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Basic Science

Increased advanced glycation end products in hypertrophied ligamentum flavum of diabetes mellitus patients

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Abstract

BACKGROUND CONTEXT: Ligamentum flavum (LF) hypertrophy plays a dominant role in lumbar spinal stenosis (LSS). Although LSS prevalence is known to be higher in patients with diabetes mellitus (DM), the underlying pathomechanisms are not well understood. Abnormal advanced glycation end products (AGEs) formation occurs in DM and promotes tissue damage in various organs through degeneration and inflammation.

PURPOSE: To analyze and compare LF histology focused on AGE status between control patients, LSS patients with DM, and LSS patients without DM.

STUDY DESIGN/SETTING: Basic research study design utilizing human LF tissue for histologic analyses.

PATIENT SAMPLE: LF tissue samples were collected from patients who underwent lumber decompression surgery for LSS in the author's institution.

OUTCOME MEASURES: Quantitative visualization of Masson's Trichrome (MT) stains, and AGE immunohistochemistry (IHC) for the three groups.

METHODS: Ten LF specimens from LSS patients with DM (DM group, mean age 71.4 years), 10 from LSS patients without DM (non-DM group, mean age 71.2 years), and 9 from patients with lumbar disc herniation or cauda equina tumor (control group, mean age 49.0 years) were harvested during surgery and histologically analyzed. Percentage of elastic fiber areas (%EF) was measured with MT staining, and the percentage of AGE immuno-positive areas (%AGEs) was measured with IHC.

RESULTS: The average %EFs were 12.8 in the DM group, 17.1 in the non-DM group, and 24.9 in the control group. The decrease in the elastic fibers was significantly more in the DM group than in the non-DM ($p<.01$) and control groups ($p<.001$). Accumulation of AGEs was found mainly in the extracellular matrix in areas of elastic fiber disruption. The %AGEs were 18.3 in the DM group, 12.1 in the non-DM group, and 4.6 in the control group. These were significantly larger in the DM group than in the non-DM ($p<.01$) and control ($p<.01$) groups. The %AGEs also positively correlated with patient age ($p<.01$, $R=0.47$).

FDA device/drug status: Not applicable.

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CONCLUSIONS: Accumulation of AGEs is significantly greater in the LF of DM patients and correlates with patient age. AGEs may accelerate degeneration and hypertrophy of LF with age and may lead to higher prevalence of LSS in patients with DM.

CLINICAL SIGNIFICANCE: The present results partly reveal the molecular mechanism of LF hypertrophy, suggesting that AGEs may be involved in the process of LF degeneration in the elderly and patients with DM.

Keywords: Advanced glycation end products (AGEs); Ageing; Diabetes mellitus; Elastic fiber; Ligamentum flavum hypertrophy; Lumbar spinal stenosis

Introduction

Lumbar spinal stenosis (LSS) is one of the most common disorders in elderly populations and is mainly caused by degeneration of spinal structures including the intervertebral discs, ligamentum flavum (LF), and facet joints. Among these, hypertrophy of LF plays a dominant role in LSS [1].

Diabetes mellitus (DM) is a multisystemic disease characterized by chronic complications and effects on the musculoskeletal system [2], with higher reported prevalence of spinal stenosis in patients with DM than in those without [3,4]. However, the increased incidence and specific pathomechanisms by which LSS occurs in DM patients have not been elucidated.

Formation of advanced glycosylation end products (AGEs) is thought to play an important role in DM and aging through tissue damage in various organs [5]. AGEs are a heterogeneous class of glycated proteins or lipids that are formed following exposure to sugars. Formation of AGEs involves a series of nonenzymatic reactions, beginning with the formation of Schiff's base and Amadori's products. During these processes, a variety of highly reactive intermediate carbonyl groups are formed, which results in further molecular rearrangement ultimately leading to AGE generation. AGEs contain a large number of chemical structures including 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), 1-alkyl-2-formyl-3,4-glycosylpyrrole (AFGP), Nε-(carboxymethyl)lysine (CML), pyrraline, and pentosidine [6]. These AGEs can lead to abnormal cross linking of extracellular and intracellular proteins and alter their normal structure and function. Furthermore, AGEs bind to receptors causing inflammation, oxidative stress, calcium deposition, and vascular smooth muscle apoptosis, which contribute to the development of atherosclerosis [7].

Based on these previous findings, we hypothesized that the AGEs may also play an important role in LF hypertrophy in patients with DM. The purpose of this study was to analyze and compare histologically determined AGE composition in the LF between control patients, LSS patients with DM, and LSS patients without DM.

