Evaluation and elucidation of the mechanisms of exercise-induced muscle damage and development of a new muscle damage marker

運動誘発性筋損傷の評価・機序解明と新規筋損傷マーカーの開発

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PREFACE

Pain and weakness are common symptoms of muscle use and muscular diseases. In exercise in which the eccentric contraction is substantial, repeated and unaccustomed, the pain, tenderness, swelling and stiffness develop slowly and are most prominent on the first and several subsequent days after the causative exercise. This is often called “delayed onset muscle soreness (DOMS)”. Muscle biopsies showed no detectable change in the concentrically contracted muscle but in the eccentrically contracted muscle, there were morphological abnormalities both immediately after the exercise and these were more pronounced after 30 hours. These included disorganized myofilaments, Z-line material distributed throughout the sarcomere and loss of Z lines; these changes could either be focal or more widespread. The appearance of intra-muscular enzymes such as creatine kinase in the plasma is an indicator of muscle damage and peaks around 4 days after the exercise. The timing of these changes suggests that the muscle weakness might be a primary consequence of the muscle damage while the pain and other features may be secondary manifestations of acute inflammation triggered by some aspect of the muscle damage. In fact, it is reported that exercise-induced muscle damage attracts leukocytes to the site of muscle damage. Neutrophils infiltrate into damaged skeletal muscle within several hours and remain up to 24 hours, and then macrophages are present from 24 hours to 14 days after exercise. These findings suggest that leucocytes play some roles in the early stages of muscle damage. However, the mechanisms of the cytoskeletal disorganization and loss of myofibrillar proteins are not well known.

A large number of researchers have investigated the relationship between muscle symptoms and biochemical changes in the plasma, however, it has been reported that there are poor correlations between changes in muscle damage markers and the magnitude of muscle symptoms. Furthermore, it is also reported that serum levels of these biomarkers depend on

gender, muscle mass, exercise intensity and duration, and there are remarkable inter-individual variations in the degree to which serum enzyme activities increase with exercise.

Based on the awareness of these issues, we aimed to re-determine the relationships among changes in muscle soreness, blood muscle damage markers, migration activity of leucocytes and inflammatory mediators in blood and urine using a local muscle damage model of one-leg calf-raise exercise, in the first experiment.

Then, we re-evaluated changes in serum leaking enzyme activities and their correlation with DOMS, and also examined concentrations of organ damage markers, pro-inflammatory cytokines and oxidative stress markers, in the second experiment. We also examined urinary excretion rates of these markers as potential surrogate non-invasive indicators of muscle damage.

Finally, we took advantage of proteomic analysis to identify urinary proteins in response to the acute eccentric exercise, and found that an N-terminal fragment of titin molecule appeared in urine after the eccentric exercise, in the third experiment. We then established a quantitative enzyme-linked immunosorbent assay (ELISA) for measurement of the urinary titin fragment, and determined whether the urinary titin fragment can be used as a biomarker for muscle damage.

CONTENTS

Chapter I

Eccentric exercise-induced delayed-onset muscle soreness and changes in markers of muscle damage and inflammation

1-1. INTRODUCTION

Unaccustomed exercise with eccentric muscle contraction and exhaustive exercise cause muscle damage, inflammation, leakage of muscle proteins into the circulation and soreness on and several days after, which is called delayed-onset muscle soreness (DOMS) (14, 21, 24, 25). Although a large number of researchers have investigated the effects of exercise on muscle damage and DOMS in humans, there were various results in inflammatory responses (13, 17, 30), which may depend on exercise mode, intensity, and duration, as well as an individual's sex and age.

Exercise disrupts skeletal muscle ultra structurally, resulting in leucocyte infiltration and release of myocellular proteins such as myoglobin (Mb) into the circulation (3, 24, 28). Systemically, marked neutrophilia with a left shift (6, 23), and enhanced capacity of neutrophils to produce reactive oxygen species (ROS) have been documented after endurance exercise (12, 23). Peripheral leucocyte count has also been shown to increase several hours after eccentric exercise (4). Histological examination of muscle biopsy in humans demonstrates leucocyte accumulation in muscle tissues (10, 29). After muscle and connective tissue damage following exercise, neutrophils are rapidly mobilized into the circulation, and soon migrate and infiltrate into the damaged tissue and produce ROS. If neutrophil functions, especially ROS production are over activated, tissue damage may occur. Within 24 h, neutrophils are replaced by macrophages which are active inflammatory cells that produce several pro-inflammatory cytokines, and promote removal of debris and remodeling of muscle tissue (9, 29). Indeed, several researchers investigated the inflammatory responses to eccentric exercise in humans (15, 19, 32). Peake *et al.* reported that changes in circulating leucocyte count after eccentric exercise are dependent on the muscle groups, or the amount of muscle mass recruited during eccentric exercise (16). Concerning changes in leucocyte receptor expression and oxidative burst activity,

there were no clear effects of different types of eccentric exercise, but they might be due to differences in the type of eccentric exercise (16).

Cytokines are proteins which regulate immune and inflammatory responses. They are classified into pro-inflammatory cytokines which promote inflammation, anti-inflammatory cytokines which inhibit inflammation, immunomodulatory cytokines which control inflammation, multifunctional cytokines, chemokines and colony-stimulating factors. Some of these substances are induced remarkably in plasma and urine following exhaustive endurance exercise (26). Other substances, involved in inflammatory responses, are neutrophil chemotactic factor complement 5a (C5a) and prostaglandin E_2 (PGE_2). Although numerous studies have investigated the effects of eccentric exercise on exercise-induced muscle damage, changes in plasma cytokines and neutrophil activation, there are several contradictory findings, partly due to 1) differences in the mode and intensity of exercise, 2) a limited range of variables measured, and 3) unreliable methods of neutrophil activity measurement.

Migration of neutrophils to the tissue microenvironment is the first step to evoke local inflammation. The production of ROS, not only of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), but also of MPO-dependent HOCl production of neutrophils, can be measured by luminol-dependent chemiluminescence (LmCL) (7, 23, 24, 27). Various neutrophil functions, such as migration and ROS production, could work as a dual-edged sword on both sides of host defence and tissue injury (23, 27). Thus, the balance between beneficial and harmful effects of neutrophil functions should be properly assessed. The use of hydrogel made it possible to mimic the *in vivo* microenvironment of neutrophil infiltration into tissues and LmCL can be detected through the transmissive gel (27). In this method, neutrophil migratory activity and ROS producing activity can be measured without the alteration of neutrophil functions by separating process in the conventional methods (7).

The aims of this study were at first to clarify relationships among muscle soreness, muscle damage markers, circulating leucocyte dynamics and changes in inflammatory mediators in blood and urine of which we tried to detect any possible changes. Especially, we applied a newly-developed measurement system of neutrophil migratory activity and ROS-producing activity by use of *ex vivo* hydrogel methodology with extracellular matrix to the investigation of the mechanisms of muscle damage (27).

1-2. METHODS

Subjects

Nine untrained healthy males participated in this study. Their mean (±SD) characteristics were as follows: age (24.8±1.3 yrs), body mass (62.3±6.3 kg), and height (1.72±0.05 m). At the time of the study, the subjects had not been involved in any hard exercise or resistance training for at least two weeks before the exercise bout, and were not taking any supplements, or participating in recovery strategies such as massage, stretching, or cryotherapy. The subjects were instructed to maintaining their usual daily schedule during the experiment. The study protocol was approved by the ethics committee of Waseda University, Japan, and the subjects provided their informed consent.

Experimental design

Subjects performed a calf-raise exercise, including repetitive eccentric muscle contractions with their right leg on a force plate. The range of motion of the ankle joint during the exercise was regulated from -20° (dorsiflexion position) to 15° (planter flexion position) using a goniometer (SG 110/A, Biometrics, Newport, UK) with its ends attached onto the skin over the tibia and calcaneus. They performed 10 sets of 40 repetitions of exercise at 0.5 Hz by the load

corresponding to half of their body weight, with a rest for 3 min between sets. DOMS was rated using a visual analogue scale (VAS) that had a 100-mm line with “no pain” on one end and “extremely sore” on the other. Blood and urine samples were collected before and 2, 4, 24, 48, 72 and 96 h after the exercise.

Blood and urine sampling and analyses

Approximately 12 ml of blood samples were drawn by a standard venipuncture technique from the antecubital vein. Blood samples were collected into serum separation tubes and vacutainers containing heparin and EDTA. A portion of whole blood was used to measure haemoglobin, haematocrit and complete blood cell counts using an automatic blood cell counter (PocH100i, Sysmex, Kobe, Japan). The serum separation tubes were left to clot at room temperature for 30 min, and the vacutainers containing EDTA for plasma separation were immediately centrifuged at 1000×G for 10 min. Serum and plasma samples were then removed and stored at –80°C for later analysis. Serum concentrations of creatinine (Cr) and Mb were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Urine samples were centrifuged immediately at 1000×G for 10 min to remove sediments, and the supernatants were stored at –80°C for later analyses. Urinary concentrations of Cr were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the plasma and urine concentrations of interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-6, IL-12p70, tumour necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) (R&D Systems; Minneapolis, MN, USA), IL-2, IL-4, IL-8, IL-10, IL-12p40, interferon-γ (IFN-γ), and C5a (Becton Dickinson Biosciences; San Diego, CA, USA), and

calprotectin and myeloperoxidase (HyCult Biotechnology; Uden, the Netherland). ELISA measurements were performed according to the instructions for each ELISA kit using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). Plasma and urine concentrations of PGE_2 (ENZO Life Sciences Inc; Farminglale, NY, USA) were measured using a chemiluminescent microplate reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany).

Preparation of peptide-bound temperature-responsive polymer (G-TRP)

Collagen peptide (24 g; SCP-5000; Nitta Gelatin Co., Osaka, Japan) was dissolved in 96 ml of distilled water at 37°C and followed by reaction with 3.26 g of N-acryloylsuccinimide (Kokusan Kagaku, Tokyo, Japan) for 4 days at 37°C to obtain polymerizable collagen peptide. N-isopropylacrylamide (108.5 g; Kojin, Tokyo, Japan) and n-butylmethacrylate (4.26 g; Wako Chemical, Osaka, Japan) were dissolved in 600 ml of ethanol and then 123 g of the above aqueous solution of polymerizable collagen peptide was added. Under nitrogen atmosphere, 1 ml of N,N,N',N'-tetramethylethylenediamine (Wako Chemical, Osaka, Japan) and 10 ml of 10 wt% ammonium persulfate (Wako Chemical, Osaka, Japan) aqueous solution were added to the mixed solution, and then reacted for 5 h at 4°C, maintaining the nitrogen atmosphere. After the reaction, 30 l of cold (4°C) distilled water were added and the mixture was concentrated to 3 l using an ultrafiltration membrane (molecular weight cut off 100,000) at 4°C. This dilution and concentration process was repeated 5 times in order to remove impurities and low molecular species. Lyophilization and sterilization of the final concentrated solution gave 105 g of peptide-bound temperature-responsive polymer (G-TRP).

Preparation of scaffold-thermoreversible gelation polymer (S-TGP) gel

Under a clean-air laminar hood workbench, 0.5 g of G-TRP and 0.5 g of TGP (Mebiol gel; Mebiol Inc, Kanagawa, Japan) were dissolved in 16.7 ml of Hank's balanced salt solution (HBSS) at 4°C for overnight, yielding a viscous transparent S-TGP gel uniform liquid without any bubbles for use in the experiments (27). Mebiol gel is a pure synthesized biocompatible copolymer composed of thermoresponsive polymer blocks and hydrophilic polymer blocks, characterized by its temperature-dependent dynamic viscoelastic properties and used as a biocompatible scaffold for three-dimensional culture without any toxicity (22). S-TGP gel is a peptide-bound thermoreversible gel formed by mixing G-TRP with the Mebiol gel. It liquefies at low temperature, turns to gel immediately upon warming, and returns to liquid state again when cooled.

Neutrophil functions

Peripheral blood samples were drawn from subjects using 2 ml Na-heparin tubes (Venoject II, Terumo Co, Tokyo, Japan). The blood samples were mixed with 2.5 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Aldrich, MO, USA) at a ratio of 1:1. Then, 150 µl luminol-blood samples were layered on 50 µl S-TGP gel prepared in a tube at 37°C, and was promptly measured by LmCL (relative light unit: RLU) using a luminometer (Gene Light 55; Microtec Co., Ltd, Funabashi, Japan). The samples were incubated at 37°C, and the production of ROS from neutrophils was monitored in a kinetic mode for 60 min. After measurement of LmCL at 60 min, luminol-blood samples were removed and the tubes with 50 µl S-TGP gel in which neutrophils migrated were washed three times with PBS warmed at 37°C. Then, the tubes with gel were cooled on ice, and 50 µl Turk solution (Wako, Osaka, Japan) were added and mixed well. The liquid obtained in this way were set on the C-Chip (Disposable haemocytometer, Neubauer improved, DHC-No.1, Digital Bio, Seoul), and the migratory cell

number was counted under the microscope. Migrated neutrophil number was calculated by 20 times multiplication of the counted cell number.

Statistical analysis

Data were analyzed using two-way analysis of variance. When significant time effects were evident, multiple comparisons were analyzed with Bonferroni adjustment. Associations between data were analyzed with Pearson's correlation coefficient (r). Statistical significance was set at $p<0.05$ and data were presented as means ± standard deviations (SD).

1-3. RESULTS

Delayed-onset muscle soreness and muscle damage markers

Muscle soreness developed on subsequent days after one-leg calf-raise exercise. It increased significantly 48 h and peaked around 72 h after exercise compared with the pre-exercise values ($p<0.01$) (Figure 1). Muscle soreness after exercise decreased at 96 h from 72 h, but remained elevated compared with the pre-exercise values ($p<0.01$) (Figure 1). Concerning blood markers of muscle damage, Mb concentration significantly increased at 72 h after exercise ($p<0.05$) as compared with the pre-exercise values (Figure 2).

Differential leucocyte count

Peripheral leucocyte counts significantly increased at 4 h after exercise, due to the increase in neutrophils ($p<0.01$), and they returned to the pre-exercise values at 24 h after the exercise. Lymphocytes and other leucocytes showed no changes (Figure 3).

Neutrophil functions

Neutrophil migratory activity increased at 4 h after exercise ($p<0.05$) and ROS producing activity showed a trend to significantly increase ($p=0.07$). They both returned to the pre-exercise value at 24 h after exercise (Figure 4).

Inflammatory substances

Plasma and urinary concentrations of inflammatory substances such as pro-inflammatory cytokines, immunomodulatory cytokines, chemokines, anti-inflammatory cytokines, colony-stimulating factors, leucocyte activation markers, neutrophil chemotactic factor C5a and algesic substance PGE_2 showed no significant changes (Table 1).

Relationships between muscle soreness and muscle damage markers

We investigated the correlations between the peak VAS value of muscle soreness, and peak value of muscle damage marker, neutrophil dynamics and inflammatory mediators (all percent changes). There was a positive correlation between the percent changes of VAS at 72 h and Mb concentration at 72 h ($r=0.73$, $p<0.05$) (Figure 5). There was also a positive correlation between the percent changes of migrated neutrophil count at 4 h and Mb concentration at 48 h ($r=0.67$, $p<0.05$) (Figure 6).

