Pentosidine Deposition Affects Biomechanical Properties of Achilles Tendon in Diabetic Rats

Yoshimasa Sakoma1*, Takayuki Furumatsu2 and Toshifumi Ozaki3

1Department of Orthopaedic Surgery, Onomichi Municipal Hospital, Onomichi, Japan
2Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan
3Corresponding author: Yoshimasa Sakoma, Department of Orthopaedic Surgery, Onomichi Municipal Hospital, 3-1170-177, Shintakayama, Onomichi, Hiroshima 722-8503, Japan, Tel: +81-848-47-1155; Fax: +81-848-47-1004; E-mail: y sakoma@gmail.com

Abstract

Pentosidine, a biomarker for advanced glycation end products (AGEs), accumulates in vessels, kidney, skin, and bone and induces serious pathological conditions in diabetic patients. However, the relationship between AGE deposition and biomechanical weakness in tendons has not been elucidated. We hypothesized that diabetic status might induce excessive pentosidine deposition in tendon tissue and that accumulated pentosidine might modify the biomechanical properties of the diabetic tendon. In this study, we assessed the effect of pentosidine on cellular viability, senescence, and gene expression of type I/III collagens in NIH3T3 fibroblasts. Pentosidine deposition and the biomechanical properties of diabetic Achilles tendon were investigated using a diabetic rat model. Pentosidine induced cellular senescence of NIH3T3 fibroblasts in a dose-dependent manner without affecting cellular viability. Moreover, pentosidine decreased gene expression of type I/III collagens in NIH3T3 fibroblasts. Diabetic rats showed a higher level of pentosidine deposition in their Achilles tendons compared with that in normal rats. In addition, the toughness and ultimate stress of the Achilles tendons were lower in diabetic rats. Our results suggest that pentosidine may induce cellular senescence and inhibit collagen synthesis in tendon fibroblasts. Moreover, the accumulated pentosidine may alter the biomechanical properties of the Achilles tendon in diabetic rats. Hence, the suppressed collagen synthesis may cause the deterioration of the tendon mechanical properties. These phenomena might be one of the reasons of the tendon deterioration in the diabetic patients.

Keywords: Advanced glycation end products; Pentosidine; Biomechanical property; Tendon; Diabetes mellitus; Senescence; Fibroblast

Introduction

In diabetic patients, advanced glycation end products (AGEs) accumulate in the vessels, kidney, skin, bones, and other tissues [1-6] and result in diabetic vascular complications such as retinopathy [5,6], nephropathy [2], and neuropathy [7]. Moreover, osteoporosis is caused by the deposition of AGEs in bone tissue [3,4,8]. However, the effect of AGE deposition on tendon tissue has not been fully investigated.

Several authors have studied the effect of AGEs on cultured cells [9-13]. AGEs are cytotoxic to human gingival fibroblasts depending on their concentration and incubation time [9]. AGEs increase inflammatory mediators such as monocyte chemotactic protein 1, interleukin 6, and vascular cell adhesion molecule 1 in vascular adventitial fibroblasts [10]. AGEs increase tumor necrosis factor-α and induce cell apoptosis by activating the nuclear factor-κ B pathway in human embryonic kidney and human mesangial cells [11]. In addition, AGEs induce fibroblast apoptosis through the activation of reactive oxygen species, mitogen-activating protein kinase, and forkhead box protein O1 transcription factor [12]. These studies indicate that AGEs induce inflammation and apoptosis by deteriorating the viability of fibroblasts.

AGEs alter the biomechanical properties of tendon tissue. Rabbit glycated Achilles tendons show decreased maximum load and increased Young's modulus in vitro [13,14]. Animal models of hyperglycemic conditions and obesity have been used for in vivo studies. The tail tendon fibrils in diabetic rats display pentosidine deposition that is 2.5 times greater than that in normal rats [15]. Conversely, Young's modulus is decreased in the patella tendons of streptozotocin (STZ)-induced diabetic rats [16]. Cross-linked AGEs increase the stiffness of myocardial and vascular tissues [17,18]. In addition, AGEs increase the stiffness of the skin and aorta and decrease the elasticity of these tissues in hyperglycemic rats [19]. These findings suggest that AGEs deteriorate the mechanical properties of tendons. However, the relationship between biomechanical properties and the level of AGE deposition in tendon tissue remains unknown.

