CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Disuse Atrophy Accompanied by Intramuscular Ectopic Adipogenesis in Vastus Medialis Muscle of Advanced Osteoarthritis Patients

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Muscle dysfunction is the most important modifiable mediating factor in primary osteoarthritis (OA) because properly contracting muscles are a key absorber of forces acting on a joint. However, the pathological features of disuse muscle atrophy in OA patients have been rarely studied. Vastus medialis muscles of 14 female patients with OA (age range, 69 to 86 years), largely immobile for 1 or more years, were obtained during arthroplastic surgery and analyzed histologically. These were compared with female patients without arthritis, two with patellar fracture and two with patellar subluxation. Areas occupied by myofibers and adipose tissue were quantified. Large numbers of myofibers were lost in the vastus medialis of OA patients. The loss of myofibers was a possible cause of the reduction in muscle strength of the operated on knee. These changes were significantly correlated with an increase in intramuscular ectopic adipose tissue, and not observed in knees of nonarthritic patients. Resident platelet-derived growth factor receptor α -positive mesenchymal progenitor cells contributed to ectopic adipogenesis in vastus medialis muscles of OA patients. The present study suggests that significant loss of myofibers and ectopic adipogenesis in vastus medialis muscles are common pathological features of advanced knee OA patients with long-term loss of mobility. These changes may be related to the loss of joint function in patients with knee OA. (*Am J Pathol* 2017, 187: 2674–2685; <https://doi.org/10.1016/j.ajpath.2017.08.009>)

Osteoarthritis (OA) is a complex syndrome and includes various symptoms and signs with multiple causes. It has been proposed that muscle dysfunction is the most important modifiable mediating factor for primary OA because properly contracting muscles are the main force absorber for a joint.¹ In addition, clinical studies support the hypothesis that exercise-related knee OA is attributable to muscle dysfunction rather than wear and tear of the articular cartilage.² Muscle function can be regained because the skeletal muscle stem cells, called muscle satellite cells, retain high regeneration potential.³ Furthermore, going beyond therapies that target impairment of joint tissues, exercise therapy, including a progressive strengthening program mainly targeting the quadriceps muscles, can reduce pain and

improve function in subjects with knee OA.^{2,4} The contribution of quadriceps muscles to functional limitations in knee OA has been studied because quadriceps muscles control knee extension during ambulation. Furthermore, greater quadriceps strength protects against cartilage loss.^{4–8} Quadriceps muscles are significantly impaired in subjects with knee OA, and the muscle strength of the quadriceps is a major determinant of physical function.⁴ In addition, patients with greater quadriceps muscle strength have less knee pain.^{5,9,10} Although quadriceps muscle

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impairment is a potential therapeutic target for knee OA, possible pathological changes in quadriceps muscles of OA patients remain largely unknown. Such studies are required to understand what happens in muscles, to improve exercise therapy, and to develop appropriate rehabilitation programs.

Preoperative quadriceps muscle strength plays a dominant role in functional ability for up to 1 year after total knee arthroplasty.⁷ This suggests that the atrophy of quadriceps muscles attributable to disuse is a critical modifiable factor that contributes to the functional limitations of patients after total knee arthroplasty. Although a rapid and substantial reduction of knee pain after total knee arthroplasty is reported in OA patients, 37% of them have limited functional improvement 1 year after surgery.¹¹ Moreover, significant deficits in quadriceps muscle strength may remain unresolved for years after surgery.^{6,8} However, a progressive strengthening program mainly targeting the quadriceps muscles can result in significant functional recovery after surgery.¹²

Prolonged periods of muscle disuse attributable to limb immobilization, chronic bed rest, and denervation result in a significant loss of muscle mass and strength. The disuse atrophy of muscles has been studied in animal models and humans.^{13,14} Rat hind-limb suspension and limb immobilization models demonstrate that disuse muscle atrophy occurs as a result of both a decrease in muscle protein synthesis and an increase in the rate of proteolysis,¹⁵ resulting in the loss of muscle mass. Recent advances in molecular and cellular biology have suggested that oxidative stress and proinflammatory cytokines also play a role.^{16,17}

Although animal studies are of much value, it is essential to study the skeletal muscles of OA patients. This is because the clinical history of muscle disuse in these patients is often more than a few years, sometimes decades, whereas animal experiments are of short duration, often only weeks or months. Therefore, the pathological features of disuse muscle atrophy in OA patients may not be similar to those of animal models. Previous studies^{18,19} have reported selective atrophy of type 2 myofibers and morphological changes with multifactorial etiology in vastus medialis muscles of OA patients.

In the present study, biopsy samples from vastus medialis muscles of OA patients with long-term loss of mobility were compared with those of nonarthritic patients and examined for evidence of pathology in relationship to muscle function. In OA patients, we observed pathological changes that included significant loss of myofibers with robust adipogenesis in these muscles associated with loss of muscle function.

Materials and Methods

Subjects

Subjects provided written informed consent for all experimental procedures. Ethical approval for the present study

was granted by the ethical committee of the National Center for Geriatrics and Gerontology and Toyota Kosei Hospital.