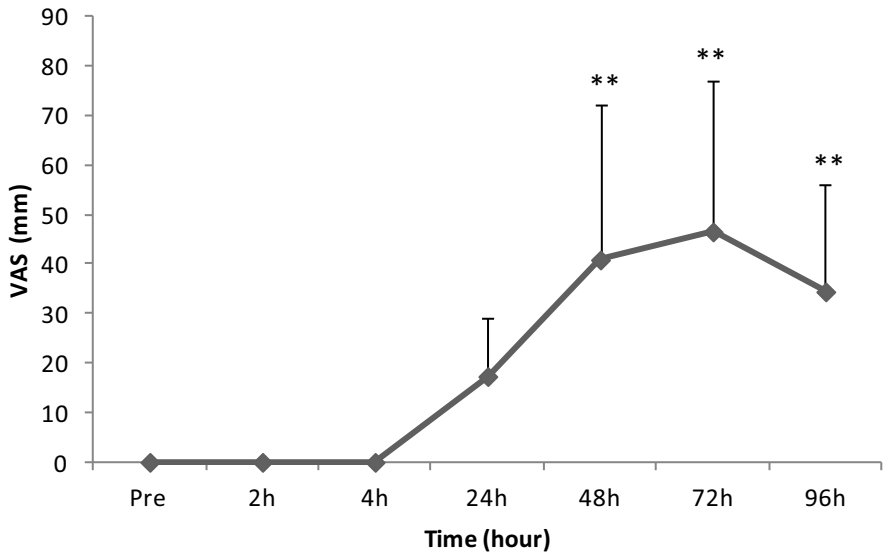

Figure 1. Time course of changes in delayed-onset muscle soreness (DOMS) following the calf-raise exercise as determined by a visual analogue scale (VAS).

Values: means±SD (n=9).

Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment.

**p<0.01: vs Pre.

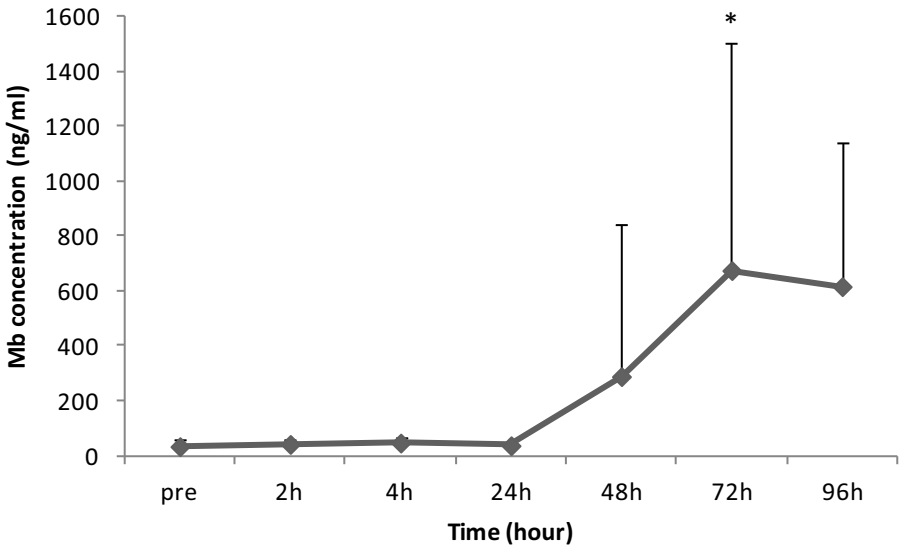

Figure 2. Changes in myoglobin (Mb) concentration following the calf-raise exercise.
Values: means±SD (n=9).
Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment.
*$p<0.05$: vs Pre.

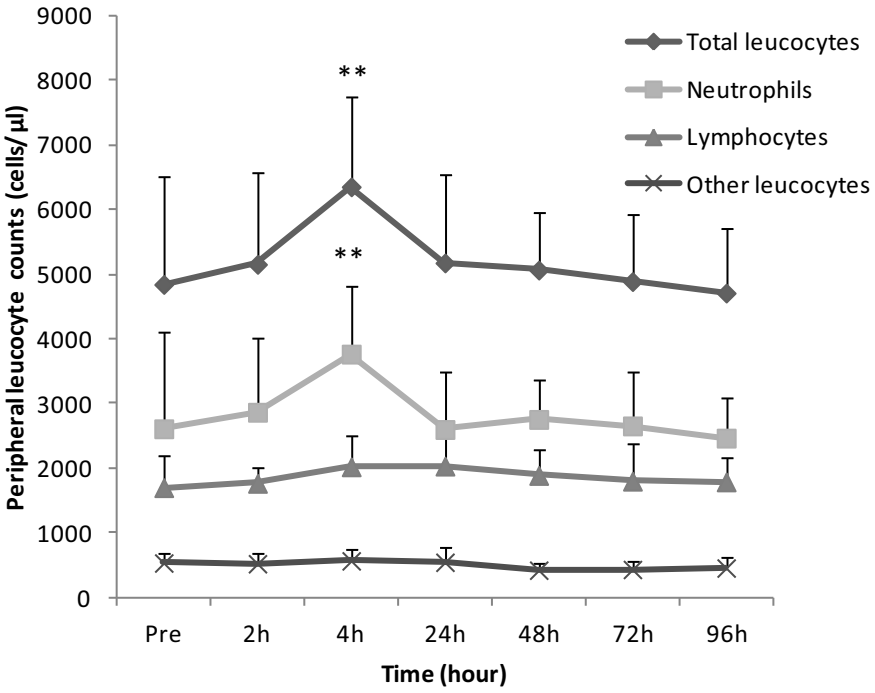

Figure 3. Changes in peripheral leucocyte counts following the calf-raise exercise.
Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment.
**p<0.01: vs Pre.

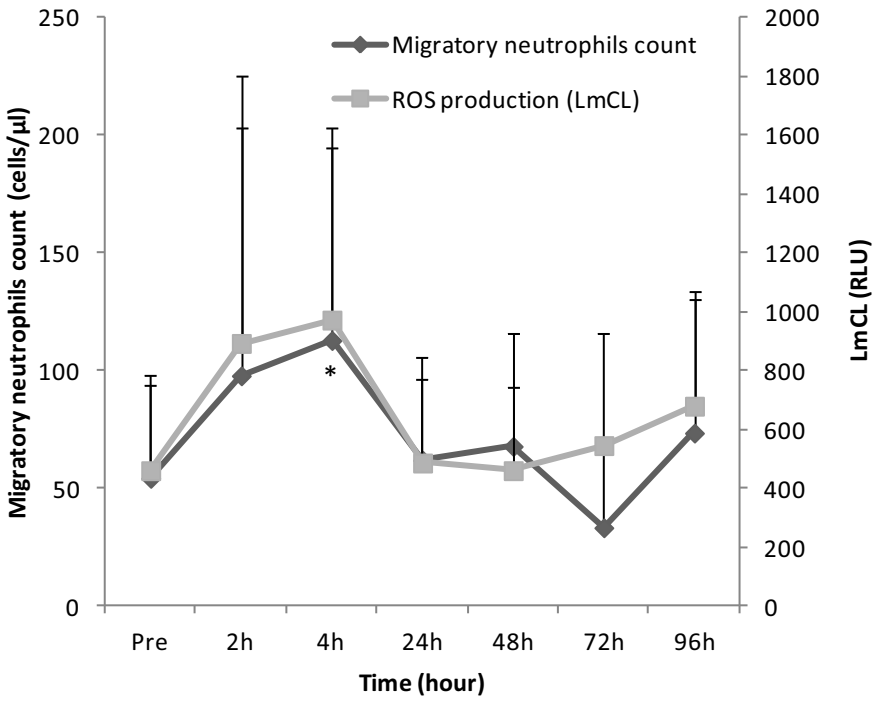

Figure 4. Changes in neutrophil migratory activity and producing activity of reactive oxygen species (ROS) as determined by Luminol-dependent chemiluminescence (LmCL) following the calf-raise exercise.

Values: means±SD (n=9). Statistics: Two-way ANOVA. Post-hoc test: Bonferroni adjustment.

LmCL p=0.07: vs Pre, migratory neutrophil counts *p<0.05: vs Pre.

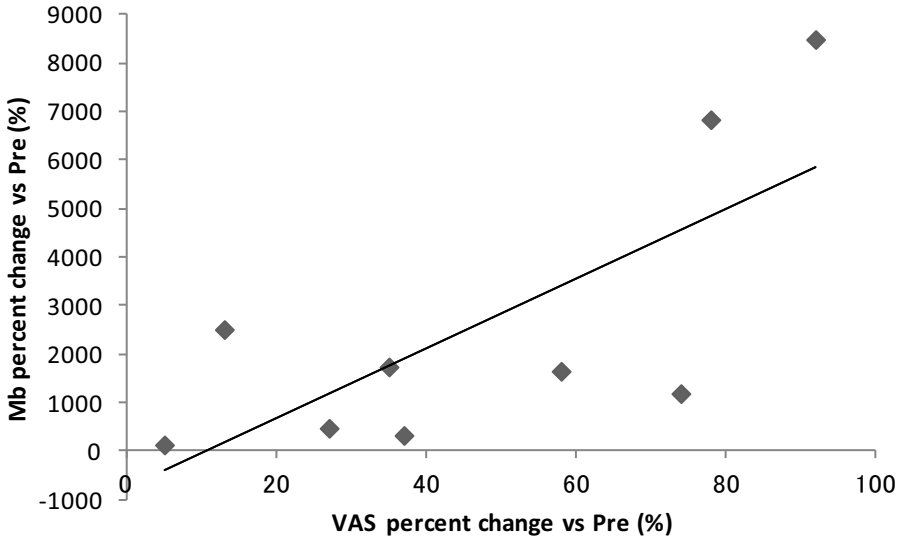

Figure 5. Associations between muscle soreness as determined by visual analogue scale (VAS) at 72 h and value of myoglobin (Mb) at 72 h.

Values: percent changes of peak values vs Pre (n=9). Statistics: Pearson's correlation coefficient.

VAS (Pre-72 h) vs Mb (Pre-72 h): $r=0.73$, $p<0.05$

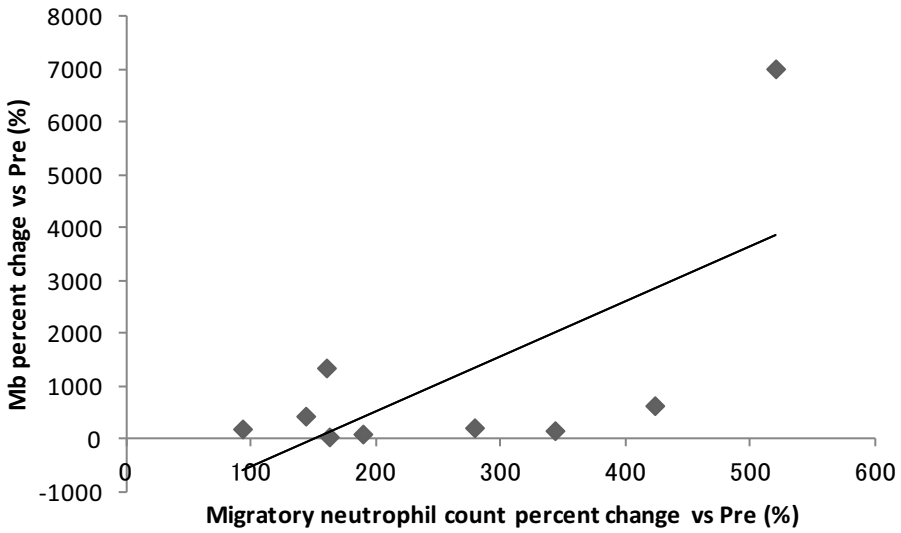

Figure 6. Associations between migratory neutrophil count at 4 h and myoglobin concentration at 48 h.

Values: percent changes vs Pre (n=9). Statistics: Pearson's correlation coefficient.

migratory nuetrophil count (Pre-4 h) vs Mb (Pre-48 h) : $r=0.67$, $p<0.05$

Table 1. Changes in plasma and urinary inflammatory substances following the calf-raise exercise.

