Contribution of oxidative stress to the degeneration of rotator cuff entheses

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Background: Rotator cuff degeneration is one of the multiple factors that lead to rotator cuff tears; however, the precise mechanism of such degeneration still remains unclear. In this study, we investigated the supraspinatus tendon enthesis to clarify the link between rotator cuff degeneration and oxidative stress in antioxidant enzyme superoxide dismutase 1 (Sod1)-deficient mice (Sod1−/−).

Methods: The supraspinatus tendon and humeral head were isolated and fixed to prepare histologic sections from wild-type and Sod1−/− male mice at 20 weeks of age. Hematoxylin-eosin staining was performed to assess the histomorphologic structure. To investigate the collagen fibers, we examined spatially aligned collagen fibers using a polarizing microscope and assessed the amount of collagen using immunohistochemical staining. To analyze the tissue elasticity, we measured the tissue acoustic properties using scanning acoustic microscopy.

Results: The Sod1−/− mice showed histologic changes, such as a misaligned 4-layered structure and fragmented tidemark, in the enthesis. Sod1 loss also decreased the amount of brightly diffracted light and type I collagen, indicating collagen downregulation. The scanning acoustic microscopy analysis showed that the speed and attenuation of sound were increased in the nonmineralized fibrocartilage of the Sod1−/− mice, suggesting decreased mechanical properties in the supraspinatus enthesis.

Conclusion: Sod1 deficiency–induced degeneration is associated with impaired elasticity in the supraspinatus tendon enthesis, recapitulating human rotator cuff degeneration. These results suggest that intracellular oxidative stress contributes to the degeneration of rotator cuff enthesis.

Level of evidence: Basic Science, Histology, Animal Model.

Keywords: Rotator cuff degeneration; enthesis; oxidative stress; Sod1; collagen fibers; scanning acoustic microscopy; mechanical property

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Rotator cuff tear is a common orthopaedic disease, resulting in shoulder pain and dysfunction. However, the causative factors of the tear are still debated. Traditionally, extrinsic factors, such as acromion morphology, the presence of spurs, and shoulder overuse, contribute to the
On the other hand, epidemiologic studies have also proposed that intrinsic factors, including aging, inflammation, oxidative stress, and hypovascularity, are attributable to rotator cuff tears. Rotator cuff degeneration is one of the characteristics of the tear and aging in rotator cuff entheses. Pathologic changes in rotator cuff degeneration have been reported to include thinning and disorientation of collagen fibers, as well as the loss of cellularity, vascularity, and fibrocartilage mass at the site of cuff insertion and so on. However, the precise mechanism of age-related degeneration remains unclear.

We previously focused on the relationship between tissue degeneration and oxidative stress, which is known to accumulate in several organs with increasing age. Oxidative stress involves an imbalance between oxidation caused by reactive oxygen species and reduction elicited by antioxidant systems, leading to the initiation and progression of age-related diseases, such as diabetes, hypertension, atherosclerosis, osteoporosis, and neurodegenerative diseases. In previous reports, we also showed that an antioxidant enzyme, superoxide dismutase 1 (Sod1), regulates the intracellular reduction-oxidation balance and its deficiency induces deleterious effects on skeletal tissue, such as low-turnover osteoporosis and the exacerbation of unloading-induced bone loss.

On the basis of this knowledge, we investigated the supraspinatus tendon enthesis in Sod1-deficient mice to elucidate the deleterious effects of oxidative stress on rotator cuff entheses.

**Methods**

**Animals**

Sod1-deficient mice (Sod1<sup>−/−</sup>) were purchased from the Jackson Laboratory (Bar Harbor, ME). The Sod1<sup>−/−</sup> mice were backcrossed with C57BL/6NCrSlc mice (Nilson SLC, Shizuoka, Japan) 5 to 6 times. The mice were maintained and housed with 5 mice per cage with a 12-hour light-dark cycle and allowed free access to food and drinking water. The spontaneous activity of the male mice was measured in the cage using an activity-monitoring system (ACTIMO; Bio Research Center, Tokyo, Japan). The mice were studied according to protocols approved by the Animal Care Committee of the authors’ institutions.