Small fragments of vastus medialis muscles were obtained from 11 patients with knee OA who underwent arthroplastic surgery, including 10 total knee arthroplasties and 1 unicompartamental knee arthroplasty at the Department of Orthopedic Surgery at the National Center for Geriatrics and Gerontology (Oobu, Japan). The average age of the patients who were subjected to quantitative analyses was 79.7 (range, 71 to 86) years. Corresponding muscle biopsy samples of nonarthritic patients were obtained from two patients with patellar fracture and two patients with patellar subluxation. Samples from patella fracture patients were obtained at the time of the removal of wiring, approximately 1 year after the initial operation for the fracture treatment. One of the cases was operated on at Toyota Kosei Hospital (Toyota, Japan). The ages of these patients were 81, 68, 60, and 40 years, respectively.

To determine the activity/mobility of patients before surgery, the activities of daily living, particularly stair climbing, were assessed by a medical interview before the surgical procedures. Questions for stair climbing were as follows: "Are you able to climb up and down stairs without the use of the handrails?" and "Do you feel pain when climbing up and down stairs?"

To examine the impact of long-term inactivity on muscle, vastus medialis muscles of arthritis patients were compared with those of patellar fracture/subluxation patients. Muscle biopsy of patients was approved by the ethical committee of the National Center for Geriatrics and Gerontology and Toyota Kosei Hospital. Written informed consent was obtained from each patient before surgery on the OA knee. A small piece of skeletal muscle, approximately 5 to 6 mm long

Figure 1 Schematic view of right knee for sampling of vastus medialis (VM) muscle. O'clock position is shown on the right. The **dashed circle** represents the patella. **Dashed lines** represent 1- and 2-o'clock positions. QT, quadriceps tendon; VL, vastus lateralis.

and 3 to 4 mm wide (average, 600 mg) was dissected from the portions within 1.5 cm from the edges of the vastus medialis muscle. It was attached at the 1- to 2-o'clock position of the right patella of the treated side during knee surgery (Figure 1). Dissected biopsy samples were kept at 4°C up to 3 hours until histological analysis. Muscle biopsy was performed unilaterally on the treated side because samples were not obtained from the untreated side for ethical reasons. Muscle biopsy provides information about correlations between pathological changes and duration of pain. In the present study, only female patients, aged 40 to 86 years, were selected to avoid possible sex differences. Vastus medialis muscle, synovium, and ligament were obtained from three OA patients: 7M15, a 69-year-old female; 7A12, a 74-year-old female; 7A26, an 85-year-old female.

For patellar fracture and patellar subluxation patients, the muscle biopsy was conducted at the time of surgery to remove the wires used for the initial treatment of the fracture and patella alignment, respectively. All OA patients revealed a grade IV change, according to the Kellgren-Laurence radiographical classification.²⁰

Measurement of Knee Extension Strength

Maximal patient knee extension strength was measured before surgery in an upright sitting position with the knee and hip flexed 90 degrees. The patient's lower leg was

wrapped with a 4-cm-wide strap, and she was instructed to extend her knee and pull the strap up as hard as possible.²¹ The strength was measured by a force gauge (product number ZP-500N; IMADA Co, Ltd, Toyohashi, Japan) connected to the strap. Knee extension strength was measured twice, and the greater value was recorded for the analyses. The intraclass correlation coefficient was 0.988. Also, significant correlations were seen between this measure and that obtained by an isokinetic dynamometer (KinCom 500H; Isokinetic International, Chattanooga, TN). Muscle strength of affected joints has been reported to be significantly reduced compared with unaffected joints.²²

Histological and Immunofluorescence Analysis

A small piece of muscle tissue (approximately 5 × 10 × 5 mm), synovium, or ligament was frozen in isopentane that was precooled with liquid nitrogen, as previously described.²³ Frozen tissues were cut into sections (7 μm thick) with a cryostat (Leica Microsystems, Wetzlar, Germany), and then subjected to immunofluorescence analysis and staining with hematoxylin and eosin. Numbers of sections obtained from different parts of the same samples were determined to show similar histological morphology. For immunofluorescence analysis, serial frozen sections were fixed with 4% para-formaldehyde in phosphate-buffered saline for 5 minutes. Then, they were incubated with mouse monoclonal antibodies

Table 1 Pathological Features and Knee Extension Strength of Patients with Knee Injury