	Unit	Pre	2h	4h	24h	48h	72h	96h
pro-inflammatory cytokines								
IL-1β-P	pg/ml	0.68 ± 0.11	0.57 ± 0.50	0.57 ± 0.39	0.72 ± 0.41	0.61 ± 0.35	0.61 ± 0.38	0.57 ± 0.38
IL-1β-U	pg/min	0.10 ± 0.09	0.58 ± 0.79	0.15 ± 0.12	0.30 ± 0.35	0.13 ± 0.07	0.13 ± 0.12	0.12 ± 0.04
TNF-α-P	pg/ml	0.46 ± 0.23	0.53 ± 0.26	0.64 ± 0.42	0.75 ± 0.35	0.44 ± 0.36	0.53 ± 0.12	0.51 ± 0.20
TNF-α-U	pg/min	0.77 ± 0.84	0.84 ± 1.05	1.33 ± 1.86	0.61 ± 0.69	1.04 ± 1.76	0.47 ± 0.67	0.27 ± 0.28
immunomodulatory cytokines								
IL-2-P	pg/ml	0.93 ± 0.17	0.83 ± 0.08	0.82 ± 0.07	0.78 ± 0.05	0.90 ± 0.30	0.84 ± 0.20	0.88 ± 0.20
IL-2-U	pg/min	0.79 ± 0.84	0.74 ± 0.43	0.40 ± 0.19	0.51 ± 0.18	0.67 ± 0.48	0.54 ± 0.57	0.30 ± 0.08
IL-12p70-P	pg/ml	0.59 ± 0.32	0.28 ± 0.27	0.59 ± 0.35	0.72 ± 0.45	0.80 ± 0.41	0.50 ± 0.23	0.55 ± 0.39
IL-12p70-U	pg/min	0.33 ± 0.24	0.34 ± 0.21	0.22 ± 0.09	0.31 ± 0.14	0.33 ± 0.23	0.56 ± 0.76	0.15 ± 0.07
IFN-γ-P	pg/ml	0.59 ± 0.16	0.60 ± 0.30	0.65 ± 0.32	0.63 ± 0.16	0.52 ± 0.16	0.54 ± 0.12	0.58 ± 0.17
IFN-γ-U	pg/min	0.79 ± 1.01	0.81 ± 0.65	0.30 ± 0.09	0.45 ± 0.16	0.42 ± 0.21	0.14 ± 0.08	0.23 ± 0.07
multifunctional cytokines								
IL-6-P	pg/ml	0.27 ± 0.16	0.38 ± 0.19	0.29 ± 0.18	0.22 ± 0.12	0.26 ± 0.12	0.30 ± 0.11	0.39 ± 0.23
IL-6-U	pg/min	0.14 ± 0.13	0.32 ± 0.15	0.15 ± 0.11	0.18 ± 0.10	0.16 ± 0.08	0.16 ± 0.14	0.29 ± 0.35
anti-inflammatory cytokines								
IL-1ra-P	pg/ml	35.6 ± 5.2	33.1 ± 5.5	34.4 ± 4.8	33.1 ± 8.4	29.3 ± 5.2	29.6 ± 2.6	32.3 ± 5.2
IL-1ra-U	pg/min	231.8 ± 173.2	401.2 ± 233.7	327.5 ± 230.7	837.6 ± 97.1	354.9 ± 192.6	204.0 ± 20.2	278.2 ± 155.0
IL-4-P	pg/ml	0.66 ± 0.35	0.70 ± 0.49	0.88 ± 0.56	1.00 ± 0.98	0.78 ± 0.66	0.76 ± 0.50	0.59 ± 0.31
IL-4-U	pg/min	0.60 ± 0.46	1.12 ± 0.72	0.95 ± 0.60	0.84 ± 0.31	0.65 ± 0.32	0.70 ± 0.69	0.77 ± 0.40
IL-10-P	pg/ml	0.71 ± 0.45	0.75 ± 0.52	0.50 ± 0.29	0.45 ± 0.21	0.57 ± 0.23	0.47 ± 0.31	0.66 ± 0.68
IL-10-U	pg/min	0.40 ± 0.44	0.56 ± 0.55	0.48 ± 0.50	0.56 ± 0.40	0.84 ± 1.11	0.46 ± 0.58	0.44 ± 0.42
IL-12p40-P	pg/ml	10.7 ± 17.8	13.2 ± 24.4	13.2 ± 24.7	10.7 ± 18.1	8.8 ± 13.7	7.7 ± 11.6	8.5 ± 13.2
IL-12p40-U	pg/min	0.81 ± 0.91	0.82 ± 0.64	0.56 ± 0.51	0.76 ± 0.83	1.06 ± 1.19	0.45 ± 0.48	0.33 ± 0.21
chemokines								
IL-8-P	pg/ml	4.5 ± 2.7	4.2 ± 2.4	4.4 ± 1.5	3.7 ± 0.9	3.7 ± 0.9	5.3 ± 4.3	4.3 ± 1.6
IL-8-U	pg/min	0.59 ± 0.42	1.20 ± 0.54	0.76 ± 0.43	1.3 ± 1.5	0.87 ± 0.69	0.65 ± 0.45	0.95 ± 0.80
MCP-1-P	pg/ml	95.4 ± 19.0	90.5 ± 13.8	94.3 ± 19.4	101.5 ± 19.1	89.8 ± 13.1	102.3 ± 13.2	96.8 ± 10.6
MCP-1-U	pg/min	87.5 ± 82.6	258.5 ± 197.9	199.4 ± 122.4	235.3 ± 146.3	189.8 ± 146.2	76.1 ± 29.2	128.3 ± 47.3
colony-stimulating factors								
G-CSF-P	pg/ml	1.8 ± 0.9	2.2 ± 1.4	2.0 ± 1.4	1.9 ± 1.0	2.3 ± 1.7	1.9 ± 1.3	2.0 ± 1.4
G-CSF-U	pg/min	0.19 ± 0.12	0.24 ± 0.13	0.27 ± 0.17	0.27 ± 0.17	0.48 ± 0.54	0.24 ± 0.24	0.16 ± 0.05
M-CSF-P	pg/ml	19.3 ± 2.5	23.1 ± 7.8	22.6 ± 5.4	21.8 ± 7.6	22.5 ± 6.3	17.0 ± 6.2	20.1 ± 3.0
M-CSF-U	pg/min	25.7 ± 23.1	130.8 ± 108.9	121.8 ± 84.9	281.4 ± 310.7	230.6 ± 298.1	40.4 ± 29.1	218.6 ± 280.9
GM-CSF-P	pg/ml	0.27 ± 0.10	0.21 ± 0.37	0.20 ± 0.06	0.28 ± 0.13	0.24 ± 0.09	0.17 ± 0.02	0.22 ± 0.09
GM-CSF-U	pg/min	0.41 ± 0.40	0.66 ± 0.61	0.54 ± 0.38	0.42 ± 0.29	0.69 ± 0.48	0.48 ± 0.50	0.41 ± 0.29
leucocyte activation markers								
calprotectin-P	ng/ml	15.7 ± 12.9	9.9 ± 6.8	10.5 ± 9.7	9.2 ± 7.0	11.7 ± 8.0	12.1 ± 6.3	12.3 ± 8.9
calprotectin-U	ng/min	0.15 ± 0.13	0.33 ± 0.19	0.27 ± 0.23	0.57 ± 0.68	0.45 ± 0.42	0.24 ± 0.23	0.20 ± 0.14
MPO-P	ng/ml	12.7 ± 7.8	12.6 ± 6.9	15.2 ± 9.8	13.2 ± 10.0	13.7 ± 8.1	12.2 ± 7.7	12.2 ± 5.9
MPO-U	ng/min	0.06 ± 0.05	0.10 ± 0.08	0.08 ± 0.06	0.10 ± 0.09	0.12 ± 0.13	0.08 ± 0.08	0.08 ± 0.07
neutrophil chemotactic factors								
C5a-P	ng/ml	3.8 ± 4.3	3.6 ± 3.6	3.2 ± 3.2	3.3 ± 3.5	3.4 ± 3.8	3.5 ± 4.0	3.5 ± 3.8
C5a-U	ng/min	0.03 ± 0.01	0.06 ± 0.02	0.09 ± 0.11	0.09 ± 0.11	0.08 ± 0.10	0.04 ± 0.02	0.06 ± 0.03
algesic substances								
PGE_2-P	pg/ml	64.1 ± 11.7	69.7 ± 12.6	79.0 ± 15.8	60.4 ± 18.0	58.9 ± 24.4	59.9 ± 7.7	58.9 ± 16.9
PGE_2-U	pg/min	1075 ± 886.4	1271 ± 732.9	1372 ± 1044	1354 ± 906.9	991.0 ± 643.2	758.3 ± 630.8	777.1 ± 535.1

Values: means±SD. Statistics: Two-way ANOVA was not significant. P: Plasma, U: Urine. Data are the gross amount in the volume of urinary excretion per one minute.

Abbreviations: IL: interleukin, TNF: tumour necrosis factor, IFN: interferon, IL-1ra: IL- 1 receptor antagonist, MPO: myeloperoxidase, MCP-1: monocyte chemotactic protein-1, G-CSF: granulocyte colony-stimulating factor, GM-CSF: granulocyte macrophage colony-stimulating factor, M-CSF: macrophage colony-stimulating factor, PGE_2: prostaglandin E_2.

1-4. DISCUSSION

The aims of this study were at first to investigate the associations among DOMS caused by calf-raise exercise, muscle damage marker in peripheral blood, and inflammatory mediators. Muscle soreness appeared at 24 h after exercise and subsequently developed from 48 h to 72 h ($p<0.01$), and decreased at 96 h though still high above compared with pre-exercise ($p<0.01$). Generally, DOMS appears from several hours or one day after exercise and peaks at two or three days after exercise (1, 14, 16). This study confirmed that calf-raise exercise with eccentric contractions caused DOMS. As a marker of muscle damage, serum Mb concentration significantly increased at 72 h after exercise compared with pre-exercise. Numerous studies have reported on muscle damage and muscle soreness, however, they did not always occur simultaneously according to the degree of muscle damage and muscle soreness (4, 5, 13, 14). In this study, we observed the similar time course and Mb was closely correlated with muscle soreness at 72 h after exercise ($r=0.73$, $p<0.05$). These results confirm that the present calf-raise exercise caused sufficient muscle damage for the purpose of the present study.

Several studies report that circulating leucocyte and neutrophil count increase within several hours after eccentric exercise, but that it dependent on the intensity, duration, and type of exercise and muscle mass (16, 20). Saxton *et al.* reported that circulating neutrophil count increased 1.48 fold (Pre:2.9±0.4, 4h:4.3±0.5) at 4 h after low systemic stress (repeated eccentric muscle action) and caused 1.76 fold (Pre:2.9±0.3, 4h:5.1±0.4) increase in neutrophils at 4 h after high systemic stress (bench-stepping) (20). Gleeson *et al*. reported the circulating leucocyte number decreased two and three days after a bench-stepping exercise, although it increased immediately after and still more increased at 1 to 4 h (5). We demonstrated that total leucocyte count increased by 31 % ($p<0.01$) and circulating neutrophil count by 44 % ($p<0.05$) above pre-exercise level, respectively. Neutrophilia returned to pre-exercise values at 24 h after

exercise. Neutrophil migratory activity was also significantly increased ($p<0.05$) at 4 h after exercise. There have been several studies that investigated the neutrophil migration into the muscle after the muscle-damaging exercise (0-6 h) (2, 3, 8, 9, 11, 33), but only Fielding *et al.* demonstrated neutrophil accumulation in the muscle (3). This could be partly attributable to the fact that biopsy samples can depict quite limited parts of the muscle tissue. Paulsen *et al.* investigated leucocyte accumulation into the exercised-muscle using radiolabeled leucocytes by scintigraphy (15). Although they measured high radioactivity in the exercised-muscle, large individual differences were observed. We demonstrated a positive correlation between the increase in neutrophil migratory activity at 4 h and the increase in Mb concentration at 48 h ($r=0.67$, $p<0.05$). This result shows the possibility that neutrophil is involved in the muscle damage and the inflammatory processes.

ROS production from neutrophils migrated into the hydrogel likened to muscle tissue was assessed with LmCL. There was a trend for this to be increased ($p=0.07$) at 4 h after exercise. Pizza *et al.* reported that neutrophil O_2^- production was increased ($p<0.05$) at 4 h following one-arm eccentric exercise (18). Suzuki *et al*. also observed increased neutrophil count with a left shift, enhanced spontaneous mobility and ROS production after 90-min bicycling, suggesting that mobilized neutrophils from the bone marrow reserve have higher activity (23). They also reported that the increases in Mb values at post 3 h were correlated closely with the raise in neutrophil count at post and LmCL response at post and 1 h post 90-min bicycling, suggesting that neutrophil mobilization and activation might affect muscle damage (24). The result in this study was similar, but we could demonstrate the enhanced neutrophil migration by use of newly-developed *ex vivo* methodology in imitation of tissue damage.

Although DOMS, muscle damage marker, circulating neutrophil counts and their functions changed significantly following the eccentric exercise, plasma and urinary

inflammatory mediators which we investigated were not changed significantly. Uchida *et al.* reported increasing serum PGE_2 following bench press exercise despite no changes in plasma IL-1β, IL-6 or TNF-α (30). Although we also investigated plasma and urinary PGE_2 concentrations, they did not change. Serum PGE_2 may be produced by leucocytes during the time left to removal of the fibrin clot and blood cells where neutrophilia occurs after exercise that might affect the higher values. These results demonstrated that the one-leg calf-raise exercise caused local inflammation and that neutrophil mobilization and migration were the most affected variables among the wide range of tested inflammatory mediators. However, further research is needed to clarify the mediators that mobilize neutrophils into the circulation and substances which are produced by migrated neutrophils and promote inflammation.

In conclusion, one-leg calf-raise exercise caused DOMS, muscle damage, increases in circulating neutrophil number and migratory activity without changes in the other inflammatory mediators. Neutrophils may be involved in the early stage of muscle damage and the inflammatory processes after eccentric exercise.

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Chapter II

Evaluation of serum leaking enzymes and investigation into new biomarkers for exercise-induced muscle damage

2-1. INTRODUCTION

Exercise-induced muscle damage has been one of the most important targets in sport science research. Direct assessment of muscle damage involves histological examination of muscle tissue via biopsy. However, there is difficulty gathering tissue samples with biopsy technique and demonstrating disruption of muscle cells (1, 3, 48), in combination with the process being an invasive experience for research participants. Therefore, the less invasive measures for the accurate assessment of exercise-induced muscle damage are needed. As indirect indicator of muscle damage, delayed-onset muscle soreness (DOMS) is a poor reflector of eccentric exercise-induced muscle damage, and changes in other indicators of muscle damage are not necessarily accompanied by DOMS (27). On the other hand, the changes of range of motion (ROM) have been used as an indirect variable of DOMS (8, 9). Although a large number of studies have reported the effects of exercise on muscle damage, DOMS and inflammatory responses in humans (26, 30, 31, 34, 41, 44, 46), the mechanisms of exercise-induced muscle damage are not fully understood at present. DOMS is characterized by tenderness and movement-related pain, that is, mechanical hyperalgesia in the exercised muscle (22), and ROM might be affected by muscle soreness (33). Therefore, it is necessary to assess the muscle symptoms in a multifaceted manner, and investigate the associations with other muscle damage markers carefully.

Serum enzyme activities such as creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aldolase (ALD) and myoglobin which leak into circulation from damaged muscle have been used as indirect markers of exercised-induced muscle damage (5, 28). Serum levels of these damage indicators depend on gender, muscle mass, exercise intensity and duration in addition to the individual training state, and there is a remarkable interindividual variability in the degree to which serum enzyme

activities increase with exercise (30). Instead of the existing leaking enzymes, fatty acid-binding protein (FABP) and neutrophil gelatinase-associated lipocalin (NGAL) have been introduced as organ damage markers. FABPs are present in almost all tissues, and were named after the tissue in which they were discovered or are prominently expressed: L-FABP, liver-type fatty acid-binding protein; I-FABP, intestinal-type fatty acid-binding protein; H-FABP, heart-type fatty acid-binding protein (50). H-FABP is mainly expressed in the heart, but to a lesser extent also in the skeletal muscle (47). NGAL was identified as a 25 kDa protein secreted by neutrophils (45), and is expressed in human tissues, including kidneys, lungs, stomach, and colon (10). NGAL is focused in recent years as a biomarker in several benign and malignant diseases, especially as a biomarker in acute kidney injuries (11), and may have the potential to protect against cellular injury mediated by reactive oxygen species (ROS) (36, 37). Although we have observed that neutrophils produce ROS following exercise (14, 42-44), NGAL might become a good alternative variable which is easier to measure simply in plasma and urine by conventional enzyme-linked immunosorbent assays (ELISA) than the existing neutrophil functional analyses that contain rather complex procedures and need to be determined *ex vivo* soon after blood sampling.

Exercise-induced muscle damage causes local inflammation which degenerates and regenerates muscle and surrounding connective tissue (30). Briefly, neutrophils are mobilized into the circulation after exercise, and soon infiltrate into the damaged tissue (2). Neutrophils are primed by the chemoattractants such as complement 5a (C5a) and interleukin (IL)-8 from the exercise-induced damaged muscle, causing rolling, arrest and transmigration which is mediated by intercellular adhesion molecules, selectins (16), initially. Neutrophils are present in muscle within a day after exercise (3, 17, 18, 19, 34, 40), and after neutrophils' infiltration, macrophages are replaced and present in muscle from 1 to 14 days after exercise (2, 3, 12, 13,

19, 32, 38). Neutrophils and macrophages produce ROS to degrade the damaged muscle tissue (23, 24), and may produce pro-inflammatory cytokines (6). The pro-inflammatory cytokines such as IL-1β, tumor necrosis factor (TNF)-α, and IL-6 are expressed in skeletal muscle following eccentric exercise (30), but we could not detect any changes of these cytokines in plasma and urine in our previous study (14). On the other hand, Sugama et al. reported that IL-17 induced by IL-6 and activated by IL-23 might promote neutrophil activation and muscle damage in a different way from the classical pro-inflammatory cytokines IL-1β and TNF-α following prolonged endurance exercise (41). Furthermore, nerve growth factor (NGF) which is involved in pathological pain conditions (15, 49) increased in the muscle after lengthening contraction in rats (22). 8-isoprostane is a prototypical biomarker of oxidative stress by exercise-induced free radical production (21). Fluctuations of these substances may be detected following exercise-induced muscle damage and inflammation, and can be one of the underlying mechanisms of it.

We have shown in a previous study that increased myoglobin concentration at 72 h after exercise was correlated with the appearance of DOMS at 72 h ($r=0.73$, $p<0.05$), and there was a positive correlation between exercise-induced increases in neutrophil migratory activity at 4 h and increases in myoglobin at 48 h ($r=0.67$, $p<0.05$) following one-leg calf-raise exercise (14), suggesting that neutrophils may be involved in muscle damage and that myoglobin is a very sensitive muscle damage marker. Based on these findings, the aims of the present study were to examine 1) changes in serum leaking enzyme activities and their correlation with DOMS, 2) concentrations of organ damage markers, novel pro-inflammatory cytokines and an oxidative stress marker and their relationship to muscle damage, and 3) urinary excretion rates of these markers as potential surrogate non-invasive indicators of muscle damage, using a local muscle damage model of one-leg calf-raise exercise.