Material and methods

Patients and samples

Ten LF specimens were harvested from LSS patients with DM, and 10 specimens were harvested from LSS patients without DM during the course of surgery for symptomatic LSS with LF hypertrophy. Control specimens without LF hypertrophy were also harvested from nine patients with lumbar disc herniation (n=5) or cauda equina tumors (n=4). Patients age at surgery, sex, and hemoglobin A1C levels were recorded. The duration of DM and insulin use was also reported. The study protocol was approved by the institutional review board of our institution (approval number: 4131).

Histologic examination

The dural and dorsal layers of the LF were examined following removal of excess connective tissue. Each LF specimen was fixed in 10% neutral-buffered formalin. The alignment of LF fibers was confirmed by microscope (Model BX50; Olympus Optical Co, Ltd, Tokyo, Japan); samples were axially cut and embedded in paraffin for sectioning. Thin serial 4 μm sections were obtained, and hematoxylin and eosin (HE) staining was performed to characterize the overall LF features. Sections were also subjected to Masson's Trichrome (MT) staining for the analysis of elastic fibers, and immunohistochemistry for determining AGEs.

AGE immunohistochemistry

Sections were deparaffinized and treated with Target Retrieval Solution (DAKO Corporation, Carpinteria, CA) for 30 minutes at 100°C. Samples were washed with phosphate buffered saline, and endogenous peroxidase activity was blocked by incubation in methanol containing 0.13% H_2O_2 for 30 minutes. After blocking with 10% goat serum (Nichirei, Tokyo, Japan) for 30 minutes, the sections were incubated overnight with primary antibodies against AGEs (1:5,000 dilution rabbit polyclonal anti-AGE, ab23722 Abcam, Cambridge, UK). Sections were then incubated with biotinylated secondary antibodies (Histofine Simple Stain PO Max Kit (Multi), Nichirei, Tokyo, Japan) for 30 minutes followed by two phosphate buffered Tween (PBT, 0.005%) washes. Color reactions were developed using 3,3-diaminobenzidine tetrachloride (DAB, Sigma Chemical Co., St Louis, MO), and nuclear counter staining was done with Mayer's hematoxylin (Wako, Pure Chemical Industries, Ltd, Osaka, Japan).

Measurements

Histologic and immunohistochemistry-stained sections were examined using light microscopy and images were captured digitally (Model BX53F; Olympus Optical Co, Ltd, Tokyo, Japan). In MT staining, the percentage of pink stained elastic fibers (%EF) was measured at 200× in five random fields in LF tissue centers using computer software (Image-J ver. 1.51, National Institutes of Health, Bethesda, MD) using the threshold technique [8,9]. The percentage of AGE immuno-positive areas in the LF extra cellular matrix (%AGEs) was evaluated similar to %EF measurements. For both datasets, 3 different researchers independently measured 15 images twice at different time points, and the measurement validity was tested. The average interclass correlations for %AGEs were 0.890 interobserver error, 0.968 intraobserver error, and for %EF were 0.905 interobserver error, 0.893 intraobserver error.

Statistical analyses

Data were analyzed using SPSS (version 22.0, IBM, NY). Multiple comparison tests were performed using one-way analysis of variance tests, Games Howell tests, and Tukey tests for comparison between the three groups. The relationship between two values was analyzed using Pearson correlation test. p Values of less than .05 were considered to be statistically significant.

Results

Patient characteristics

Demographic data for the patients are shown in Table 1. The mean ages at surgery were 71.4 years in the DM group, 71.2 years in the non-DM group, and 49.0 years in the control group. Ages were significantly lower in the control group compared to the other groups (p<.01), but no significant differences in the age between DM and non-DM groups were observed.

Table 1
Patient demographics

Category	Control	non-DM (LF)	DM (LF)
Sample numbers	9	10	10
Mean age in years	49.0±17.2	71.2±5.5*	71.4±11.1*
Sex			
Male	2	7	6
Female	7	3	4
DM status			
HbA1c (%)	5.2±2.3	5.7±0.4	6.5±0.7
Disease duration (y)	-	-	24.7±16.9
Insulin self-injection use (n)	-	-	2

DM, diabetes mellitus. HbA1C, glycated hemoglobin A1c levels.
Average±one standard deviation.
* p<.01 when compared to controls.