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**Figure 1** Preparation of supraspinatus enthesis. (A) After the skin and subcutaneous tissue of the upper body were removed, the pectoralis major (m), deltoid (d), and trapezius (t) muscles were observed. (B) After the deltoid and trapezius muscles were removed by use of microscopy, the clavicle (c), acromion (a), and humeral head (h) were identified. (C) After the clavicle and acromion were removed, the supraspinatus (s) and infraspinatus (i) muscles and tendons were observed. (D) The supraspinatus and infraspinatus muscles and tendons and the humeral head were removed in 1 lump.
Tissue preparation

The Sod1–/– and wild-type (WT) male mice were killed at 20 weeks of age (n = 4 in each group). After the skin and subcutaneous tissue of the upper body were removed (Fig. 1, A), we stripped the deltoid and trapezius muscles to identify the clavicle and acromion using microscopy (Fig. 1, B). Maintaining an abducted position to protect the supraspinatus tendon, we removed the clavicle and acromion (Fig. 1, C). The complexes of the supraspinatus and infraspinatus muscles and tendons and the humeral head were removed in 1 lump (Fig. 1, D); fixed in 4% paraformaldehyde at room temperature overnight; decalcified with 10% EDTA in 10-mmol/L phosphate buffer (pH 7.4) for 1 week; and then embedded in paraffin blocks. The paraffin blocks were cut on a standardized frontal plane and stained with hematoxylin-eosin and toluidine blue (TB).

Histologic analysis of supraspinatus enthesis

We analyzed the sections using an optical microscope to assess the overall histologic structure and microstructure of the supraspinatus enthesis, such as the 4-layered structure, including the tendon, the nonmineralized and mineralized fibrocartilage and bone, and the tidemark, which forms a boundary between 2 fibrocartilages, as reported previously.5

Histologic analysis of collagen fibers in enthesis

To analyze the collagen fiber structure in the enthesis, we observed the sections using a polarizing microscope. Polarizing light directed at spatially oriented collagen fibers in tissue sections is diffracted and shines brightly against a dark background.1,5 The slides were rotated 360° on the microscope tray to find the position of maximum brightness.2,5

Histologic evaluation of supraspinatus enthesis

Quantitative histologic measurements comprised the following: (1) the number of chondrocytes, (2) the number of non-chondrocytes, (3) the percentage of aligned chondrocytes, (4) the spatial arrangement of collagen fibers, and (5) the area of metachromasia, as reported previously.5

Number of chondrocytes

At the enthesis, the number of chondrocytes was counted in a standardized rectangular field on hematoxylin-eosin–stained sections (Fig. 2).

Number of non-chondrocytes

Non-chondrocytic cells were counted in the same fields as the chondrocytes.
Percentage of aligned chondrocytes
In the same field used for the number of chondrocytes, the number of chondrocytes forming rows was counted. A row was defined as 3 or more chondrocytes aligned longitudinally. The number of chondrocytes aligned in rows divided by the total number of chondrocytes provided the percentage of chondrocytes aligned in rows.

Area of metachromasia
Metachromasia binds basic blue dyes, such as TB, changing its color to reddish blue, a property known as metachromasia. The area of intense metachromasia was quantified using the same image-analysis software. On the TB-stained slides, a standardized field starting at the bone-tendon junction was captured. Intense metachromatic areas within the standardized field were measured automatically and interpreted as fibrocartilage.

Scanning acoustic microscopy analysis
A scanning acoustic microscopy (SAM) system specially developed at Tohoku University operating in the frequency range of 50 to 150 MHz was used in this study. The SAM analysis was performed as previously reported. The tissue sound speed measured with SAM was directly proportional to the square value of its Young modulus. In other words, the sound speed was used as a parameter of the tissue material properties, particularly elasticity. Although it is not a pure physical parameter, the attenuation constant measured with SAM has a close relationship with the absorption of the ultrasound waves in the material. The absorption of tissue is known to be affected by the tissue’s molecular weight and viscosity.

Immunohistochemical analysis
Type I and III collagen fibers in the reparative tissues were identified and examined immunohistochemically using murine monoclonal antibodies (anti-collagen I antibody [ab21285] and anti-collagen III antibody [ab7778]; Abcam, Cambridge, UK). To quantify the protein expression, stained sections were analyzed by computer-assisted image analysis with the Qwin analysis system (Leica Microsystems, Wetzlar, Germany).