2-2. METHODS

Subjects

Nine untrained healthy males (age 24.8 ± 1.3 (mean ± SD) yrs, body mass 62.3 ± 6.3 kg and height 1.72 ± 0.05 m) volunteered for this study. At the time of the study, the subjects had not been involved in any hard exercise or resistance training for at least two weeks before the exercise bout, and were not taking any supplements, or participating in recovery strategies such as massage, stretching or cryotherapy. The subjects were instructed to maintain their usual daily schedule during the experiment. All subjects completed a medical questionnaire and gave written informed consent. The experimental procedure was approved by the ethics committee of Waseda University.

Experimental design

Subjects performed a calf-raise exercise, including repetitive eccentric muscle contractions with their right leg on a force plate. The range of motion of the ankle joint during the exercise was regulated from -20° (dorsiflexion position) to 15° (plantar flexion position) using a goniometer (SG 110/A, Biometrics, Newport, UK) with its ends attached onto the skin over the tibia and calcaneus. They performed 10 sets of 40 repetitions of exercise at 0.5 Hz with a load corresponding to the half of their body weight, with 3 min rest between sets. The tenderness of the exercised muscle correlative to DOMS was assessed using the FP meter (SN-402, Navis, Japan) at 1 kg and rated subjectively using a visual analogue scale (VAS) that has a 100-mm line with “no pain” on one end and “extremely sore” on the other. The points of measurements were the proximal, the middle and the distal points of medial gastrocnemius (MG) and lateral gastrocnemius (LG), the middle points between MG and LG, and the middle and the distal

points of soleus (SOL). The ankle active ROM was assessed using the goniometer. The tenderness of the exercised muscle and the ankle active ROM were assessed before, immediately after and 24 h, 48 h, 72 h, 96 h and 168 h after exercise. The blood and urine samples were collected before and 2 h, 4 h, 24 h, 48 h, 72 h and 96 h after the exercise. Participants were supposed to urinate 2 h before each sampling, and the urine samples were collected in measuring cylinders.

Blood and urine sampling and analyses

Approximately 12 ml of blood was drawn by standard venipuncture technique from the antecubital vein. Blood samples were collected into serum separation tubes and vacutainers containing heparin and EDTA. A portion of whole blood was used to measure haemoglobin, haematocrit and complete blood cell counts using an automatic blood cell counter (PocH100i, Sysmex, Kobe, Japan). The serum separation tubes were left to clot at room temperature for 30 min, and the vacutainers containing EDTA for plasma separation were immediately centrifuged at 1000×G for 10 min. Serum and plasma samples were then removed and stored at –80°C for later analyses. Serum CK, AST, ALT, LDH and ALD activities were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Urine samples were centrifuged immediately at 1000×G for 10 min to remove sediments, and the supernatants were stored at –80°C for later analyses. Urinary concentration of creatinine was measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure the plasma and urine concentrations of H-FABP, I-FABP, L-FABP and NGAL (Human H-FABP, I-FABP, L-FABP and NGAL ELISA kit, Hycult biotechnology, Uden, The Netherlands), IL-17A (Human IL-17A ELISA kit, Gen-Probe Diaclone SAS, Besancon, France), IL-23 (Human IL-23, R&D Systems, Inc. MN, USA), nerve

growth factor (NGF) (Human Nerve Growth Factor ELISA kit, Cusabio Biotech Co. Ltd., Wuhan, China), E-selectin (Soluble), sL-selectin and sP-selectin (Life technologies Co. CA, USA) and 8-isoprostane (Detroit R&D, Inc., MI, USA). The measurements were performed according to the instructions for each ELISA kit using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). The urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Statistical analysis

Data are presented as mean ± SD. Statistical validation was made using Friedman's test. When significant time effects were evident, multiple comparisons were analyzed with Scheffe test. Associations between data were analyzed with Pearson's correlation coefficient (r). Statistical significance was accepted at $p<0.05$.

2-3. RESULTS

Changes in the tenderness of the exercised muscle, and the ankle joint active range of motion (ROM)

As shown in Table 1, tenderness of the exercised muscle developed on subsequent days after calf-raise exercise and was principally sensed on the MG. It increased significantly 72 h (proximal MG; $p<0.05$, middle MG; $p<0.01$) after exercise compared with the pre-exercise values. The ankle joint active ROM in the dorsal flexion decreased significantly at 48 h ($p<0.05$) and 72 h ($p<0.01$). The lowest ROM was shown at 72 h after exercise in dorsal flexion, whereas there was no significant difference in plantar flexion compared with the pre-exercise values.

Changes in the muscle and the organ damage markers

As shown in Table 2, CK and ALD activities significantly increased at 72 h after exercise ($p<0.05$), thereafter, they remained elevated for 96 h after exercise ($p<0.01$). LDH activity significantly increased at 96 h after exercise ($p<0.01$). However, AST and ALT activities showed no significant difference when compared with pre-exercise values. As listed in Table 3, plasma concentrations of H-FABP, I-FABP and L-FABP were below the detectable levels of the assays and urinary concentrations exhibited no significant change. Plasma and urinary NGAL were not significantly different compared with pre-exercise values.

Changes in the pro-inflammatory cytokines and other indicators

As shown in Table 4, plasma concentration of IL-17A was not significantly changed and urinary excretion rate of IL-17A was below the detectable level. Plasma concentration of IL-23 was below the detectable level, whereas urinary excretion rate of IL-23 showed no significant change. Plasma sE-selectin concentration was below the detectable level, and sL-selectin and sP-selectin concentrations were not significantly different. Plasma concentration of 8-isoprostane was below the detectable level, and urinary excretion rate of 8-isoprostane and plasma concentration of NGF did not change significantly.

Relationships between enzyme activities and the tenderness of exercised muscle and the ankle active range of motions (ROM)

As shown in Table 5, there were no significant correlations between the tenderness of proximal MG 72 h and CK, LDH and ALD for the percent changes of the peak values. The tenderness of middle MG 72 h was significantly correlated with ALD 72 h ($r=0.78$, $p<0.05$). The dorsal

flexion of ankle active ROM was not significantly correlated to the tenderness of MG or to CK, LDH and ALD.

Table 1. Changes in the tenderness of exercised muscle and active range of motion (ROM).

	unit	Pre	0 h	24 h	48 h	72 h	96 h	168 h
medial gastrocnemius								
proximal	mm	16 ± 9	21 ± 10	35 ± 20	40 ± 31	54 ± 24*	36 ± 18	20 ± 15
middle	mm	19 ± 10	23 ± 12	34 ± 18	48 ± 24	64 ± 22**	49 ± 18	23 ± 14
distal	mm	20 ± 11	19 ± 14	30 ± 14	45 ± 23	49 ± 31	40 ± 19	19 ± 12
medial/lateral gastrocnemius								
proximal	mm	15 ± 15	14 ± 12	26 ± 16	24 ± 18	25 ± 23	19 ± 15	11 ± 14
middle	mm	16 ± 13	15 ± 12	18 ± 10	25 ± 24	26 ± 28	16 ± 15	11 ± 11
distal	mm	15 ± 9	16 ± 11	23 ± 15	21 ± 15	24 ± 18	18 ± 15	15 ± 14
lateral gastrocnemius								
proximal	mm	11 ± 12	8 ± 10	15 ± 13	14 ± 11	26 ± 24	18 ± 15	8 ± 12
middle	mm	14 ± 12	15 ± 12	18 ± 13	26 ± 18	39 ± 29	26 ± 19	10 ± 13
distal	mm	13 ± 10	10 ± 11	15 ± 12	19 ± 16	21 ± 17	16 ± 14	6 ± 11
soleus								
middle	mm	21 ± 14	18 ± 13	24 ± 15	18 ± 10	21 ± 14	14 ± 12	14 ± 15
distal	mm	7 ± 8	10 ± 10	11 ± 11	7 ± 11	9 ± 12	10 ± 14	6 ± 8
ROM of ankle joint								
plantar flexion	degree of an angle	33 ± 4	34 ± 4	32 ± 3	32 ± 10	32 ± 5	32 ± 6	33 ± 4
dorsal flexion	degree of an angle	-25 ± 5	-20 ± 5	-20 ± 5	-15 ± 4*	-12 ± 7**	-16 ± 5	-23 ± 7

Data are presented as means ± SD (n=9). Statistics: * $p<0.05$, ** $p<0.01$, significantly different from each Pre values. before exercise (Pre), immediately post-exercise (0 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), 168 hour post-exercise (168 h), range of motion (ROM).

Table 2. Changes in the muscle damage markers following the calf-raise exercise.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
CK	U/l	131.7 ± 94.0	133.0 ± 84.2	139.3 ± 81.4	172.6 ± 150.7	766.1 ± 1474.0	3245 ± 4648*	6069 ± 6498**
AST	U/l	19.4 ± 5.9	18.4 ± 5.1	18.6 ± 4.8	19.2 ± 7.0	29.3 ± 26.2	75.9 ± 88.1	126.2 ± 130.2
ALT	U/l	16.8 ± 6.0	16.3 ± 5.9	15.8 ± 5.0	16.0 ± 6.3	17.7 ± 7.1	27.8 ± 19.6	40.0 ± 29.4
LDH	U/l	139.8 ± 26.9	137.8 ± 24.7	138.1 ± 21.9	137.9 ± 26.9	146.3 ± 24.7	187.8 ± 79.9	241.7 ± 103.8**
ALD	U/l	3.7 ± 0.7	3.7 ± 0.1	3.8 ± 0.6	3.8 ± 0.6	6.2 ± 6.1	21.0 ± 26.7*	47.2 ± 44.7**

Data are presented as means ± SD (n=9). Statistics: * $p < 0.05$, ** $p < 0.01$, significantly different from each Pre values. before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aldolase (ALD).

Table 3. Changes in plasma and urinary novel markers of organ damage.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
H-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
H-FABP-U	pg/min	1242 ± 970	1156 ± 1114	1106 ± 1011	3261 ± 4243	2559 ± 3815	3857 ± 4835	3072 ± 2523
I-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
I-FABP-U	pg/min	45.8 ± 47.8	53.7 ± 35.7	87.9 ± 95.2	54.8 ± 48.2	81.9 ± 83.0	37.8 ± 31.8	40.5 ± 19.2
L-FABP-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
L-FABP-U	pg/min	3988 ± 3280	5323 ± 5031	4378 ± 2609	6846 ± 6631	5847 ± 6369	5539 ± 5480	6727 ± 3595
NGAL-P	ng/ml	13.2 ± 5.0	14.3 ± 7.7	13.8 ± 8.2	14.2 ± 9.6	13.6 ± 7.0	17.1 ± 12.9	15.5 ± 11.2
NGAL-U	ng/min	0.36 ± 0.51	0.76 ± 1.13	0.35 ± 0.20	0.39 ± 0.38	0.52 ± 0.55	0.35 ± 0.33	0.35 ± 0.29

Data are presented as means ± SD (n=9). before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), not detected below the detection limits (ND), plasma (P), urine (U), heart (H), Intestine (I), liver (L), fatty acid-binding protein (FABP), neutrophil gelatinase-associated lipocalin (NGAL). Urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Table 4. Changes in plasma and urinary inflammatory biomarkers.

	unit	Pre	2 h	4 h	24 h	48 h	72 h	96 h
IL-17A-P	pg/ml	31.1 ± 36.7	29.9 ± 35.1	29.1 ± 33.4	27.8 ± 32.7	30.9 ± 31.8	33.3 ± 33.5	30.1 ± 29.9
IL-17A-U	pg/min	ND	ND	ND	ND	ND	ND	ND
IL-23-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
IL-23-U	pg/min	0.9 ± 1.2	1.2 ± 1.5	1.4 ± 1.8	1.4 ± 2.0	1.3 ± 2.0	0.8 ± 1.3	0.4 ± 0.4
8-isoprostane-P	pg/ml	ND	ND	ND	ND	ND	ND	ND
8-isoprostane-U	pg/min	243.9 ± 151.9	501.3 ± 397.2	443.7 ± 293.5	792.5 ± 867.7	688.0 ± 969.8	267.1 ± 116.3	490.5 ± 372.3
sE-selectin-P	ng/ml	ND	ND	ND	ND	ND	ND	ND
sL-selectin-P	ng/ml	1341.0 ± 158.0	1348.0 ± 254.1	1276.0 ± 250.9	1263.0 ± 353.1	1370.7 ± 275.9	1440.0 ± 208.7	1332.0 ± 133.9
sP-selectin-P	ng/ml	32.7 ± 2.3	31.9 ± 9.1	33.6 ± 10.9	31.6 ± 5.3	29.1 ± 7.3	27.9 ± 5.5	30.8 ± 8.5
Nerve growth factor-P	pg/ml	2.4 ± 3.1	4.2 ± 5.9	2.6 ± 2.7	9.7 ± 17.6	9.3 ± 17.7	6.1 ± 8.2	9.7 ± 21.1

Data are presented as means ± SD (n=9). before exercise (Pre), 2 hour post-exercise (2 h), 4 hour post-exercise (4 h), 24 hour post-exercise (24 h), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h), not detected below the detection limits (ND), plasma (P), urine (U), soluble Endothelial (sE), soluble Leukocyte-Endothelial (sL), soluble Platelet (sP). Urinary data were corrected for the gross amount (raw concentration × urine volume) per minute (excretion rate).

Table 5. Pearson's correlation coefficient matrix of muscle damage markers, tenderness of medial gastrocnemius and ankle active range of motion in dorsal flexion.

	CK-72 h	CK-96 h	LDH-96 h	ALD-72 h	ALD-96 h	PMG-72 h	MMG-72 h	dROM-48 h	dROM-72 h
CK-72 h		0.24	0.32	0.94	0.48	0.22	0.59	-0.34	-0.43
CK-96 h	0.24		0.94	0.38	0.93	-0.04	0.13	0.19	-0.05
LDH-96 h	0.32	0.94		0.46	0.95	-0.04	0.20	0.31	0.13
ALD-72 h	0.94	0.38	0.46		0.64	0.37	0.78*	-0.15	-0.25
ALD-96 h	0.48	0.93	0.95	0.64		0.06	0.42	0.22	0.04
PMG-72 h	0.22	-0.04	-0.04	0.37	0.06		0.37	0.25	0.03
MMG-72 h	0.59	0.13	0.20	0.78*	0.42	0.37		0.02	0.12
dROM-48 h	-0.34	0.19	0.31	-0.15	0.22	0.25	0.02		0.89
dROM-72 h	-0.43	-0.05	0.13	-0.25	0.04	0.03	0.12	0.89	

All data are calculated as percent changes for the pre-exercise values.

Statistics: * Significant correlation between serum leaking enzyme activity and muscle tenderness score ($p<0.05$), creatine kinase (CK), lactate dehydrogenase (LDH), aldolase (ALD), proximal medial gastrocnemius (PMG), middle medial gastrocnemius (MMG), ankle range of motion in dorsal flexion (dROM), 48 hour post-exercise (48 h), 72 hour post-exercise (72 h), 96 hour post-exercise (96 h).