Histologic and immunohistochemistry findings

HE stains showed EFs that are organized, parallel, and rich in LF of the control group compared to the DM and non-DM groups (Fig. 1). MT staining showed that the EF was sparse with more disruptions and irregular distributions in LF of the DM group compared to the control and non-DM groups (Fig. 2). The average %EFs were 12.8% in the DM group, 17.1% in the non-DM group, and 24.9% in the control group. Fig. 3 showed that EF was significantly decreased in the DM group compared to the non-DM (p<.01) and control groups (p<.001). Positive accumulation of AGEs was found mainly in the extracellular matrix of areas with disrupted elastic fibers and fibrosis, and also in vascular endothelial cells (Fig. 4). The average %AGEs were 18.3% in the DM group, 12.1% in the non-DM group, and 4.6% in the control group, and were significantly increased in the DM group compared to the non-DM and control groups (p<.01 and p<.001, respectively, Fig. 5). However, there were no significant differences in the %AGEs between insulin and noninsulin users in the DM group. No correlation between %AGEs and duration of DM or preoperative HbA1C levels was observed. Interestingly, with or without DM, there was an observed correlation between %AGEs and patient age (p<.01, R=0.47, Fig. 6).

Discussion

Histologic finding in this study showed that elastic fibers significantly decreased in the LF of LSS patients with increased differences in patients with DM. This study, to the best of our knowledge, is the first to examine AGEs in the LF. Our results showed that the AGE accumulation was increased in the DM group compared to the other groups, and AGEs density correlated with patient age.

There have been few studies focused on characterizing LF histology in DM patients. Ghadri-Anari et al. investigated the LF histology in DM and non-DM patients, and reported no significant differences in fibrosis, loss of elastin fibers, calcification, and cellular distribution between groups [10]. On the other hand, a similar comparative study of LF histology by Shemesh et al. demonstrated significant decreases in elastic fibers of DM patients [11]. We quantified elastic fiber area by using MT staining and found a significant decrease of elastic fibers in DM patients. This discrepancy may be explained by differences in study definitions of DM and staining methods. The Ghadri-Anari study included patients with high blood sugar, which may have included patients with short disease duration. The previously mentioned study used Verhoeff van Gieson staining for elastic fibers, whereas MT staining was used in both our and the Shemesh studies. It is difficult to conclude that elastic fibers decrease more in DM patients, but the higher prevalence of LSS in DM patients and other histologic studies indicate the presence of a specific degeneration mechanism in the LF of DM patients.

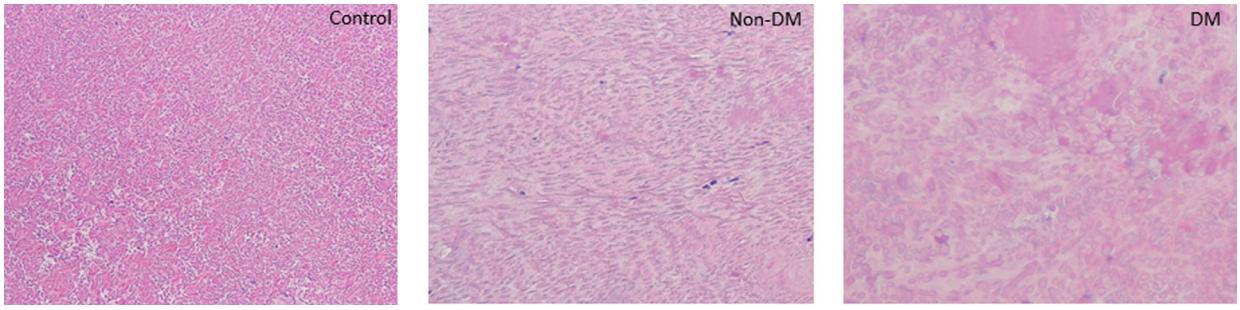

Fig. 1. Histologic findings after hematoxylin and eosin (HE) staining at 200× magnification (Left; control, Middle; non-DM, Right; DM). Images show the elastics fibers exhibit parallel and organized pattern and are not disrupted in the control group compared to DM and non-DM groups.

