | Almost equal loss in fat and muscle mass | Reduction in muscle protein mass, function and muscle quality |
| Type-dependent changes in muscles | - Mixed atrophy of type I and II muscles (depend on a severity of disease) | - Selective atrophy of type II muscles<br>- Shifting toward type I fiber |
| Intervention | - Resistance exercise<br>- Hormonal changes (e.g. testosterone, IGF)<br>- Nutritional supplementation (high protein meals) | - Pharmacological treatment (e.g. Eicosapentaenoic acid, thalidomide, megesterol) |

baseline. Recently, heat-killed mycobacteria dissolved in CFA induced a transient phenomenon similar to inflammation, with cytokine secretion (Raghavendra *et al.*, 2004).

Skeletal muscle consists of complex cytoskeletal networks (e.g., sarcomeres). The basic contractile protein is composed of filament systems and regulatory proteins. The actin-containing thin filaments are attached in the Z-lines and extend toward the M-line, where they interact with the myosin-containing thick filaments for muscle contraction. An important filament system involves titin. These filaments span half sarcomeres, with their N-termini overlapping in the Z-lines and their C-termini overlapping in the M-lines, thus forming a continuous filament system among adjacent myofibrils (Mues *et al.*, 1998).

Both RA mice and OA mice showed muscle fiber type-dependent atrophy. In the present study, we selected atrophic markers containing MAFbx and MuRF1. Both proteins are well-known ubiquitin E3 ligases, act as regulators of protein turnover in contractile myofilaments, and are upregulated in atrophic conditions (Sassoon and Caiozzo, 2009). MuRF1, which consists of a tripartite RING, B-box, and coiled-coil domain, is needed not for normal muscle growth, but for rapid muscle atrophy. The activity of MuRF1 can be controlled in a titin-dependent manner. On the other hand, atrogin-1/MAFbx participates in MHC-selective degradation. Li *et al.* suggested that atrogin-1/MAFbx-mediated muscle wasting could be induced by TNF-α via the ROS-sensitive kinase p38 MAPK (Li *et al.*, 2005). These results showed a variable change in MAFbx expression compared with that of MuRF1 in the gastrocnemius. RA-induced muscle atrophy is considered to affect muscle composition as opposed to sarcomeric structure. The expression of MHC isoforms induced by RA showed a significant alteration of MHC-Iβ, a marker of slow-twitch muscle, and a relatively slight change in MHC-IIb, a marker of fast-twitch muscle. In RA mice, MHC-Iβ fibers readily respond in the gastrocnemius, reportedly inducing prominent changes (Nordemar *et al.*, 1976). From these results, we suggest that careful intervention is needed in RA patients because of the possibility of fiber type-dependent changes.

Recent studies have suggested that several cytokines, including IL-1, IFNγ, and TNFα, induced cachexia. In the United States, 67% of patients suffer from cachexia with RA. Clinically, it manifests as weight loss caused by severe muscle wasting in the setting of ongoing disease (Table 3). In this study, the decreased expression of MHC-Iβ in both RA and OA was considered a type of cachexia; further study is required to further examine this.

In conclusion, the purposes of this study were to search the literature, focusing on pathological mechanisms for RA and OA at the molecular level, compare differences between RA and OA, and summarize the differences. Our results suggest that alterations in the muscle atrophy genes occurred in RA- and OA-induced mice, resulting in a shift from type II-prominent muscles towards type I-dominant muscles. Based on these results, we suggest that exercise intervention focusing on increasing endurance might be helpful in the treatment of RA patients.

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(Received: 1 June 2011 / Accepted: 20 June 2011)