Patient identification	Age, years	Occupied area, %			Maximal knee extension strength, kg		Body weight, kg	Disease	Duration of pain, years	Stair climbing*	Operation
		Myofiber	Adipose tissue	Interstitial tissue	Operated on side	Untreated side					
3F04	68	82.6	6.5	10.9	8.1	15.8	47.4	Patellar fracture	1	Yes; no	Wire removal
5A17T	60	80.5	4.1	15.4	ND	ND	ND	Patellar fracture	1	Yes; no	Wire removal
4J23	81	88.5	2.7	8.8	ND	ND	ND	Patellar subluxation	0	Yes; no	Patella alignment
5M20	40	78.6	2.4	19.0	ND	ND	ND	Patellar subluxation	3	Yes; yes	Patella alignment
2A29	77	72.0	13.8	14.2	13.6	13.9	51.8	Osteoarthritis	6	No; no	TKA
2S12	80	61.7	24.7	13.5	16.8	14.3	59.3	Osteoarthritis	5	No; yes	TKA
2003	79	46.6	29.6	23.8	13.9	13	51.1	Osteoarthritis	5	No; yes	TKA
3F13	71	62.8	37.2	Other†	14.7	19	92.8	Osteoarthritis	4	Unable	TKA
3A03	82	72.3	27.7	Other†	25.6	20.9	46.9	Osteoarthritis	1.5	No; yes	TKA
3A17	78	68.8	31.2	Other†	14.5	13.8	54.6	Osteoarthritis	10	No; yes	TKA
3A24	82	26.2	47.2	26.6	8	12.1	49.8	Osteoarthritis	10	No; yes	TKA
3M29	73	79.6	20.4	Other†	16.1	14.9	53.6	Osteoarthritis	3	No; no	TKA
3J03	83	61.0	39.0	Other†	15.4	18.5	39.6	Osteoarthritis	20	No; yes	TKA
3J24	86	72.0	28.0	Other†	6.6	9.3	43.2	Osteoarthritis	5	No; yes	UKA
3J31	86	44.2	35.7	20.1	13.2	12.3	37.4	Osteoarthritis	20	No; no	TKA

All osteoarthritis patients revealed grade IV change, according to the Kellgren-Laurence radiographical classification.
*Without handrails; pain (reported by patients in medical interviews).
†Not determined because the occupied area was <5%.
ND, not determined; TKA, total knee arthroplasty; UKA, unicompartmental knee arthroplasty.

to the following: human CD31 (clone WM59; 1:100; eBioscience, San Diego, CA), α -smooth muscle actin (clone 1A4; 1:400; Sigma, St. Louis, MO), myosin heavy chain type IIa and IIx (clone WB-MHCf; 1:20; Leica, Nussloch, Germany), myosin heavy chain type I (clone WB-MHCs; 1:20; Leica), goat antibody to platelet-derived growth factor receptor α (PDGFR α ; 1:200; R&D Systems, Minneapolis, MN), and rabbit antibody to perilipin (1:250; Sigma) at 4°C overnight. They were then incubated with Cy3- or Alexa Fluor 488-labeled antibody to mouse IgG, Cy3- or Alexa Fluor 488-labeled antibody to goat IgG (Jackson ImmunoResearch Laboratories, Bar Harbor, ME), and Cy3- or Alexa Fluor 488-labeled antibody to rabbit IgG (Invitrogen, Carlsbad, CA) at recommended dilutions for 1 hour. The sections were mounted in SlowFade Gold antifade reagent with DAPI (Invitrogen). In indicated experiments, specimens were processed for immunofluorescence analysis, followed by staining with hematoxylin and eosin. Samples were examined using a model BX50 Olympus microscope with a charge-coupled device (DP70; Olympus, Tokyo, Japan) or a confocal laser scanning microscope system LSM700 (Carl Zeiss, Oberkochen, Germany). Tiling images were captured using a fluorescence microscope BZ-9000 (Keyence, Osaka, Japan). Areas occupied by myofibers, lipids, and interstitial tissue were quantified from the images of specimen stained with hematoxylin and eosin using Lumina Vision software version 3.7 (Mitani Co, Tokyo, Japan). Correlation with the occupied areas was examined using the Pearson product-moment correlation coefficient (Pearson's coefficient) test.

Results

Patient Characteristics

In medical interviews, the two patellar fracture patients reported little pain after the initial fracture surgery. One of the two patellar subluxation patients did not report pain before the surgery, and the other reported slight pain when standing a long time at work. The duration of inactivity of one of the patellar subluxation patients was 3 years, and those of the other three patients were <1 year. In contrast, 11 OA patients reported pain for >1 year (average, 8.1 years) and greatly reduced activity before the arthroplastic surgical procedures. The medical interviews revealed that the most significantly impaired activity of daily living in OA patients was stair climbing (Table 1). Nonarthritic patients (two patellar fracture and two patellar subluxation patients) climbed stairs without handrails, although one of the patellar subluxation patients reported pain on descending stairs. One OA patient was unable to climb stairs. Ten OA patients reported that they needed to use handrails to climb stairs. These patients also reported putting both feet on each step of the stairs. In addition, 7 of the 10 OA patients also reported pain when stair climbing, whereas 3 OA patients did not report this pain. Thus, mobility was impaired in OA patients. Therefore, it is hypothesized that the skeletal muscles we examined of patients who experienced much reduced activity for >1 year would be affected by prolonged immobility.