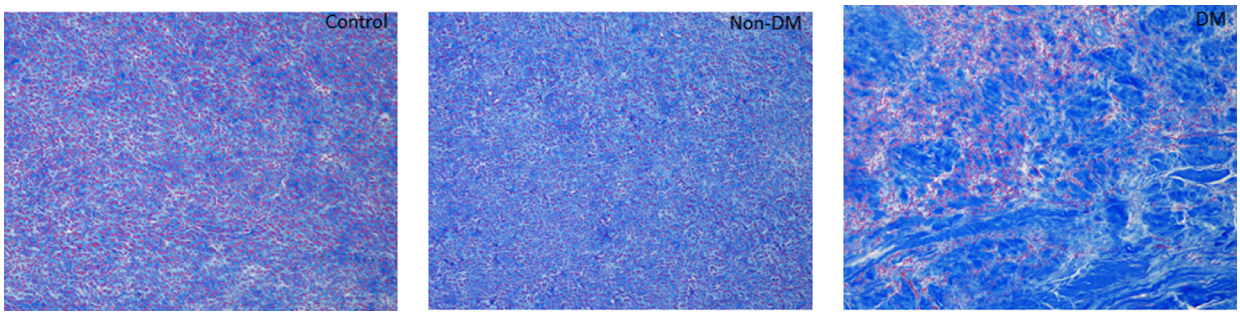

Fig. 2. Histologic finding after Masson' s Trichrome (MT) staining at 200× magnification (Left; control, Middle; non-DM, Right; DM). The control group shows rich abundance of elastic fibers (pink) without fibrotic areas, whereas the elastic fibers are decreased and disrupted with high area of fibrosis (blue) in the non-DM group. DM group shows a more severe decrease in elastic fibers with greater disruption and further increases in fibrotic areas.

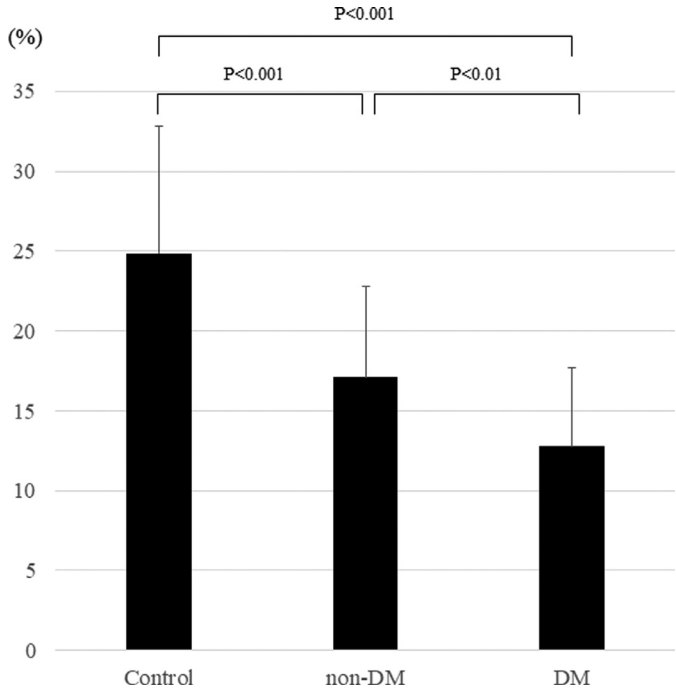

Fig. 3. Quantification of (%EF) on histopathologic staining of MT. The percentage of elastic fibers is significantly higher in the control group compared to the non-DM and DM groups. *DM indicates diabetes mellitus.

Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases that play an important role in degradation of extracellular matrix and tissue remodeling. There have been several studies showing a relationship between expression of MMPs and LF hypertrophy. Park et al. reported that levels of activated MMP-2 and MMP-13 were significantly higher in the LF of patients with spinal stenosis compared to those with disc herniation [12]. Lakemeire et al.