2-4. DISCUSSION

Muscle-derived leaking enzymes such as CK, AST, ALT, LDH and ALD have been used in many studies as indirect markers of exercise-induced muscle damage. However, there are generally poor correlations between DOMS and muscle damage indicators (27). In this study, CK and ALD were significantly increased earlier than LDH. Also, ALD showed relatively lower interindividual variations and was significantly correlated with tenderness of middle MG at 72 h, whereas there were only moderate, non-significant correlations between tenderness and the other leaking enzyme activities. These results suggest that ALD rather than CK and LDH might be a more accurate and objective muscle damage indicator.

There are a few studies that have investigated the organ damage markers, FABPs and NGAL following exercise-induced muscle damage. FABPs are present in almost all tissues (50), but H-FABP is present in skeletal muscle (47). Sorichter et al. reported that plasma H-FABP and myoglobin increased earlier (30 min) than CK (2 h) following 20 min of downhill running (39). These results suggest that plasma H-FABP concentrations reflect exercise-induced muscle damage earlier, but plasma H-FABP seems to have no advantage in view of its similarity of changes in the appearance of myoglobin. Also, the change might be attributed to not only eccentric exercise-induced local muscle damage, but also systemic factors such as dynamic exercise-induced haemoconcentration. In this study, one-leg calf-raise exercise caused severe muscle damage as shown by the precise local assessment of the muscle symptoms, but we could not detect any changes in FABPs and NGAL nor any correlations with the symptoms. In any case, these organ damage markers are not considered to be indicative of muscle damage resulting from eccentric exercise loading on the calf, thus they cannot be used as local muscle damage markers at least.

Endurance exercise induces peripheral blood neutrophilia (41-44), and enhances the capacity of neutrophils to produce ROS (29, 42-44). In a study using myeloperoxidase (MPO) knockout mice, exercise-induced muscle damage was facilitated by MPO-containing neutrophils and their activating factors such as proinflammatory cytokines (25). On the other hand, Maruhashi et al. reported that the antioxidant capacity was affected by the type and intensity of exercise, specifically, low-load eccentric exercise did not reduce antioxidant capacity, but conversely low-load concentric exercise temporarily reduced antioxidant capacity (20). We investigated neutrophil activation-related markers such as selectins, 8-isoprostane, IL-17A and IL-23, but they were not significantly changed, suggesting that inflammatory responses and oxidative stress were not changed, at least, in the examined markers following one-leg calf-raise exercise. The enhanced eccentric exercise-induced neutrophil migratory activity independent of ROS production and MPO degranulation observed in our previous study (14) might be due to the mobilization of functionally different heterogeneous neutrophils possibly from the bone marrow reserve (42, 44). Since we could not detect any significant changes of a wide range of proinflammatory and oxidative stress markers in this exercise mode other than neutrophil mobilization and migration (14), it is necessary to focus more on the involvement of this earlier step of inflammation as the underlying mechanisms and the point of target for potential preventive countermeasures against exercise-induced muscle damage in the future studies.

In conclusion, it is confirmed in the present study that not only are serum CK but also ALD activities more reliable indicators for exercise-induced muscle damage than the other examined variables, but there are lower correlations with muscle symptoms, and organ damage markers of FABPs and NGAL could not be alternative indicators for muscle disruption, neutrophil mobilization and migration. Also, we could not detect any perturbations of novel

proinflammatory cytokines and soluble adhesion molecules, and the inflammatory mechanisms are still not clear. Therefore, further research is needed to determine whether there are more sensitive indicators including urinary biomarkers as non-invasive assessment of exercise-induced muscle damage and the underlying mechanisms as well.

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Chapter III

Urinary titin fragment as a new biomarker for muscle damage

3-1. INTRODUCTION

Myopathies such as muscular dystrophy, myositis, and rhabdomyolysis are known as diseases that cause muscle tissue disruption with progressive muscle weakness and wasting. During the past 20 years, an increasing number of genes have been identified that cause different forms of myopathy. The discovery of novel genes encoding proteins with different subcellular localization, which cause various types of myopathy, has shed new light on the pathogenic mechanisms of this heterogeneous group of diseases. On the other hand, pathophysiological mechanisms of myopathy are not fully understood, yet. To make the diagnosis of these myopathies an invasive pathomorphological test is required. Identification of a non-invasive biomarker would be advantageous to make an earlier diagnosis of these diseases, evaluate disease progression and monitor treatment effects.

To understand the mechanisms of myopathy experimentally, an experimental model of physiological muscle disruption, such as exercise-induced muscle damage, is commonly used (8, 21, 24). In fact, muscle damage is particularly induced by eccentric exercise (6), and causes skeletal myofiber disruption, inflammatory cell infiltration and muscle soreness (15, 16), similar to the pathology of myopathy. The direct assessment of muscle damage is morphological evaluation of skeletal muscle (1, 2, 33). However, since eccentric muscle contraction induces sporadic muscle disruption, it is sometimes difficult to find damaged tissue. In addition, muscle biopsy is invasive for research participants. Non-invasive methods to assess muscle disruption are needed in the absence of muscle damage.

Muscle-derived enzyme activities in serum such as creatine kinase (CK), lactate dehydrogenase (LDH) and myocellular protein myoglobin (Mb) which leak into circulation from damaged muscle have been used as indirect markers for muscle damage (5). In addition, delayed-onset muscle soreness (DOMS) and the changes of range of motion (ROM) have also

been used as indicators of muscle symptoms (6, 7). However, it has been reported that there is poor correlation between changes in muscle damage markers and the magnitude of muscle symptoms (20). Furthermore, it is reported that serum levels of these biomarkers depend on gender, muscle mass, exercise intensity and duration. There is a remarkable inter-individual variation in the degree to which serum enzyme activities increase with exercise (24). Taken together, there are very few noninvasive and sensitive biomarkers that reflect exercise-induced muscle damage.

Recent advance of proteomic tools offer a strategy to undertake comprehensive protein expression analysis that can be applied to search for biomarkers. In fact, many proteomic studies have already been performed to identify biomarkers of serum, urinary and salivary proteins for early diagnosis of various diseases including cancer (32) and Alzheimer's disease (4). In relation to exercise-induced muscle damage, Malm et al. reported that several Z-band related protein expressions were detected in serum after eccentric exercise (17). Sietsema et al. reported that alpha 1-antichymotrypsin and C-1 protease inhibitor peptides increased earlier post-exercise than CK, and thus these proteins were suggested as a new biomarker of muscle injury (29). To date, no study has analyzed urinary proteins after eccentric exercise using proteomics.

In this study, we performed proteomic analysis to identify urinary proteins in response to acute eccentric exercise. We found that a N-terminal fragment of titin (also known as connectin) is detectable in urine after eccentric exercise. We then established a quantitative enzyme-linked immunosorbent assay (ELISA) for measurement of the urinary titin fragment, and determined whether the urinary titin fragment can be used as a biomarker for muscle damage.

3-2. METHODS

Subjects

Out of the nine healthy males participated in the original investigation (11), urine samples were used from the subjects with the Mb rated during the experimental period. Their mean (±SD) characteristics were as follows: age 24.8±1.3 yrs, body mass 62.3±6.3 kg, and height 1.72±0.05 m. The subjects were instructed to maintain their usual daily schedule during the experiment. The study protocol was approved by the ethics committee of Waseda University, Japan, and the subjects provided their informed consent.

Experimental design

Subjects performed a calf-raise exercise, including repetitive eccentric muscle contractions with their right leg on a force plate as described previously (11). The range of motion (ROM) of the ankle joint during the exercise was regulated from -20° (dorsiflexion position) to 15° (planter flexion position) using a goniometer (SG 110/A, Biometrics, Newport, UK) with its ends attached onto the skin over the tibia and calcaneus. DOMS was rated using a visual analogue scale (VAS) that had a 100-mm line with “no pain” on one end and “extremely sore” on the other. Blood and urine samples were collected before and 2, 4, 24, 48, 72 and 96 h after the exercise. Participants were supposed to urinate 2 h before each sampling, and the urine samples were collected in measuring cylinders. Serum Mb measurement was described previously (11), serum CK, LDH, ALD and FABPs measurement were described in Kanda et al. (12).

Human urine protein concentrates

Urine samples were centrifuged immediately at 1000×G for 10 min to remove sediments, and the supernatants were stored at –80°C for later analyses. The samples were transferred to Ultra-4 membrane concentrators (MWCO 3k, Cat No.UFC800324, Millipore, Billerica, MA, USA) and spun at 7000×G for 6 h to reduce the volumes to 20 ml. Then 20 ml of Lysis buffer

(4% (w/v) CHAPS, 2 M thiourea, 8 M urea, 10 mM Tris-HCl pH 8.8) were added to the samples. The protein amount in urine concentrates was measured using the BCA assay (Pierce, Rockford, IL, USA) and frozen at –80°C for later analyses.

Gel electrophoresis and imaging

Immunobilized pH gradient (IPG) strips (pH 3–10L, 24 cm) were rehydrated and mixed samples were applied with cup loading. Isoelectric focusing (IEF) was performed using a Multiphor II (Amersham Biosciences, Little Chalfont, Bucks, UK) for 54 kVh at 20°C in the dark (27, 28). Strips were equilibrated for 10 min in 50 mM Tris-HCl pH 8.8, 6 M urea, 30% v/v glycerol, 1% w/v SDS containing 65 mM DTT and then for 10 min in the same buffer containing 240 mM iodoacetamide. Equilibrated IPG strips were transferred onto 24 cm x 20 cm, 12% T, 7.5% C polyacrylamide gels made between low fluorescence glass plates. Strips were overlaid with 0.5% w/v low melting point agarose in 25 mM Tris-base, 0.1% SDS, 192 mM glycine containing 0.1% bromophenol blue. Gels were run in Ettan DALT twelve (Amersham Biosciences) with 2 W/gel at 20°C, until the dye front had run off the bottom of the gels. The 2-D gels between low fluorescence glass plates were scanned directly with a Tyhoon 9400 (Amersham Biosciences). Normalization among three Cy™ dyes was accomplished by adjusting the maximum pixel values to 55.000 counts with changing the PMT voltage. The images generated were exported as tagged image (Amersham Biosciences).

Image analysis

The differential in-gel analysis (DIA) of DeCyder™ was used to merge the Cy2, Cy3 and Cy5 images for each gel, and detect spot boundaries for the calculation of normalized spot volumes/protein abundance. At this stage, features resulting from non-protein sources (e.g. dust

particles, streaks) and faint spots (e.g. spot area, ≤300, spot volume, ≤10.000) were filtered out. The analysis was used to calculate abundance differences between samples run on the same gel. The biological variation analysis (BVA) of DeCyder™ was then used to match all pair wise image comparisons from DIA for a comparative cross-gel statistical analysis. Comparison of normalized Cy3 and Cy5 spot volumes with the corresponding Cy2 standard spot volumes within each gel gave a standardized abundance. This value was compared across all gels for each matched spot and a statistical analysis (t-test or ANOVA method) was performed using the triplicate values from each experimental condition. All analyzed gels were matched to one "master gel" to assign the same number to the same protein spot. The master gel image was obtained from the pooled samples made from all vehicle control and all chemical dosed samples.

In-gel digestion and peptide extraction

Gel electrophoresis for MS analysis was performed with the same procedures as described in the "gel electrophoresis and imaging". After gel electrophoresis, gel was fixed in 10% v/v methanol: 7% v/v acetic acid and stained with Sypro® Ruby, and then this gel for MS analysis were matched to the master gel for expression analysis by BVA software. Spots of interest were excised from 2-D gels using an automated spot picker (Amersham Biosciences) following the manufacturer's instructions. Spots were collected in 200 mL of water in 96-well plates. The recovered gel pieces were washed with aqueous 50 mM ammonium bicarbonate and ACN, then incubated with 12.5 ng/mL trypsin (Promega, Southampton, UK) at 30°C for 15 h. The generated peptides were eluted with 50 mM ammonium bicarbonate followed by 10% v/v formic acid and ACN. The combined fractions were dried in a Speedvac and dissolved in 0.1% v/v formic acid.

MS analysis

MS analysis was carried out by LC-MS/MS (18, 34). HPLC (CapLC, Waters, Milford, MA) was coupled with the quadrupole (Q)-TOF micro mass spectrometer (Micromass, Man-chester, UK). Instrument operation, data acquisition and analysis were performed using MassLynx 3.2 software (Micromass). The tryptic peptides were concentrated and desalted on a 300 mm id/5 mm length C18 PepMap column (LC Packings, San Francisco, CA). The eluted peptide was analyzed by MS/MS sequencing with an automated MS-to- MS/MS switching protocol. Online determination of precursor-ion masses was performed over the m/z range from 400 to 1600 amu in the positive charge detection mode with a cone voltage of 50 V. The cone voltage, extraction voltage, microchannel plate detector voltage and collision energy were optimized before the measurement of samples. The database search was performed with MASCOT Deamon (Matrix Science, London, UK) (9, 25). The generated pkl files were submitted to Swiss-Prot (release 47.4) and NCBInr (14-Jul-2005). Search parameters were as follows: fixed modifications – carbamidomethyl, variable modifications – oxidation (M), missed cleavages: up to 1, monoisotopic, peptide tolerance 1.0 Da, MS/MS tolerance 0.5 Da. The ions score cut-off was set to 20. The automatically identified proteins were manually checked individually to remove the redundancy.

Immunoblotting analysis

The collected urine was mixed with a complete protein-loading buffer containing 50 mM Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 10% glycerol, 20 mM dithiothreitol, 127 mM 2-mercaptoethanol, and 0.01% bromophenol blue, supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). The urine samples

were transferred to microfuge tubes, heated for 5 min at 100 °C, and centrifuged in a microfuge for 5 min at 12,000 × g at room temperature. Then, urine samples were loaded onto 7.5%–15% gels (depending on the molecular weight of the protein) for SDS–polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and immunodetected with an enhanced chemiluminescence kit (Amersham) using a LAS-3000 imaging system (Fuji Film, Japan). The antibodies directed against titin fragments were used for the immunoblotting analysis: anti-TTN antibodies clone 7D3, clone 2B3, clone 2F12 (Abnova, Taipei, Taiwan), and rabbit anti-titin/CMD1G polyclonal antibody (Bioss, Boston, USA). The secondary antibodies used were sheep anti-mouse IgG–horseradish peroxidase (HRP) (NA931, Amersham) and goat anti-rabbit IgG–HRP (Amersham). Protein quantification was performed with the ImageJ software (NIH, Bethesda, MD).

Enzyme-linked immunosorbent assay (ELISA)

The titin fragment concentrations were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, a 96-well microtiter plate (Immulon II, Dynex Technologies, Chantilly, VA) was coated with an anti-human titin monoclonal antibody (clone 7D3, Abnova) overnight at 4°C. After adding 250 µl phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma, St. Louis, MO), wells were blocked for 2 h. Urine samples were thawed, centrifuged at 10,000 rpm for 5 min and diluted (1/3) with PBS containing 1% BSA, and 100 µl of each were added and incubated for 1 h. Using recombinant human titin fragment (1-111 a.a., Q01, Abnova), known concentrations of N-terminal titin fragment were also plated to establish standard values. After washing the plate with PBS-Tween, another anti-human titin monoclonal antibody (clone 2B3, Abnova) was added to the wells and incubated for 1 h. After washing the plate with PBS-Tween, a rat anti-mouse IgG1 conjugated with HRP (ab99603, Abcam) was

added to the wells and incubated for 1 h. After washing, substrate solution was added and the color intensity produced after 15 min was measured by a microplate reader (ARVO MX; PerkinElmer, Waltham, MA) at 490 nm. All samples were assayed in duplicate and the average of absorbance values was used as the representative value. Regression analysis using the relationship of standard titin fragment concentrations and amount of absorbance was used to interpolate the concentration of titin fragment in the samples. To avoid interassay variability, all samples from each subject were assayed on the same plate.