Figure 2 Macroscopic view of lower extremity muscles. **A–C:** Vastus medialis obtained from a patient with patellar fracture (3F03; **A**) and patients with osteoarthritis [OA; 3F13 (**B**) and 2S12 (**C**)]. Patient information was shown in Table 1. **C:** Dotted line represents a muscle bundle. Specimens were stained with hematoxylin and eosin. **D:** Quantification of area occupied by myofibers (MFs) and adipose tissue (Ad) in vastus medialis muscles of patellar fracture/subluxation patients (open column) and OA patients (closed column). Data expressed as means $\pm$ SD (**D**). * P < 0.05, *** P < 0.001. Scale bars: 500 μ m (**A–C**). Original magnification: $\times 6$ (**A**); $\times 8$ (**B**); $\times 40$ (**C**). Endo, endomysial adipose tissue; In, interstitial tissue; Peri, perimysial adipose tissue.

Loss of Myofibers in Vastus Medialis Muscle of Knee OA Patients

Areas occupied by myofibers were determined by a low-power microscopic view of the pathological features of vastus medialis muscles (Figure 2, A and B, and Supplemental Figure S1). Myofibers occupied $82.6\% \pm 4.3\%$ of the muscles of patellar fracture/subluxation patients (Figure 2, A and D, Supplemental Figure S1, and Table 1). In contrast, myofiber-occupied areas significantly decreased to $60.7\% \pm 15.7\%$ of the muscles of OA patients compared with nonarthritic patients ($P = 0.019$) (Figure 2, B and D, Supplemental Figure S1, and Table 1).

Ectopic interstitial adipogenesis was present in the perimysium and endomysium of OA patients (Figure 2C). Muscles of OA patients included adipose (fatty) tissue that occupied $30.4\% \pm 9.2\%$ of the whole muscle area (Figure 2, B and D, and Table 1). In contrast, adipose tissue occupied only $3.9\% \pm 1.9\%$ of the area in the patellar fracture/subluxation patients ($P = 0.00009$) (Figure 2, A and D, and Table 1). Necrotic and regenerating myofibers were rarely found in muscles of OA patients and nonarthritic patients. In addition, infiltrating immune cells were rarely identifiable in the muscles in either group. Collectively, there was no sign of acute muscle degeneration/regeneration or inflammation in the vastus medialis muscles of OA patients.

Loss of Muscle Fibers in OA Patients and Onset of Adipogenesis

The muscle areas that lost myofibers were occupied mostly by ectopic adipose tissue in OA patients (Figure 3A). The Pearson product-moment correlation coefficient between the area of myofibers and the area of adipose tissue was -0.82 ($P = 0.00019$) (Figure 3B). The results indicated that loss of myofibers was accompanied by ectopic interstitial adipogenesis in OA patients. The area occupied by muscle fibers in OA patients decreased, depending on the duration of pain (Figure 3C). The Pearson product-moment correlation coefficient between the area occupied by myofibers and the duration of pain was -0.61 ($P = 0.016$). The results suggest that long-term loss of mobility decreased the number of myofibers.

Muscle fiber type was determined in vastus medialis muscle accompanied by ectopic adipogenesis in OA patients. Muscles of patellar fracture/subluxation patients (3F04, 5A17T, 4J23, and 5M20) and OA patients (3A17, 7M15, 7A12, and 7A26) were analyzed by immunofluorescence staining. Type 2 myofibers occupied $72.3\% \pm 16.6\%$ of myofibers in muscles of patellar fracture/subluxation patients (Figure 4, A–D). In contrast, type 2 myofiber-occupied areas decreased to $55.5\% \pm 7.6\%$ of the whole myofiber-occupied area of OA patients (Figure 4, E–L). However, selective decrease of fast myofibers was not significant in vastus medialis muscles of OA patients

Figure 3 Correlations between quantities of myofibers and ectopic adipogenesis. **A:** Occupied area of vastus medialis muscles with myofibers (black), adipose tissue (gray), and interstitial tissue (white). Muscles obtained from 4 nonarthritic patients and 11 osteoarthritic (OA) patients were pathologically analyzed. Identification numbers of patients are shown. **B:** The area occupied by adipose tissue decreased along with the increase of the area occupied by myofibers in vastus medialis muscles of 4 nonarthritic (closed circle) and 11 OA (open circle) patients. The Pearson product-moment correlation coefficient between the area occupied by myofibers and the area occupied by adipose tissue was -0.82 . $P = 0.0009$. **C:** The area occupied by myofibers in vastus medialis muscles of 4 nonarthritic (closed circle) and 11 OA (open circle) patients decreased along with the increase of the duration of pain of the damaged knee. The Pearson product-moment correlation coefficient between the area of myofiber and the duration of pain was -0.61 . $P = 0.016$.