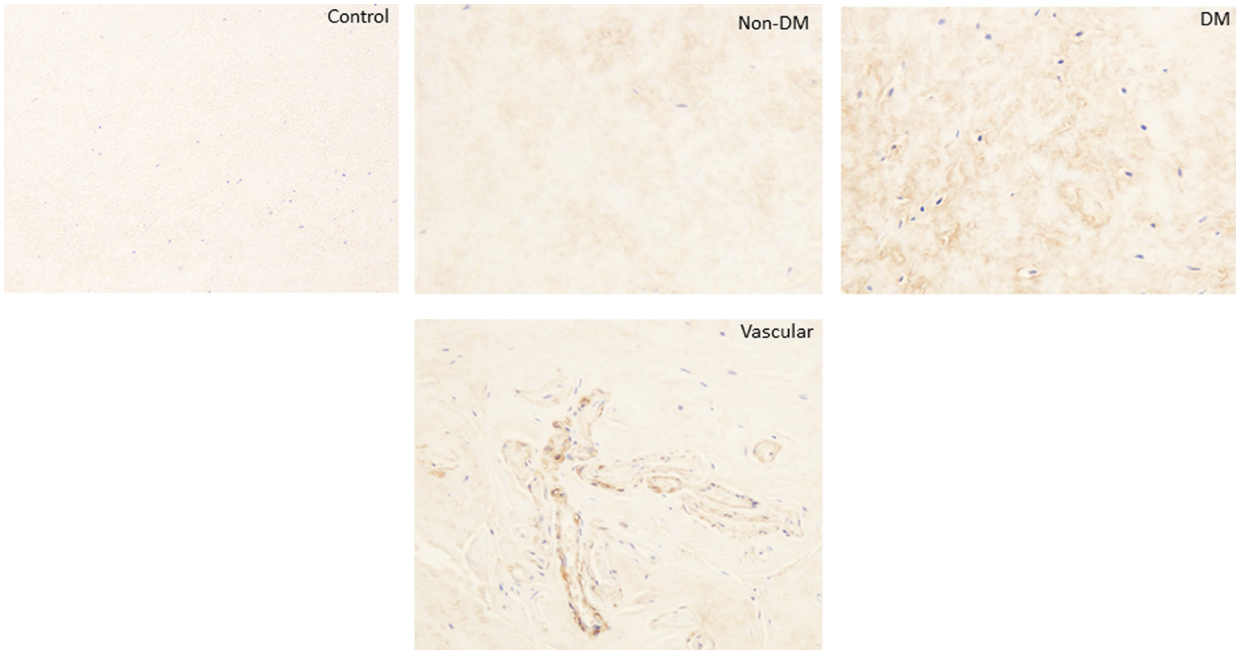

Fig. 4. Immunohistochemistry findings for AGEs at 200× magnification. The immune-positive area for AGEs in the disrupted LF elastic fibers and ECM is much greater in the DM group compared to the other groups. AGE staining is also seen in vascular endothelial cells. *ECM indicates extracellular matrix component.

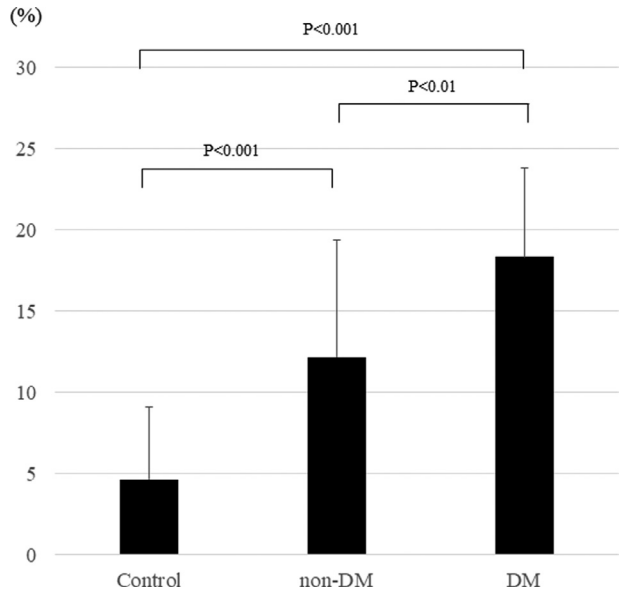

Fig. 5. Quantification of %AGEs according to IHC. The percentage of AGE immune-positive areas is significantly increased in the LF of the DM group compared to the other two groups. The non-DM group also has increased positive areas compared to the control group. *LF indicates ligamentum flavum. *%AGEs indicates percentage of advanced glycation end products.

demonstrated that hypertrophied LF showed higher expression of MMPs-1, -3, and -9 compared to control LF [13]. With regards to DM studies, Cui et al. compared the histology of LF in DM patients and reported that elastin degradation and fibrosis were more severe with higher expression of MMP-13 compared to non-DM patients. They also demonstrated glucose addition promotes MMP-13 expression in NIH/3T3 fibroblasts in vitro implying that DM may promote LF degeneration through MMP activity [14].