Statistical analysis

Data were analyzed using one-way analysis of variance. When significant time effects were evident, multiple comparisons were analyzed with Bonferroni adjustment. Associations between data were analyzed with Pearson's correlation coefficient (r). Statistical significance was set at $p<0.05$ and data are presented as mean ± standard deviation (SD).

3-3. RESULTS

Proteomics analysis

A typical 2D-DIGE gel image was shown as Figure 1. We found that some proteins were differentially expressed after the eccentric exercise.

Approximately 99 proteins (156 spots) were identified as unique from 2D-DIGE gel patterns. As specified for each protein in Table 1.

A titin fragment

We identified N-terminal titin fragment (molecular weight; 28kDa) as one of 99 proteins shown to have altered expression after the eccentric exercise (Figure 2 and Figure 3). As shown in

Figure 4, a primary mouse monoclonal antibodies specific for titin, 2B3 IgG1 (1:1000, Abnova) and 7D3 IgG2a (1:1000, Abnova) detected a recombinant N-terminal titin fragment (1-111 a.a.; 0.5 µl, 5 µl. H00007273-Q01, Abnova), whereas 2F12 IgG2b (1:1000, Abnova) indicated lower level expression to Titin recombinant protein using goat anti-mouse IgG-HRP (1:5000, Santa Cruz Biotechnology). Next, we determined if these antibodies could detect the urinary titin fragment in human urine samples. As shown in Figure 5, 2B3 and 7D3 signals were detected from the urine samples that were obtained after the eccentric exercise but not from pre-exercise samples.

ELISA

We attempted to establish an ELISA to measure urinary titin fragment. Based on the Western blot analysis, ELISA was performed using monoclonal antibody 7D3 and 2B3. To confirm specificity of the detection, reactivities to the recombinant N-terminal titin fragment (1-111 a.a.) were measured. The typical reactivities are presented in Figure 6. We used the ELISA to measure the titin fragment in urine from 9 subjects before and after the eccentric exercise. As shown in Figure 7 (individual values) and Figure 8 (means), urinary titin fragment concentration significantly increased at 96 h after the exercise ($p<0.01$).

Relationships between urinary titin fragment concentrations and muscle damage markers

As shown in Figure 9 A to H, there were positive correlations between the percent changes of the titin fragment and CK at 48 h after the eccentric exercise ($r=0.98$, $p<0.01$) (A), the titin fragment and CK at 72 h ($r=0.81$, $p<0.01$) (B), the titin fragment and Mb at 48 h ($r=0.98$, $p<0.01$) (C), the titin fragment and Mb at 72 h ($r=0.67$, $p<0.05$) (D), the titin fragment and Mb (48 h and 72 h) ($r=0.88$, $p<0.01$) (E), the titin fragment and LDH (48 h and 72 h) ($r=0.91$,

$p<0.01$) (F) , the titin fragment and ALD at 48 h ($r=0.98$, $p<0.01$) (G), the titin fragment 48 h and ALD at 72 h after the eccentric exercise ($r=0.94$, $p<0.01$) (H), the titin fragment and H-FABP at 72 hours ($r=0.85$, $p<0.05$) (I), and the titin fragment at 72 h after the eccentric exercise and neutrophil producing activity of ROS at 4 h ($r=0.89$, $p<0.01$) (J).

Relationships between urinary titin fragment concentrations and muscle soreness (MMG-VAS) and range of motion (ROM)

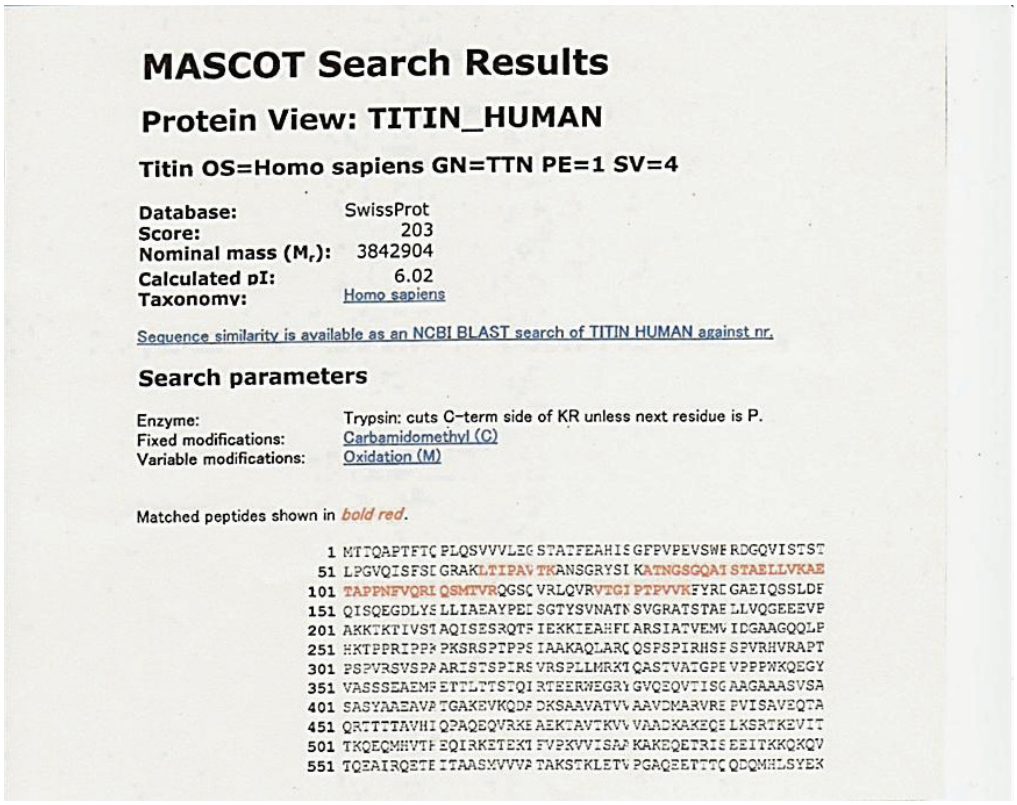
As shown in Figure 10 A to D, there were positive correlations between the percent changes of the titin fragment and MMG-VAS at 48 h ($r=0.91$, $p<0.01$) (A), the titin fragment 48 h and MMG-VAS 72 h ($r=0.78$, $p<0.05$) (B), the titin fragment and DOMS at 48 h ($r=0.84$, $p<0.01$) (C) and the titin fragment 48 h and DOMS 72 h ($r=0.74$, $p<0.05$) (D). As shown in Figure 10 E and F, there were negative correlations between the percent changes of the titin fragment 72 h and ROM 48 h ($r=-0.71$, $p<0.05$) (E) and the titin fragment 72 h and ROM at 72 h after the eccentric exercise ($r=-0.76$, $p<0.05$) (F).

Table 1. Specified 99 proteins from 2D-DIGE patterns.

No.	Protein Name	Ac.No.	Mw
1	14 kDa phosphohistidine phosphatase	PLMN_HUMAN	93247
2	3-mercaptopyruvate sulfurtransferase	CO6A1_HUMAN	109602
3	Abhydrolase domain-containing protein 14B	TRFE_HUMAN	79294
4	Acid ceramidase	CAD11_HUMAN	88367
5	Actin; cytoplasmic 1	ANT3_HUMAN	53025
6	Acyl-coenzyme A oxidase-like protein	APOD_HUMAN	21547
7	Alpha-1-antitrypsin	CATA_HUMAN	59947
8	Alpha-2-HS-glycoprotein	A1AT_HUMAN	46878
9	Alpha-enolase	IGHG1_HUMAN	36596
10	Aminoacylase-1	KNG1_HUMAN	72996
11	Antithrombin-III	FIBB_HUMAN	56577
12	Apolipoprotein D	ARSA_HUMAN	54409
13	Arylsulfatase A	FIBB_HUMAN	56577
14	Basement membrane-specific heparan sulfate proteoglycan core protein	VTDB_HUMAN	54526
15	Beta-2-microglobulin	CLIP2_HUMAN	116223
16	Cadherin-1	ENOA_HUMAN	47481
17	Cadherin-11	GNS_HUMAN	62840
18	CAP-Gly domain-containing linker protein 2	TPP1_HUMAN	61723
19	Carbonic anhydrase 1	A2GL_HUMAN	38382
20	Carbonic anhydrase 2	ACY1_HUMAN	46084
21	Carcinoembryonic antigen-related cell adhesion molecule 8	IDHC_HUMAN	46915
22	Cartilage intermediate layer protein 2	A2GL_HUMAN	38382
23	Catalase	GALK1_HUMAN	42702
24	Cathepsin D	ACTB_HUMAN	42052
25	Cathepsin Z	PEPA3_HUMAN	42349
26	CD59 glycoprotein	FIBA_HUMAN	95656
27	Collagen alpha-1(VI) chain	ZAGL1_HUMAN	23080
28	Copper transport protein ATOX1	ZA2G_HUMAN	34465
29	Cystatin-A	ALDOA_HUMAN	39851
30	Cystatin-M	ZA2G_HUMAN	34465
31	Ester hydrolase C11orf54	ZA2G_HUMAN	34465
32	Fatty acid-binding protein; heart	CAPG_HUMAN	38760
33	Fatty acid-binding protein; liver	ZA2G_HUMAN	34465
34	Fibrinogen alpha chain	DHSO_HUMAN	38927
35	Fibrinogen beta chain	TRFE_HUMAN	79294
36	Fibrinogen gamma chain	CD14_HUMAN	40678
37	Fructose-bisphosphate aldolase A	CK054_HUMAN	35608
38	Fructose-bisphosphate aldolase B	ALBU_HUMAN	71317
39	Galactokinase	G3P_HUMAN	36201
40	Ganglioside GM2 activator	DDAH1_HUMAN	31444
41	Gelsolin	TRFE_HUMAN	79294
42	Glutamine-rich protein 2	CD14_HUMAN	40678
43	Glutaminyl-peptide cyclotransferase	ITIH4_HUMAN	103521
44	Glyceraldehyde-3-phosphate dehydrogenase	THTM_HUMAN	33443
45	GTP cyclohydrolase 1 feedback regulatory protein	ITIH4_HUMAN	103521
46	Heat shock cognate 71 kDa protein	LDHB_HUMAN	36900
47	Hemoglobin subunit beta	MDHC_HUMAN	36631
48	Ig gamma-1 chain C region	ITIH4_HUMAN	103521
49	Ig kappa chain C region	AMBP_HUMAN	39886
50	Ig lambda-2 chain C regions	PTGDS_HUMAN	21243

51	Integrin-linked kinase-associated serine/threonine phosphatase 2C	PTGDS_HUMAN	21243
52	Inter-alpha-trypsin inhibitor heavy chain H4	CATD_HUMAN	45037
53	Isocitrate dehydrogenase [NADP] cytoplasmic	IGKC_HUMAN	11773
54	Kininogen-1	IGKC_HUMAN	11773
55	Leucine-rich alpha-2-glycoprotein	IGKC_HUMAN	11773
56	Leukocyte elastase inhibitor	CAH1_HUMAN	28909
57	L-lactate dehydrogenase B chain	RET4_HUMAN	23337
58	Lysosomal alpha-glucosidase	RET4_HUMAN	23337
59	Macrophage-capping protein	PEBP1_HUMAN	21158
60	Malate dehydrogenase; cytoplasmic	ABHEB_HUMAN	22446
61	Mannan-binding lectin serine protease 2	PGBM_HUMAN	479253
62	Matrix-remodeling-associated protein 8	QRIC2_HUMAN	181228
63	Monocyte differentiation antigen CD14	PRDX2_HUMAN	22049
64	Multimerin-2	SAP3_HUMAN	21281
65	Myoglobin	HSP7C_HUMAN	71082
66	N(G); N(G)-dimethylarginine dimethylaminohydrolase 1	ALBU_HUMAN	71317
67	N(G); N(G)-dimethylarginine dimethylaminohydrolase 2	LAC2_HUMAN	11458
68	N-acetylglucosamine-6-sulfatase	AMBP_HUMAN	39886
69	Napsin-A	TITIN_HUMAN	3842904
70	Neurofilament heavy polypeptide	MMRN2_HUMAN	105028
71	Nuclear transport factor 2	SAP3_HUMAN	21281
72	Pancreatic secretory trypsin inhibitor	AMBP_HUMAN	39886
73	Pepsin A-3	AMBP_HUMAN	39886
74	Peroxiredoxin-2	PRDX6_HUMAN	25133
75	Peroxiredoxin-5; mitochondrial	CYTM_HUMAN	16785
76	Peroxiredoxin-6	CD59_HUMAN	14795
77	Phosphatidylethanolamine-binding protein 1	IGKC_HUMAN	11773
78	Phosphoglycerate kinase 1	ILKAP_HUMAN	43450
79	Plasminogen	ACOXL_HUMAN	62383
80	Prostaglandin-H2 D-isomerase	PRDX5_HUMAN	22301
81	Prostate-specific antigen	ABHEB_HUMAN	22446
82	Protein AMBP	CEAM8_HUMAN	38415
83	Protein DJ-1	PHP14_HUMAN	13995
84	Protein S100-A6	CYTA_HUMAN	11000
85	Putative zinc-alpha-2-glycoprotein-like 1	IGKC_HUMAN	11773
86	Retinol-binding protein 4	CYTA_HUMAN	11000
87	Secreted Ly-6/uPAR-related protein 2	HBB_HUMAN	16102
88	Serotransferrin	IGKC_HUMAN	11773
89	Serum albumin	GFRP_HUMAN	9749
90	SH3 domain-binding glutamic acid-rich-like protein 3	SLUR2_HUMAN	10723
91	Sorbitol dehydrogenase	S10A6_HUMAN	10230
92	Superoxide dismutase [Cu-Zn]	RS27A_HUMAN	18296
93	Syntenin-1	ATOX1_HUMAN	7568
94	Titin	CAH2_HUMAN	29285
95	Tripeptidyl-peptidase 1	RS27A_HUMAN	18296
96	Ubiquitin-40S ribosomal protein S27a	NFH_HUMAN	112639
97	Vesicular integral-membrane protein VIP36	PRDX2_HUMAN	22049
98	Vitamin D-binding protein	ITIH4_HUMAN	103521
99	Zinc-alpha-2-glycoprotein	DDAH2_HUMAN	29911

Figure 1. A typical image of 2D-DIGE before and after eccentric exercise.
Urine samples were labeled with Cy3(before) or Cy5(after) . We found some proteins were differentially expressed after eccentric exercise. Red colored spots reflect protein expression increase compared with pre and after exercise.



MASCOT Search Results

Protein View: TITIN_HUMAN

Titin OS=Homo sapiens GN=TTN PE=1 SV=4

Database: SwissProt
Score: 203
Nominal mass (M_r): 3842904
Calculated pI: 6.02
Taxonomy: Homo sapiens

Sequence similarity is available as an NCBI BLAST search of TITIN_HUMAN against nr.