($P = 0.11$), probably because fast myofibers decreased in number age dependently (Figure 4M). The Pearson product-moment correlation coefficient between the area occupied by fast myofibers and the age of patients was -0.77 ($P = 0.024$). Two muscles with severe atrophy (7A12 and 7A26) contained 48.6% and 49.5% of type 2 myofibers, whereas vastus medialis muscles with moderate atrophy (3A17 and 7M15) contained 61.0% and 63.0%. The decrease of type 2 myofibers was likely to depend on the severity of muscle atrophy accompanied by ectopic adipogenesis in OA patients. The results indicate that selective atrophy of type 2 myofibers in vastus medialis muscles significantly depends on age of patients and is also

Figure 4 Selective loss of type 2 myofibers in vastus medialis muscles of osteoarthritic (OA) patients. **A–L:** Sections of the vastus medialis obtained from patients with patellar fracture (5M20; **A–D**) and OA [7M15 (**E–H**) and 7A12 (**I–L**)] were stained with hematoxylin and eosin (**A**, **E**, and **I**) and processed for immunofluorescence analysis (**B–D**, **F–H**, and **J–L**). Patient information is given in [Table 1](#) and [Materials and Methods](#). **B**, **F**, and **J:** Adipocytes were identified with specific antibodies to perilipin. Fast (type 2) myofibers and slow (type 1) myofibers were identified with specific antibodies to myosin heavy chain type IIa and IIx (**C**, **G**, and **K**) and type I (**D**, **H**, and **L**), respectively. **C**, **G**, and **K:** Percentages of type 2 myofiber-occupied areas are shown. **A** and **B**, **C** and **D**, **E** and **F**, **G** and **H**, **I** and **J**, and **K** and **L:** Neighboring sections were analyzed. **M:** The area occupied by type 2 myofibers in vastus medialis muscles of four nonarthritic (closed circle) and four OA (open circle) patients decreased along with the age of patients. The Pearson product-moment correlation coefficient between the area of fast myofiber and the age was -0.771 . $P = 0.024$. Scale bars: 0.5 mm (**A–L**). Original magnification, $\times 6$ (**A–L**).

involved in the loss of myofibers accompanied by ectopic adipogenesis.

In addition, associations of pathological features with muscle strength were analyzed in 11 OA patients ([Table 1](#)). Muscle strength is primarily dependent on the amounts of myofibers. The loss of myofibers may be related to the decrease of muscle strength. However, we did not find a significant correlation between maximal knee extension strength of the operated on side with the area of myofibers for 11 OA patients. Other possible factors, including nutrition, muscle weakness by aging, and comorbidity, may also affect muscle performance of the patients in a subject-dependent way.

Sites of Ectopic Adipogenesis

Ectopic adipogenesis in the vastus medialis muscles ([Figure 5](#), **A–L**) of OA patients was observed exclusively in the perimysium and endomysium of muscles. Oil red O staining showed no lipid deposition within myofibers (data

not shown). Sporadic adipogenesis was found between intact myofibers ([Figure 5](#), **A–C**), and ectopic adipose tissue was increased in the endomysium ([Figure 5](#), **D** and **E**) and perimysium ([Figure 5, **E** and **F**\). Ectopic adipose tissue exhibited similar morphologies in both sporadic and collective adipogenesis \(\[Supplemental Figure S2\]\(#\), **B–D**\). In contrast, normal muscle did not contain ectopic adipose tissue \(\[Figure 2A\]\(#\) and \[Supplemental Figure S2A\]\(#\)\). Interstitial tissue with unidentified cells and a mass of extracellular matrix was often found adjacent to ectopic adipose tissue \(\[Figure 5\]\(#\), **G–I**\). Ectopic adipose tissue was robustly developed and occupied the vacant space left by the loss of myofibers \(\[Figure 5\]\(#\), **J–L**\). Intramuscular adipose tissue did not connect with peripheral adipose tissues, suggesting that ectopic adipose tissue is derived from resident adipogenic cells in skeletal muscles.](#)

Small atrophic myofibers were also found in the area without adipogenesis ([Figure 5M](#)). Therefore, it is unlikely that ectopic adipogenesis triggers muscle atrophy. In contrast, ectopic adipose tissue was always

Figure 5 Ectopic lipid accumulation in skeletal muscles of osteoarthritic (OA) patients. Vastus medialis muscles of OA patients were analyzed. **A–C:** Ectopic adipogenesis was induced sporadically at perimysium/endomysium of muscles. Three independent specimens are shown. **Arrows** represent sporadic accumulation of ectopic adipose tissue. **D–F:** Ectopic adipose tissue was accompanied by small abortive myofibers. Three independent specimens are shown. **G–I:** Ectopic adipose tissue was often accompanied by interstitial tissue (**asterisks**). Three independent specimens are shown. **J–L:** Most myofibers were lost, and remnant myofibers showed atrophic morphology in ectopic adipose tissue. Three independent specimens are shown. **M–O:** Small atrophic (**white arrow**) and abortive (**black arrow**) myofibers appeared in the absence (**M**) or presence (**N**) of ectopic adipogenesis, whereas the size of myofibers remained constant in the nonatrophic area of the muscle (**O**). Identification numbers of patients are shown. Scale bars: 100 μ m (**A–O**). Original magnification: $\times 50$ (**A–L**); $\times 200$ (**M–O**).

accompanied by clusters of small atrophic myofibers (Figure 5N). The grouped atrophic myofibers were not found in the area without adipose tissue (Figure 5O). The results suggest that atrophy of myofibers is triggered independently of ectopic adipogenesis. In addition, ectopic adipogenesis may play a role in the exacerbation of muscle atrophy.