Composition of AGEs has been associated with production of MMPs. Cipollone et al. demonstrated overexpression of AGE receptor (RAGE) in diabetic atherosclerotic lesions, and that RAGE increases the biosynthesis of MMP-2 and -9 in monocytes through COX2/prostaglandin E2 pathways [15]. Nah et al. showed that AGE-BSA increases MMP-1, -3, and -13 expressions in human osteoarthritic chondrocyte through JAK/STAT pathways [16]. AGEs also play a dominant role in secretion of inflammatory cytokines, such as tumor necrotizing factor and interleukin-1, and these cytokines could account for the coordinated removal and replacement of senescent extracellular matrix components [17]. Our results show significantly higher AGE immunopositive areas and more disrupted elastic fibers in hypertrophied LF samples in patients with DM. Overall, the previous reports and the results of this study suggest that AGE accelerates LF degeneration in DM patients possibly through MMP-mediated elastic fiber degradation.

Ishibashi et al. reported that plasma levels of HbA1C are linearly correlated with AGEs, MMP-9, ACE expression, and cell apoptosis in coronary autopsy specimens which suggested that AGE/RAGE promotes cell apoptosis in the coronary artery through MMP-9 activity [18]. However, our present results did not show correlations between AGE accumulation and HbA1C levels at time of surgery. This may be a result of preoperative blood sugar control protocols. On the other hand, AGE accumulation in the LF was significantly correlated with age at time of surgery. Multiple studies have

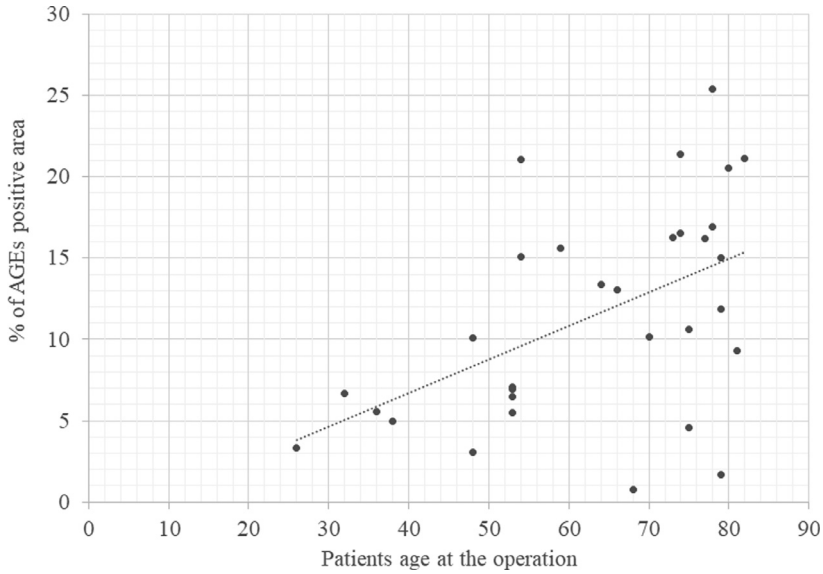

Fig. 6. Correlation between patient age and AGEs. There is a positive correlation between the concentration of percent area of AGEs in the LF and patient age (r=0.47, p<.01).

demonstrated the relationship between age and AGE accumulation in various organs [19–22] and age-related disorders including arterial sclerosis, osteoporosis, and chronic renal failure [23–25]. Other studies have reported that LF hypertrophy and thickness correlates with age [26–29], and these findings highlight the important role of AGE in LF hypertrophy in the patients regardless of DM status.

There are several limitations that can be discussed in this study. First, we examined AGEs in the LF using only immunohistochemistry. AGEs are comprised of a wide range of compounds and the antibody used in this study broadly detected AGEs and was unable to distinguish between a particular compound. Recently, it has been reasoned that some AGEs and related downstream signaling pathways can be leveraged as potential therapeutic target for various disorders [30,31]. To extend this to the therapeutic target potential of AGEs in LF degeneration, it is necessary to identify specific proteins that are increased in degenerated LF. Second, we have not directly examined the function of AGEs in LF. The accumulation of AGEs was found in the extracellular matrix, especially in areas with elastic fiber disruption, suggesting a catabolic function for AGEs. However, AGEs also play an important role in other pathologic conditions including calcification, fibrosis, and inflammation [32,33]. Therefore, future in vitro studies using LF specific cell types will be needed to clarify the function of these AGEs.

Conclusions

In conclusion, the comparison of LF histology between patients without LSS and those with LSS with or without DM revealed that elastic fibers were significantly decreased in LSS patients. This decrease was more substantial in LSS patients with DM. AGE accumulation was shown to be higher in the LF of DM patients, and accumulation was also correlated with patient age. AGEs may accelerate the pathways related to degeneration and hypertrophy of LF with age which in turn can lead to the higher prevalence of LSS in the patients with DM.

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