Search parameters

Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)

Matched peptides shown in *bold red*.

```
    1 MTTQAPTFTQ PLQSVVVLEG STATFEAHIS GFPVPEVSWF RDGQVISTST
   51 LPGVQISFSE GRAKLTIPAV TKANSGRYSI KATNGSGQAI STAELLVKAE
  101 TAPPNFVQRL QSMIVRQGSQ VRLQVRVTGI PTPVVKFYRD GAEIQSSLDF
  151 QISQEGDLYS LLIAEAYPED SGTYSVNATN SVGRATSTAE LLVQGEEEVP
  201 AKKTKTIVST AQISESRQTP IEKKIEAHFD ARSIATVEMV IDGAAGQQLP
  251 HKTPPRIPPP PKSRSPTPPS IAAKAQLARQ QSPSPIRHSP SPVRHVRAPT
  301 PSPVRSVSPA ARISTSPIRS VRSPLLMRKT QASTVATGPE VPPPWKQEGY
  351 VASSSEAEMP ETTLTTSTQI RTEERWEGRY GVQEQVTISG AAGAAASVSA
  401 SASYAAEAVA TGAKEVKQDP DKSAAVATVV AAVDMARVRE PVISAVEQTA
  451 QRTTTTAVHI QPAQEQVRKE AEKTAVTKVV VAADKAKEQE LKSRTKEVIT
  501 TKQEQMHVTP EQIRKETEKT FVPKVVISAP KAKEQETRIS EEITKKQKQV
  551 TQEAIRQETE ITAASMVVVP TAKSTKLETV PGAQEETTTQ QDQMHLSYEK
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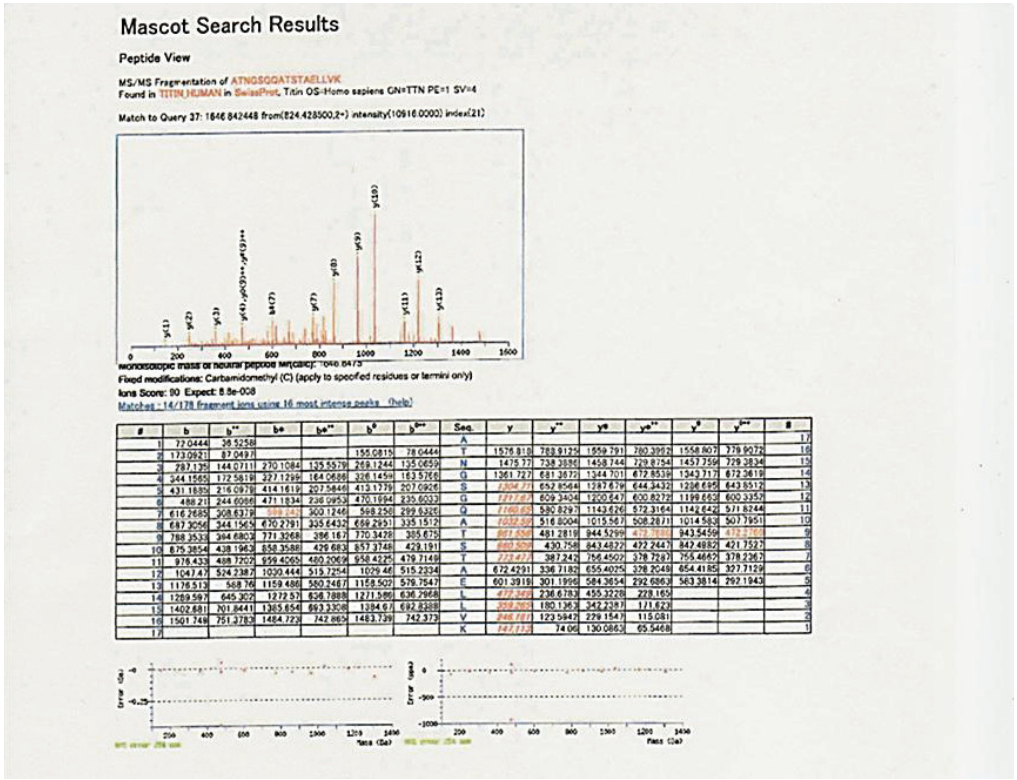


Mascot Search Results

Peptide View

MS/MS Fragmentation of ATNGSGQATSTAELLVK
Found in TITIN_HUMAN in SwissProt, Titin OS=Homo sapiens GN=TTN PE=1 SV=4

Match to Query 37: 1646.842448 from(824.428500,2+) intensity(10916.0000) index(21)

Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Ions Score: 90 Expect: 8.8e-008
Matches: 14/178 fragment ions using 16 most intense peaks (help)

#	b	b**	b*	b***	b°	b°**	Seq.	y	y**	y*	y***	y°	y°**	#
1	72.0444	36.5258					A							17
2	173.0921	87.0497			155.0815	78.0444	T	1576.810	788.9125	1559.791	780.3962	1558.807	779.9072	16
3	287.1291	144.0711	270.1084	135.5579	269.1244	135.0659	N	1475.77	738.3886	1458.744	729.8754	1457.759	729.3834	15
4	344.1565	172.5819	327.1299	164.0686	326.1459	163.5766	G	1361.727	681.3672	1344.701	672.8539	1343.717	672.3619	14
5	431.1835	216.0957	414.1619	207.5846	413.1779	207.0926	S	1304.71	652.8564	1287.679	644.3432	1286.695	643.8512	13
6	488.21	244.6096	471.1834	236.0953	470.1994	235.6033	Q	1217.67	609.3404	1200.647	600.8272	1199.652	600.3353	12
7	616.2685	308.6379	[illegible]	300.1246	598.258	299.6326	Q	1160.65	580.8291	1143.626	572.3164	1142.642	571.8244	11
8	687.3056	344.1565	670.2791	335.6432	669.2951	335.1512	A	1032.59	516.8004	1015.567	508.2871	1014.583	507.7951	10
9	788.3533	394.6803	771.3268	386.167	770.3428	385.675	T	961.556	481.2819	944.5299	472.7686	943.5459	472.2766	9
10	875.3854	438.1963	858.3588	429.683	857.3748	429.191	S	860.509	430.758	843.4822	422.2447	842.4982	421.7527	8
11	976.433	488.7202	959.4065	480.2069	958.4225	479.7149	T	773.47	387.242	756.4502	378.7287	755.4662	378.2367	7
12	1047.47	524.2387	1030.444	515.7254	1029.46	515.2334	A	672.4291	336.7182	655.4025	328.2049	654.4185	327.7129	6
13	1176.513	588.76	1159.486	580.2467	1158.502	579.7547	E	601.3918	301.1996	584.3654	292.6863	583.3814	292.1943	5
14	1289.597	645.302	1272.57	636.7888	1271.586	636.2968	L	472.349	236.6783	455.3228	228.1650			4
15	1402.681	701.8441	1385.654	693.3308	1384.67	692.8388	L	359.265	180.1363	342.2101	171.623			3
16	1501.749	751.3783	1484.723	742.865	1483.739	742.373	V	246.181	123.5942	229.1547	115.081			2
17							K	147.113	74.06	130.0863	65.5468			1

Figure 2. The N-terminal titin fragment was identified by protein database search engines MASCOT.

Figure 3. N-terminal titin fragments expression after eccentric exercise.

P1: Pre exercise, P2: 2 hours after exercise, P3: 4 hours after exercise, P4: 24 hours after exercise, P5: 48 hours after exercise, P6: 72 hours after exercise, P7: 96 hours after exercise

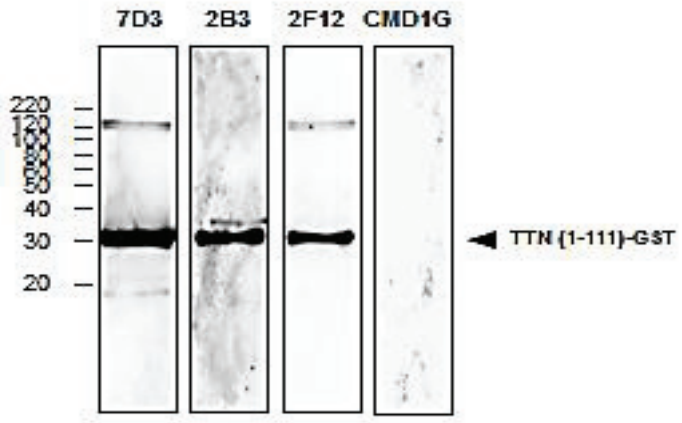
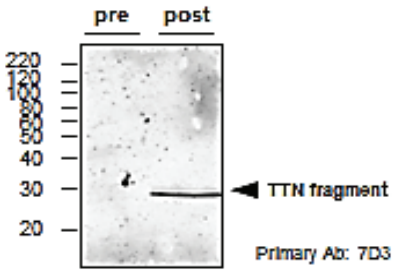


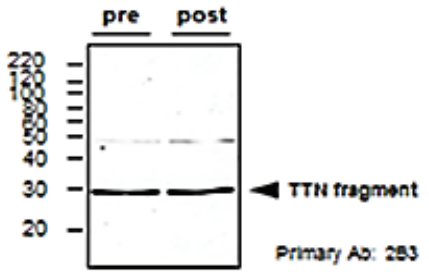
Figure 4. Screening of antibodies against the N-terminal fragment of human titin.

To screen antibodies that can react with the N-terminal fragment of human titin, a recombinant human titin fragment (1-111 a.a.) was loaded to SDS-PAGE gel, and the reactivities were evaluated by Western blot with adequate secondary antibody.

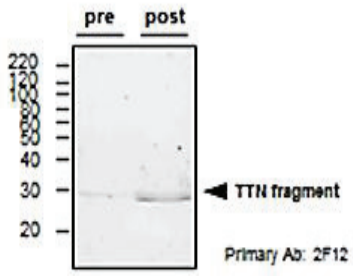
Anti-human titin antibody clone: 7D3 (1:1000), 2B3 (1:1000), 2F12 (1:1000) detected signals from the titin recombinant protein. Anti-human titin antibody; CMD1G (1:500) did not detect signals.



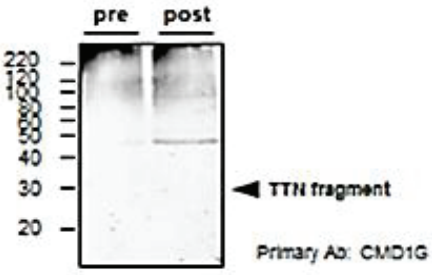
A: anti-human titin antibody/ clone: 7D3 detected signals from urine samples



B: anti-human titin antibody/ clone: 2B3 detected signals from urine samples



C: anti-human titin antibody/ clone: 2F12 detected signals from urine samples



D: rabbit anti-titin polyclonal antibody: CMD1G did not detect signals from urine samples

Figure 5. Screening of antibodies against urine samples.

To screen antibodies that can react with the N-terminal fragment of human titin, urine samples were loaded to SDS-PAGE gel, and the reactivities were evaluated by Western blot with adequate secondary antibody.

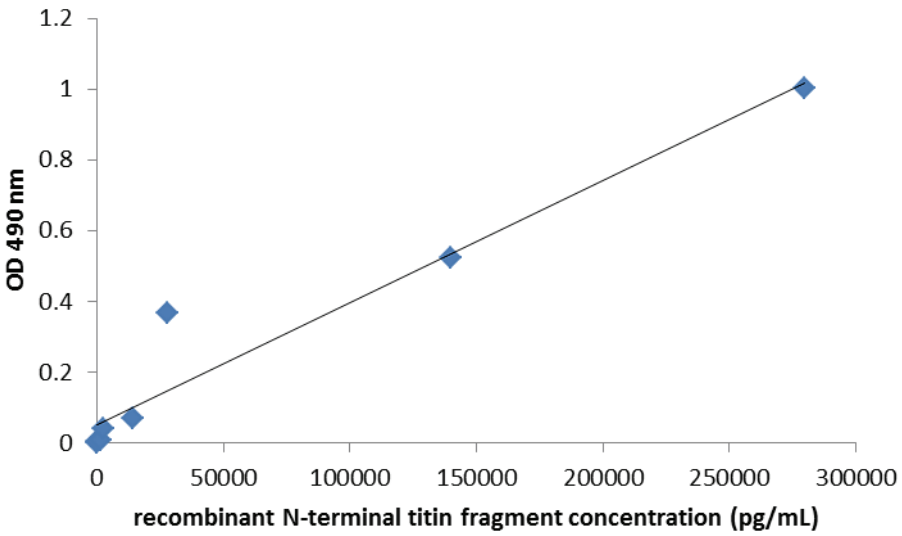


Figure 6. Recombinant N-terminal titin concentrations measured by ELISA.

Based on Western blot analysies, ELISA was performed using an anti-human titin monoclonal antibody 7D3 and 2B3, a rat anti-mouse IgG1 conjugated with HRP. Color intensity was measured by a microplate reader at 490 nm.

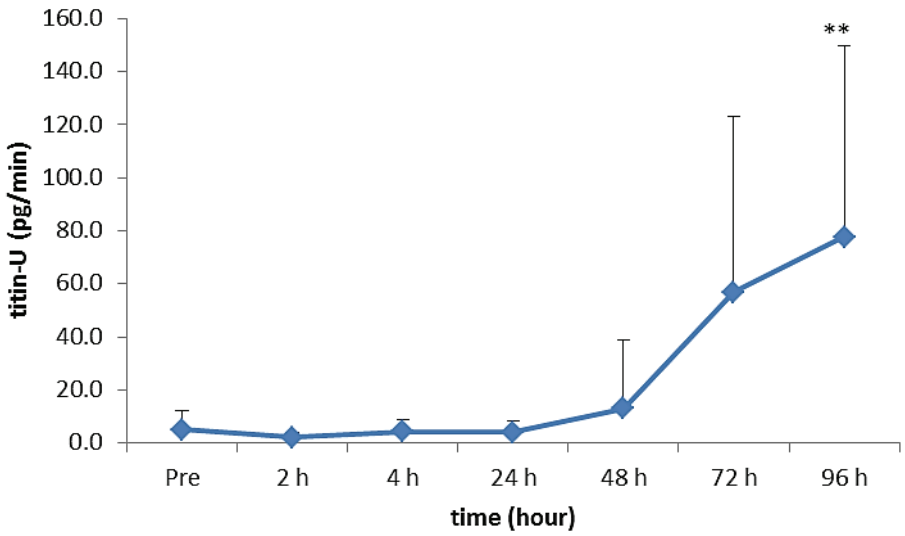


Figure 7. Urinary titin fragment excretion rate after eccentric exercise.
Urinary titin fragment concentrations significantly increased at 96 h after the exercise. Data were corrected for the gross amount of urine per minute. Values were expressed as means ± SD (n=9). ** $p<0.01$: vs Pre.

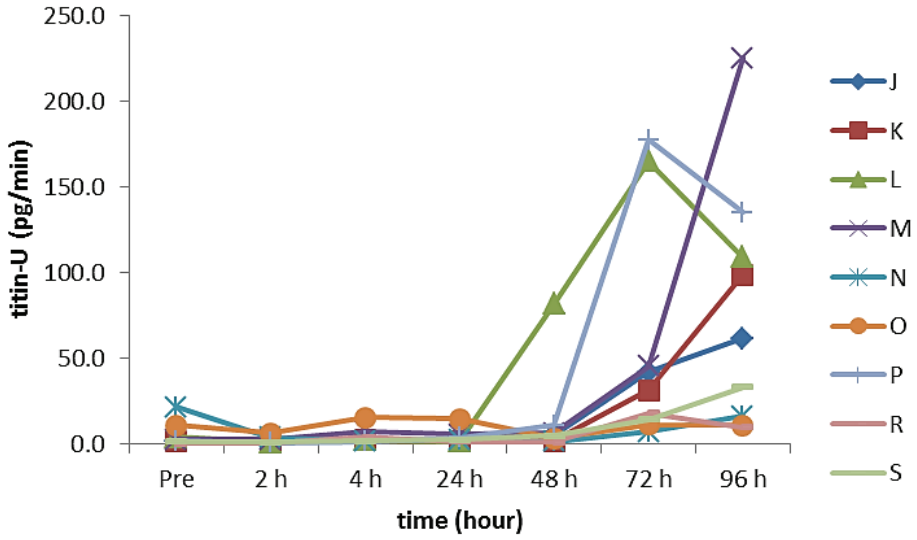


Figure 8. Individual urinary titin fragment excretion rate after eccentric exercise. Data were corrected for the gross amount of urine per minute.

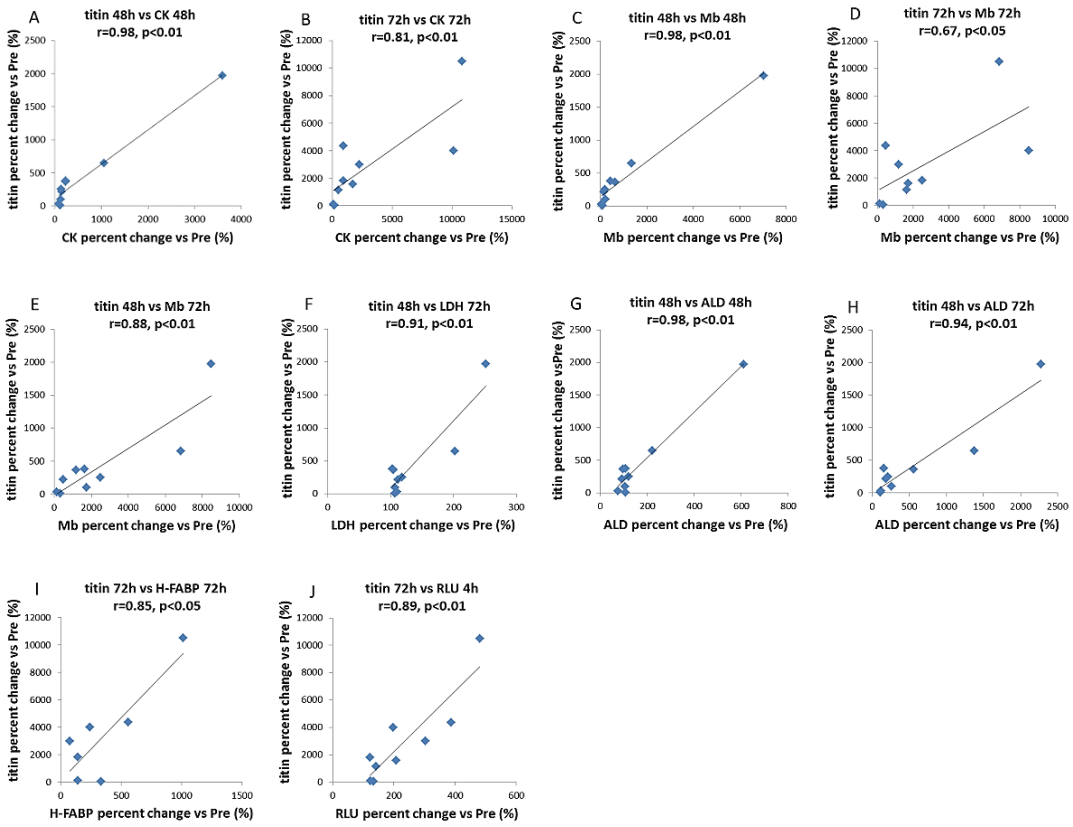


Figure 9. Relationships between changes in urinary titin fragment excretion rate and changes in other biomarkers.

Values were expressed as relative to Pre (n=9). There were positive correlations between changes in the titin fragment and CK at 48 hours: r=0.98, p<0.01 (A), the titin fragment and CK at 72 hours: r=0.81, p<0.01 (B), the titin fragment and Mb at 48 hours: r=0.98, p<0.01 (C), the titin fragment and Mb 72 hours: r=0.67, p<0.05 (D), the titin fragment 48 hours and Mb72 hours: r=0.88, p<0.01 (E), the titin fragment and LDH (48 hours and 72 hours): r=0.91, p<0.01 (F), the titin fragment 48 hours and ALD at 72 hours after eccentric exercise: r=0.94, p<0.01 (H), the titin fragment and H-FABP at 72 hours: r=0.85, p<0.05 (I), the titin at 72 hours after the eccentric exercise and neutrophil producing activity of ROS: r=0.89, p<0.01 (J).

Abbreviations: CK: creatine kinase, Mb: myoglobin, LDH: lactate dehydrogenase, ALD: aldolase, H-FABP: heart-type fatty acid-binding protein, RLU: relative light unit (Luminol-dependent chemiluminescence measured as neutrophil producing activity of reactive oxygen species)

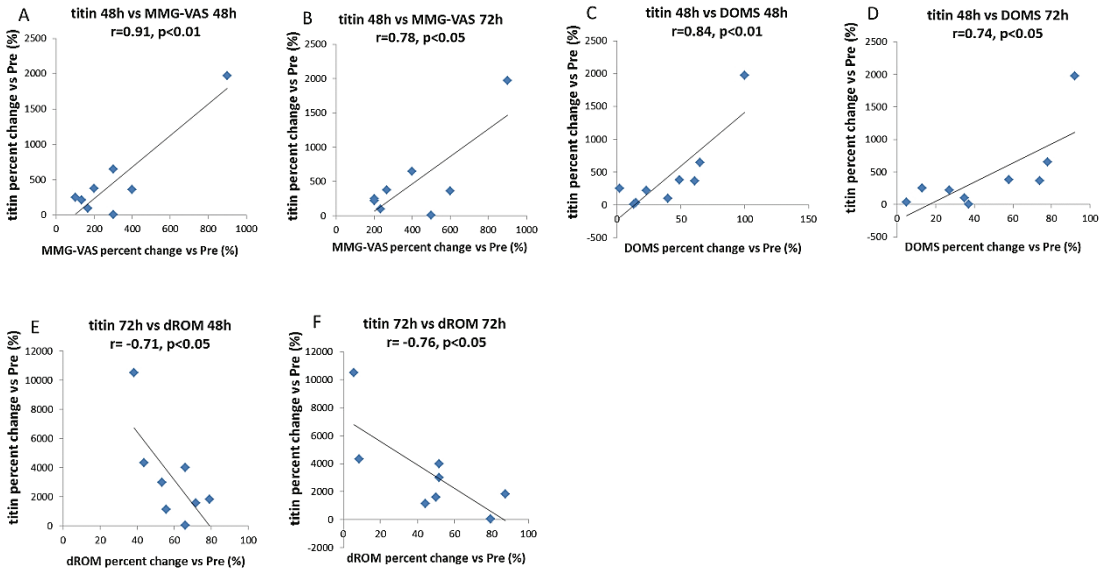


Figure 10. Relationships between changes in urinary titin fragment excretion rate and changes in muscle soreness (MMG-VAS and DOMS) and range of motion of ankle joint (dROM).

Values were expressed as relative to Pre (n=9). There were positive correlations between changes in the titin fragment and MMG-VAS at 48 hours: r=0.91, p<0.01 (A), the titin fragment 48 hours and MMG-VAS 72 hours: r=0.78, p<0.05 (B), the titin fragment and DOMS at 48 hours: r=0.84, p<0.01 (C) the titin fragment 48 hours and DOMS 72 hours: r=0.74, p<0.05 (D). There were negative correlations between the titin fragment 72 hours and dROM 48 hours: r=-0.71, p<0.05 (E) and the titin fragment and dROM at 72 hours after the eccentric exercise: r=-0.76, p<0.05 (F).