Hyperplasia of Smooth Muscle of Blood Vessels

The diameter of blood vessels that had the appearance of arteries in the perimysium of the vastus medialis muscles of patellar fracture/subluxation patients was $<100 \mu$ m (Figure 6, A and B). In contrast, extraordinarily large blood vessels, of which diameters were $>100 \mu$ m, were

Figure 6 Hyperplasia of smooth muscle layer of blood vessels in vastus medialis muscles of osteoarthritic (OA) patients. **A** and **B**: Small vessels were found in the muscle perimysium of the patellar fracture patient (3F04). **A** and **B**: The boxed area (**A**) is magnified fourfold (**B**). **C–F**: Large vessels with thick smooth muscle layers developed in ectopic adipose tissue in muscles of four OA patients. **A** and **C–F** are shown at the same magnification. **G–I**: Serial sections of the vastus medialis of an OA patient (3A24) were stained with hematoxylin and eosin (**G**) and processed for immunofluorescence analysis (**H** and **I**). Smooth muscle cells and endothelial cells of enlarged vessels were identified with specific antibodies to smooth muscle actin (**H**) and CD31 (**I**), respectively. **F** and **G**: Interstitial tissue (asterisk) surrounded smooth muscle layers. Identification numbers of patients are shown. Scale bars: 100 μ m (**A–G**). Original magnification: $\times 35$ (**A** and **C–I**); $\times 150$ (**B**). α -SMA, α -smooth muscle actin.

found in perimysium/endomysium occupied by ectopic adipose tissue in muscle of four OA patients (Figure 6, C–F). Immunofluorescence analyses using specific antibodies against CD31 and smooth muscle actin confirmed that these structures were, in fact, blood vessels (Figure 6, G–I). Hyperplasia of smooth muscle layers was often accompanied by connective tissue surrounding vessels (Figure 6, F and G). The maximal diameter of OA patients' vessels was >1 mm (Figure 6F).

Ectopic Adipogenesis of Resident Mesenchymal Progenitor Cells in Vastus Medialis Muscles of Knee OA Patients

Possible involvement of the resident mesenchymal progenitor cells in the fatty degeneration of the vastus medialis muscles of OA patients was determined by immunofluorescence analysis because adipogenesis within skeletal muscle has been shown to derive from resident PDGFR α^+ -mesenchymal progenitor cells.^{24,25} Specific

antibodies to PDGFR α and perilipin identified PDGFR α ⁺-mesenchymal progenitors and perilipin⁺-differentiated adipocytes in the vastus medialis muscles of OA patients. Perilipin⁺-adipocytes were seen in ectopic adipose tissues, and many PDGFR α ⁺ cells were found in interstitial tissues adjacent to perilipin⁺-adipocytes (Figure 7, A and B). The results indicated that interstitial tissue adjacent to ectopic adipose tissue (Figure 3A and Figure 5, G–I) contained PDGFR α ⁺-mesenchymal progenitors. PDGFR α ⁺ cells also accumulated in perivascular connective tissue (Figure 7, C and D). In contrast to the muscle of OA patients, aberrant PDGFR α ⁺ cells did not accumulate in the vastus medialis of the patellar fracture patient (Figure 7, E and F). Moreover, perilipin⁺/PDGFR α ⁺-differentiated adipocytes were not found in muscles of the patellar

fracture patient. These results imply that resident PDGFR α ⁺-mesenchymal progenitor cells contribute to ectopic adipogenesis in vastus medialis muscles of OA patients.

Ectopic Adipogenesis of Mesenchymal Progenitor Cells in Joint Tissues Other than Vastus Medialis Muscles of Knee OA Patients

Adipose-rich phenotype is well known in tissues around the knee joint of OA patients. Ectopic adipogenesis in vastus medialis muscle raised a possibility of adipose deposits in nonmuscle tissues in and around the knee joint of OA patients. To explore whether the disuse atrophy of OA patients is accompanied by ectopic adipogenesis in joint tissues other than vastus medialis muscle, synovium and ligament were obtained from three OA patients.

Ectopic adipose tissue occupied a large area of vastus medialis muscle (Figure 8A). Adipose tissue also occupied a limited area of synovium of the same patient (Figure 8B). The subintimal adipose tissue in synovium was unlikely to be caused by inactivity of the patients because synoviocytes overlie loose connective tissue consisting of fat, collagen, and blood vessels in normal synovium.²⁶ In addition, ectopic adipose tissue was not found in ligament (Figure 8C). The results suggest that the inactivity of OA patients induces ectopic adipogenesis exclusively in vastus medialis muscle but not in synovium and ligament.