Statistical analysis
The statistical analyses were performed by use of the Student t test for comparisons between the Sod1−/− and WT mice. All data are expressed as mean ± standard deviation.

Results
Sod1 deficiency induced histologic changes in supraspinatus enthesis
In the histologic analyses, the WT mice were found to have a well-organized 4-layered structure of supraspinatus enthesis (tendon proper, nonmineralized fibrocartilage, mineralized fibrocartilage, and bone) and tidemark, with a boundary of nonmineralized and mineralized fibrocartilage (Fig. 2, A). In contrast, the Sod1−/− mice showed a misaligned 4-layered structure and fragmented tidemark in the enthesis (Fig. 2, B). With TB staining, we found that the WT mice clearly showed a TB-positive area in the enthesis (Fig. 2, C) whereas the Sod1−/− mice exhibited weak staining with TB in the supraspinatus enthesis compared with the WT mice (Fig. 2, D). These findings indicated that the Sod1−/− mice displayed a misaligned 4-layered structure and fragmented tidemark in the enthesis.

Next, to clarify whether Sod1−/− mice showed decreased locomotive activity, we measured the spontaneous activity of Sod1−/− and WT mice in the cage using an activity-monitoring system. The activity analysis showed that no difference was observed between Sod1−/− and WT mice (data not shown), suggesting that decreased mechanical loading could not affect the histologic changes in the supraspinatus enthesis of Sod1−/− mice.
Sod1 deficiency resulted in formation of misaligned collagen fibers in enthesis

Next, we evaluated the alignment of the collagen fibers in the supraspinatus enthesis using polarizing microscopy. The WT mice exhibited brightly diffracted light at the enthesis along the tendon (Fig. 3, A). In contrast, the Sod1−/− mice showed markedly decreased brightly diffracted light in the enthesis compared with the WT mice (Fig. 3, B). These results indicated that Sod1 loss resulted in the deterioration of spatially aligned collagen fibers in the supraspinatus enthesis.

Quantification of histologic changes of supraspinatus enthesis

To quantify the histologic changes of the supraspinatus enthesis, we analyzed 5 measurements: the number of chondrocytes (Fig. 4, A) and non-chondrocytes (Fig. 4, B), the percentage of chondrocyte aligned in rows (Fig. 4, C), the area of diffracted polarized light (Fig. 4, D), and the area of metachromasia (Fig. 4, E). Sod1−/− mice showed significant reduction in all quantitative histologic measurements compared with WT mice, except for the number of non-chondrocytes (Fig. 4).

Sod1 deficiency decreased expression of type I collagen in supraspinatus tendon enthesis

To analyze the collagen levels in the enthesis, we performed immunohistochemical staining of type I and III collagen. We found that the amount of type I collagen was significantly decreased in the entheses of the Sod1−/− mice compared with that observed in the WT mice (Fig. 5, A) whereas the amount of type III collagen was not significantly different between the two groups (Fig. 5, B).

Sod1 deficiency impaired elasticity in supraspinatus tendon enthesis

Finally, to investigate mechanical properties, we measured the speed and attenuation of sound using SAM. These two parameters reflected both the histologic architecture and the structure of the collagen fibers. The SAM analysis comparing the same portion of the supraspinatus enthesis in the Sod1−/− and WT mice is shown in Figure 6, A. Statistical analysis showed that Sod1 deficiency increased the speed and attenuation of sound in the nonmineralized fibrocartilage and decreased these parameters in the layer of bone (Fig. 6, B and C). On the other hand, these parameters were not different in the mineralized
fibrocartilage between the Sod1−/− and WT mice (Fig. 6, B and C).

**Discussion**

In this study, we found that Sod1 deficiency in mice induced several histopathologic changes in the supraspinatus enthesis (Figs. 2-5), recapitulating some of the features of human rotator cuff degeneration in elderly individuals. These data strongly suggest that SOD1 constitutively protects against rotator cuff degeneration during aging. Using a human tendon, Wang et al. reported that an antioxidant enzyme, peroxiredoxin 5 (PRDX5), was significantly upregulated in degenerative supraspinatus tendon compared with normal subscapularis tendon, suggesting a compensated response to oxidative stress in the degenerative tendon. In vitro studies also showed that exogenous oxidative stress by H₂O₂ loading induced cellular apoptosis in human tendon fibroblasts. In contrast, overexpression of PRDX5 effectively protected against H₂O₂-induced apoptosis in the tendon fibroblasts. Taken together with our results, these data show that cellular reduction-oxidation regulation plays a crucial role in the homeostasis of connective tissues including the enthesis and tendon.