Abbreviations: MMG: the middle point of medial gastrocnemius, VAS: visual analogue scale, DOMS: delayed-onset muscle soreness, dROM: range of motion in the dorsal flexion.

3-4. DISCUSSION

In the present study, we identified the N-terminal fragment of titin molecule in urine by proteomic analysis after an acute eccentric exercise. Then, we verified the existence of the titin fragment by Western Blotting and established an ELISA to measure the urinary titin fragment concentrations. As a result, we found that the urinary titin fragment significantly increased >48 hours after the eccentric exercise, and the changes in the titin fragment were strongly associated with changes in both muscle symptoms and serum muscle damage markers.

To our knowledge, this is the first report showing a titin fragment in urine. Titin is the largest protein, with a molecular weight up to 3,700 kDa, in humans, and is known as a sarcomere structural protein of cardiac and skeletal muscle (13). The I-band region of titin underlies the myofibril elasticity, and links the Z-line to the M-line, keeping the A-line in the center of sarcomere of the appearance of cross-striations in skeletal and cardiac muscle. Titin filaments overlap with N-terminal ends of Z-lines, and also the C-terminal titin regions from adjacent half-sarcomeres overlap in the M-line (2, 10). An understanding of the functions of titin has recently been rapidly advanced. It is suggested that titin has various functions such as a molecular ruler for sarcomere assembly, the main source of passive elasticity in the sarcomere and a hub for signal transduction (14, 31). Since, titin plays an important role in passive and active skeletal muscle contractility, titin contributes to muscle weakness in titin-associated skeletal muscle diseases such as spasticity and disuse atrophy (23).

The urinary excretion rates of the titin fragment were significantly correlated with muscle damage symptoms such as DOMS and reduced range of motion. We showed significant correlations between changes in the urinary excretion rate of the titin fragment and changes in serum CK, Mb, LDH and ALD after the acute eccentric exercise. The increase in circulating muscle enzymes may indicate a level of skeletal myofiber necrosis and tissue damage following

muscle injury (19, 30). Using immunohistochemical analysis Yu et al. reported focal loss of some myofibrillar proteins, including titin, in human soleus muscle 2-3 days after eccentric exercise (35). Thus, our finding suggests that urinary titin fragment may come from damaged muscle. These results suggest that the urinary titin fragment has potential as a sensitive biomarker for muscle damage. However, further research is needed to clarify whether presence of the urinary titin fragment depends on gender, muscle mass, exercise intensity and duration, and if there is a remarkable inter-individual variation in the urinary titin fragment concentration.

It has been discovered that titin isoforms are expressed differently in cardiac and skeletal muscles, N2A (up to 3700 kDa) in skeletal muscle (2), N2B (300 kDa) and N2BA (3300 kDa) (10) in cardiac muscle. The titin fragment detected in this study, was estimated 200 amino acids long from the N-terminal based on their amino acid sequences and molecular weights. Since there are no variations of amino acid sequences in these isoforms in the N-terminal region, we could not exclude the possibility that the fragment comes from cardiac muscle. However, it is very likely that the titin fragment was released from damaged skeletal muscle as following a marathon plasma cardiac creatine kinase (CK-MB) remained within the normal range, whereas total CK activity significantly increased (22).

The mechanism of urinary excretion of the titin fragment is currently unknown. During urine formation, glomerular filtration allows for the passage of water and solutes into the urinary space while retaining plasma proteins. It may be possible that the N-terminal fragment of titin was excreted into urine due to fragmentation of the titin molecule in damaged muscle, as the molecular size is reduced. Further research is needed to clarify the mechanisms of the titin cleavage and urinary excretion of the titin fragment.

In conclusion, the urinary titin fragment has potential as a sensitive and noninvasive biomarker for muscle damage, since it reflects changes in the serum muscle damage markers

and muscle symptoms after exercise-induced muscle damage.

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Chapter IV

Concluding remarks

In this study, we investigated changes in markers of muscle damage and inflammation in plasma and urine after the acute eccentric one-leg calf-raise exercise. We found changes in muscle damage markers without changes in the other inflammatory mediators, suggesting the exercise-induced muscle damage does not cause systemic inflammation. We confirmed that there are poor correlations between changes in muscle damage markers and the magnitude of muscle symptoms. We could not detect any changes in the pro-inflammatory cytokines and the soluble adhesion molecules in response to the eccentric exercise. Neither, plasma/urine levels of the organ damage markers including fatty-acid binding proteins (FABPs) nor neutrophil mobilization and migration were also changed by the acute eccentric exercise. These findings suggested that further researches are necessary to establish sensitive indicators for the exercise-induced muscle damage.

We then tried to search for urinary proteins that are responsible to the eccentric exercise using proteomic analysis, and identified the N-terminal fragment of titin, which increased after the eccentric exercise. We established an ELISA to measure the urinary titin fragment. We measured the titin fragment in the urine samples the eccentric exercise from 9 individuals and found that the titin fragment increased after the eccentric exercise. These findings may suggest that the urinary titin fragment has potential as a sensitive and non-invasive biomarker for muscle damage.

Chapter V

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研究業績書

種 類 別	題名、　発表・発行掲載誌名、　発表・発行年月、　連名者（申請者含む）
a:論文	○Eccentric exercise-induced delayed-onset muscle soreness and changes in markers of muscle damage and inflammation: Exerc Immunol Rev. 19: 72-85, 2013. **Kazue Kanda**, Kaoru Sugama, Harumi Hayashida, Jun Sakuma, Yasuo Kawakami, Shigeki Miura, Hiroshi Yoshida, Yuichi Mori, Katsuhiko Suzuki. ○Evaluation of serum leaking enzymes and investigation into new biomarkers for exercise-induced muscle damage: Exerc Immunol Rev. **Kazue Kanda**, Kaoru Sugama, Jun Sakuma, Yasuo Kawakami, Katsuhiko Suzuki. (Exerc Immunol Rev. 20: 39-54, 2014)
e:その他（論文）	運動による自覚的疲労度とストレスホルモン・サイトカインとの関連性. 日本補完代替医療学会誌. 8 (2): 67-73, 2011. 石渡智子, 森藤雅史, 石島寿道, 青山友子, 菅間薫, **神田和江**, 鈴木克彦, 樋口満. 月経周期と持久性運動による唾液の酸化ストレス指標の変動. 日本補完代替医療学会誌. 7 (2): 125-128, 2010. 林田はるみ, 志村まゆら, 菅間薫, **神田和江**, 鈴木克彦.
e:研究報告書	好中球機能･サイトカイン産生能からみた各種人工血小板素材の in vitro 評価.H12(ADP)リポゾームの人工血小板としての前臨床評価（効力と安全性）. 平成 21 年度厚生労働科学研究費補助金（創薬基盤推進研究事業：政策創薬総合研究事業）分担研究報告書. 鈴木克彦, 鈴木洋子, 菅間薫, **神田和江**, 沢田秀司, 勝野俊介, 武岡真司. 好中球機能を用いた血小板減少・外傷性出血における人工血小板投与の in vivo 評価. H12(ADP)リポゾームの人工血小板としての前臨床評価（効力と安全性）. 平成 22 年度厚生労働科学研究費補助金（創薬基盤推進事業：政策創薬総合研究事業）分担研究報告書. 鈴木克彦, 木下学, 西川可穂子, 宮崎裕美, 齊藤大蔵, **神田和江**, 沢田秀司, 溝上翼, 菅間薫, 勝野俊介, 武岡真司. 血小板減少ウサギモデルにおける外傷性出血に対する人工血小板投与が好中球機能に及ぼす影響. 平成 23 年度厚生労働科学研究費補助金（政策創薬総合研究事業）H12(ADP)リポソームの人工血小板としての前臨床評価（効力と安全性）（分担）研究報告書：その 2, 71-75, 2012.　鈴木克彦, 木下学, 西川可穂子, 宮崎裕美, 齊藤大蔵, 沢田秀司, **神田和江**, 溝上翼, 新井愛美, 土井麻実, 藤山敦史, 武岡真司.
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