Next, possible involvement of the resident mesenchymal progenitor cells in adipogenesis of nonmuscle tissues around the knee joints of OA patients was determined by immunofluorescence analysis. Perilipin⁺-adipocytes were found in adipose tissues, and many PDGFR α ⁺ cells were found in interstitial tissues adjacent to perilipin⁺-adipocytes in synovium and vastus medialis muscles (Figure 8, D and E). In contrast, perilipin⁺-adipocytes or PDGFR α ⁺ cells were not found in ligament of the same OA patient (Figure 8F). The results suggest that resident PDGFR α ⁺-mesenchymal progenitor cells also contributed to adipose tissue that synovium contained in OA patients. However, the subintimal adipose tissue that is usually found in synovium of a normal healthy person²⁶ did not increase in OA patients. Collectively, inactivity of osteoarthritis patients may induce ectopic adipogenesis exclusively in vastus medialis muscles.

Figure 7 Platelet-derived growth factor receptor α -positive (PDGFR α ⁺) mesenchymal progenitor cells in vastus medialis muscles of osteoarthritic (OA) patients. **A–F:** Specimens of an OA patient (3A24; **A–D**) and a patellar fracture patient (3F04; **E** and **F**) were stained with hematoxylin and eosin (**A**, **C**, and **E**) after processing for immunofluorescence analysis (**B**, **D**, and **F**). Mesenchymal progenitor cells and differentiated adipocytes were identified with specific antibodies to PDGFR α (red) and perilipin (green), respectively. Nuclei were stained with DAPI (blue). **Boxed areas** in **A**, **C**, and **E** represent the fields shown in **B**, **D**, and **F**, respectively. **C** and **D:** **Arrows** represent blood vessels. Identification numbers of patients are shown. Scale bars: 50 μ m (**A–F**). Original magnification: $\times 60$ (**A**, **C**, and **E**); $\times 120$ (**B**, **D**, and **F**).

Discussion

Correlations between physical function and quadriceps muscle strength are seen in OA patients.^{6,7} The vastus medialis muscle of OA patients exhibits selective atrophy of type 2 fibers that may reflect pain-related immobilization.¹⁸ However, correlations between histopathological changes and muscle strength in the quadriceps muscle have been unknown in OA patients.

Figure 8 Platelet-derived growth factor receptor α -positive (PDGFR α^+) mesenchymal progenitor cells in joint tissues of osteoarthritic (OA) patients. **A–F:** Macroscopic view of vastus medialis muscles (**A** and **D**), synovium (**B** and **E**), and ligament (**C** and **F**) of an OA patient (7A12) were shown. Specimens of an OA patient were stained with hematoxylin and eosin (**A–C**) and processed for immunofluorescence analysis (**D–F**). Mesenchymal progenitor cells and differentiated adipocytes were identified with specific antibodies to PDGFR α (red) and perilipin (green), respectively. Nuclei were stained with DAPI (blue). **Arrows** represent adipose tissue (Ad) (**B**). Identification numbers of patients are shown. Similar results were also obtained from samples of the other two OA patients (7M15 and 7A26). Scale bars: 0.5 mm (**A–C**); 100 μ m (**D–F**). Original magnification: $\times 9$ (**A**); $\times 5$ (**B**); $\times 10$ (**C**); $\times 60$ (**D–F**).

The present study revealed a significant loss of myofibers in the OA knees. The loss of myofibers may result in attenuation of muscle strength, possibly attributable to disuse atrophy.^{18,27} The present results suggest that the area of myofibers and the muscle strength were not themselves correlated in elderly patients with end-stage OA of the knee, probably because other factors (ie, nutrition, muscle weakness by aging, and comorbidity) also affected the muscle strength in a patient-dependent manner. However, the loss of myofibers may be associated with the decrease of muscle strength.

Previous studies^{13,18,19} report selective atrophy of type 2 myofibers in vastus medialis and vastus lateralis muscles of patients with long-term loss of mobility, and one of the studies also described lipid accumulation in muscles of patients with end-stage OA of the knee.¹⁸ Present studies showed that the loss of myofibers was accompanied by and significantly correlated with ectopic adipogenesis in the vastus medialis muscles of elderly patients with advanced knee OA. In addition, loss of myofibers was selectively induced in type 2 myofibers in vastus medialis muscles of OA patients. However, present results suggest that this is primarily because of aging. We also observed a lack of extended ectopic adipogenesis in the vastus medialis muscles in four nonarthritic patients (Figure 3A). Collectively, the present studies suggest that the loss of myofibers accompanied by ectopic adipogenesis is related to patient inactivity, which is known to accompany loss of knee joint function in OA.^{18,19}

Necrotic or regenerating myofibers were uncommon in muscles of OA patients. No sign of an acute phase of muscle degeneration and inflammation was found in muscles of these patients, although myofibers were

occasionally reduced in size. Therefore, the chronic loss of myofibers in patients may not be attributable to repeated muscle degeneration and/or inflammation, but to long-term disuse atrophy caused by arthritic knee pain with loss of knee function.

In association with the loss of myofibers, ectopic adipogenesis was observed in the perimysium/endomysium of the vastus medialis muscles in OA patients. Small abortive atrophic myofibers were found in areas without adipogenesis. Thus, it is likely that atrophy of myofibers is triggered independently of ectopic adipogenesis, although it may play a role in exacerbating the atrophy of myofibers.