We also showed that Sod1 deficiency reduced the spatial arrangement of collagen fibers on polarizing microscopy and decreased the amount of type I collagen on immunohistochemistry, indicating the downregulation of collagen fibers in the supraspinatus enthesis (Figs. 3, 4, D and 5). We previously reported that Sod1 loss significantly enhanced intracellular reactive oxygen species and suppressed...
the transcriptional expression of the type I collagen gene in dermal fibroblasts and osteoblasts.\textsuperscript{10,12} Because disruption of collagen metabolism is known to be associated with age-related changes in connective tissues,\textsuperscript{3,15,17} SOD1 may protect against age-related degeneration in connective tissues by collagen anabolism. Furthermore, Wang et al.\textsuperscript{18} have reported that phosphorylated c-Jun N-terminal kinase and metalloproteinase 1 were significantly upregulated in ruptured human supraspinatus tendons and in human tendon cells under an oxidative condition, suggesting that oxidative stress--induced c-Jun N-terminal kinase activation promotes metalloproteinase 1 expression, leading to tendon matrix degradation. Taken together, these results indicate that oxidative stress impairs collagen metabolism, resulting in the disorganization and degradation of collagen fibers in entheses.

The SAM analysis showed that Sod1 deficiency increased the speed and attenuation of sound in the non-mineralized fibrocartilage, indicating that the tissue elasticity of the area changed to that of a solid (Fig. 6). This result may be due to a reduction in the number of collagen fibers in the fibrocartilage, which is composed of collagen fibers and cartilage matrix. On the other hand, the speed and attenuation of sound in the bone areas were significantly lower in the Sod1\textsuperscript{−/−} mice. These results are considered to reflect the decrease in bone mass and quality in mice.\textsuperscript{12} Sano et al.\textsuperscript{14} reported that nonmineralized fibrocartilage exhibits the lowest elastic modulus among the 4 zones at the insertion site, which may be interpreted as reflecting an adaptation to various types of biomechanical stress. In this study, the Sod1\textsuperscript{−/−} mice exhibited decreased tissue elasticity in the nonmineralized fibrocartilage. These changes in tissue elasticity might impair the inherent dispersion function of the enthesis to various biomechanical stresses and result in tissue damage in the supraspinatus entheses of the mutant mice.

There were several limitations in this study. First, we investigated the shoulders of Sod1-deficient mice. It is unclear whether oxidative stress causes rotator cuff degeneration in human beings. Next, we could not perform tensile testing to specifically elucidate the relationship between the structural changes and mechanical properties of the supraspinatus enthesis in microscopy. Therefore, the true effectiveness of oxidative stress with regard to mechanical strength could not be judged accurately. Although we assume that this study clarified the area of decreased tissue elasticity using SAM, further tensile analysis of supraspinatus entheses will provide us with definitive conclusions to show the mechanical properties in rotator cuff degeneration. In addition, because we used systemic Sod1-deficient mice for analysis of “gain of function” in this study, we cannot exclude the possibility that systemic changes, such as hormonal or skeletal abnormalities,\textsuperscript{9,12} adversely affected the tendon enthesis in Sod1\textsuperscript{−/−} mice. Finally, this study had a relatively small sample size. Further analyses using transgenic mice for analysis of “gain of function” and human materials are needed to clarify the protective role of SOD1 in rotator cuff degeneration.

**Conclusion**

We showed that a deficiency of Sod1, an intracellular antioxidant enzyme, causes human rotator cuff degeneration--like histologic changes and altered tissue elasticity in the supraspinatus enthesis. These results strongly suggest that intracellular oxidative stress induces the degeneration of the supraspinatus enthesis, resulting in rotator cuff tears. To our knowledge, this is the first study to analyze and demonstrate the pathology in the enthesis of the supraspinatus using gene-targeting transgenic mice. This protocol has the potential for use in further basic research on the supraspinatus enthesis.

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