This intramuscular adipogenesis is observed ectopically in several pathological conditions, including advanced cases of Duchenne muscular dystrophy and Bethlem myopathy.^{28,29} Ectopic lipid production outside myofibers may depend on ectopic adipogenic differentiation of resident mesenchymal progenitor cells in skeletal muscles.^{24,25} In the present study, neither lipid accumulation in myofibers^{30,31} nor adipose tissue infiltration^{30,32–34} was detected in the vastus medialis muscles of OA patients.

Previous studies suggest that necrosis of myofibers stimulates adipogenesis of resident mesenchymal progenitor cells.^{24,35} Then, in turn, ectopic adipose tissue may exacerbate the degeneration of myofibers, resulting in a severe loss of myofibers. The fact that we observed that the loss of myofibers was inversely correlated with the appearance of ectopic interstitial adipose tissue clearly demonstrates that there is a close link between these two events. PDGFR α has been known to be a useful marker of adipogenic progenitor cells.³⁶ The present study suggests that PDGFR α^+ -mesenchymal progenitors are involved in fatty degeneration in vastus medialis muscle of OA patients. PDGFR α^+

progenitors may be a target for the intervention for attenuation of the exacerbation of muscle atrophy of OA patients.

We also found hyperplasia of smooth muscle layers of blood vessels in ectopic adipose tissue in skeletal muscles of OA patients. Because the enlarged blood vessels appeared to have an abortive morphology, it may be that the hyperplasia of smooth muscle layers impairs local blood circulation in skeletal muscles. Indeed, hyperplasia of smooth muscle layers might enhance the deterioration of skeletal muscles.

There are some limitations in the present study. Histopathological analysis of cryosections revealed morphological features of muscles in OA patients. However, a possible variation in histological appearance was noted going from the proximodistal aspect of a muscle biopsy and vastus medialis muscle. Muscles derived from patellar fracture/subluxation patients were analyzed as control samples and compared with those of OA patients. However, we were able to conduct knee extension muscle strength analysis on only one nonarthritic patient, thus preventing a comparison of this parameter between the two groups. Other control samples, including age-matched healthy muscles, were not obtained because of ethical reasons in Japan. The number of samples analyzed was small, partly because it was so difficult to make histological sections from atrophied muscle containing large amounts of adipose tissue. Also, all samples came from a Japanese population. Moreover, we did not investigate the relation of pathological changes to the intensity of symptoms in arthritis patients because of the difficulty of quantifying them, considering that the intensity of pain varies over time. The relationships between the pathological images of the vastus medialis muscle and the magnetic resonance imaging/computed tomography images and involvement of ectopic adipogenesis in insulin resistance or fiber type-dependent atrophy are issues for further investigations. Magnetic resonance imaging or computed tomography evaluation at the site of biopsied muscle was not possible from the ordinary section images of knee joints because the vastus medialis muscle at the sites was too thin and round and, thus, not suitable for evaluation.

Our pathological findings revealing fiber loss in the vastus medialis muscle in elderly patients with advanced knee OA are of interest because the loss of muscle fibers is a possible cause of the reduced muscle strength. Although a decrease in muscle mass is supposed to be a main cause of skeletal muscle disability in patients, the loss of muscle fibers would be another significant cause, even if muscle mass was not reduced. Thus, the involvement of muscle fiber loss with dysfunction of skeletal muscle in OA patients should be determined in a future study.

Adipose-rich phenotype is well known in OA patients. Infrapatellar fat pad that is situated intracapsularly and extrasynovially in the knee joint is considered to be an active OA joint tissue.³⁷ Therefore, it would be interesting to determine whether PDGFR α ⁺ progenitors are present in adipose deposits in other joint tissues, such as synovium,

joint capsule, and ligament. Adipose tissue occupied a large area of vastus medialis muscle of OA patients. However, synovium contained limited amounts of adipose tissue, whereas ligament did not exhibit lipid deposition. We did not find any signs showing that inactivity of OA patients increased adipose tissue in synovium because limited amounts of lipid deposition are usually included, even in the normal synovium.²⁶ The present study suggests that inactivity of OA patients strongly induces ectopic adipogenesis, exclusively in vastus medialis muscle, not in other joint tissues.

The present study explored adipose tissue in synovium-involved PDGFR α ⁺ progenitors as well. PDGFR α ⁺ progenitors in ectopic adipose tissue of vastus medialis muscle are possibly derived from the similar origin of PDGFR α ⁺ progenitors in adipose tissues of subintimal layer of synovium. It is possible that PDGFR α ⁺ progenitors are involved in the OA adipose-rich phenotype in general.

We investigated the pathological features of vastus medialis muscles from advanced knee OA patients. Our results strongly suggest that significant loss of myofibers and ectopic adipogenesis in the perimysium/endomysium of the vastus medialis muscle are common pathological features in elderly OA patients with 1 or more years of loss of mobility. The findings of the present study provide an improved understanding of knee-related muscle pathology in knee OA. They provide a basis for improvement of therapeutic strategies and the optimization of rehabilitation programs for the locomotive activity of elderly OA patients with disuse atrophy of skeletal muscles.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.08.009>.

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