Pentosidine is a well-characterized AGE found in increased levels in diabetes. We hypothesized that (1) diabetic rats have a greater amount of pentosidine in tendon tissue than that of the normal rats, and (2) the amount of pentosidine deposited modifies the biomechanical properties of the tendon tissue. In the present study, we assessed the effect of pentosidine on cultured fibroblasts and investigated the relationship between biomechanical tissue properties and the amount of pentosidine in rat Achilles tendons.
Methods

In vitro study

Quantitative real-time polymerase chain reaction (PCR) analysis

NIH3T3 cells were seeded in a 12-well plate and cultured in the manner described above. After 1 week of culture, total RNA was extracted using a QuickGene SP kit (Fujifilm, Tokyo, Japan). RNA samples (1 μg) were reverse transcribed to complementary DNA. SYBR-Green (Agilent Technologies, Tokyo, Japan) PCR was performed using these complementary DNAs as templates. Mean cycle threshold (Ct) values were used to calculate gene expression with normalization to hypoxanthine phosphoribosyltransferase as an internal control. The primer sequences were as follows: forward, 5′-GTGGTTCTGACCGTGACC-3′, and reverse, 5′-GAGTGGCACATCTGTAGGTC-3′ for the α1 chain of type I collagen (Col1a1); forward, 5′-CTGGAAGGATGGTGCACG-3′, and reverse, 5′-TCCTGAGTGCTCTTGTGC-3′, for the α1 chain of type III collagen (Col3a1) [20]. After 3 minutes of initial denaturation at 95°C, PCR was performed with 45 cycles of 10 seconds of denaturation at 95°C and 30 seconds of annealing and extension at 55°C. Quantification was achieved using the comparative Ct method according to the manufacturer protocol. Expression was quantified using the delta-delta Ct method.

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In vivo study

Animals and plasma glucose measurements

Four male Otsuka Long-Evans Tokushima fatty (OLETF) rats and 2 male Long-Evans Tokushima Otsuka (LETO) rats aged 20 weeks were supplied by Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan). The OLETF rat is an animal model of human non-insulin-dependent diabetes mellitus established from an outbred Long-Evans strain [21]. Nearly 100% of male OLETF rats develop a diabetic syndrome at 25 weeks of age. LETO rats served as normal controls. Body weight and blood sugar levels were measured at the time of sacrifice. Plasma glucose levels were measured in a central vein using a blood glucose meter (ONETOUCH UltraVue, LIFESCAN Inc., Tokyo, Japan). The bilateral Achilles tendons were collected—8 from OLETF rats and 4 from LETO rats—and stored at -20°C until analysis.

Biomechanical analysis

The biomechanical properties of the Achilles tendons were carried out using an Instron Testing Device, Model 5965 (Instron Japan Co. Ltd., Kanagawa, Japan). The specimens were thawed at room temperature and secured between 2 clamps (Figure 1). The circumference of each Achilles tendon (mm) was measured at the midpoint of the fixed tendon, and the cross-sectional area (mm²) was calculated. The length of the tendon (mm) was measured as the distance between the clamps. Then the tendon was tugged to rupture at a rate of 1,000 mm/min. The data were digitized and imported into a computer. From the analysis of the stress-strain relationship, ultimate stress (N/mm²), Young’s modulus of elasticity (N/mm²), toughness (N/mm²), and strain to failure (%) were calculated for each specimen. The ruptured specimens were placed into saline and stored for histopathological assessment.

Histopathological analysis

After 24 hours of fixation in formalin, the specimens were embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin, and the degree of tissue degeneration was assessed with light microscopy using the Bonar scale [22]. Specimens with a Bonar score of 3 or higher were characterized as having tissue degeneration and excluded from the study. The sections were also stained with an anti-pentosidine monoclonal antibody, KH012 (Trans Genic Inc., Kobe, Japan). After reacting with primary antibody, the sections were reacted with 3,3′-diaminobenzidine solution without counterstaining. All control sections were completely negative. The percentage of stained area was calculated using the following method.
The images of the stained sections were obtained in 3 high-power fields and imported into a computer. The total pentosidine-positive area (%) and the pentosidine-positive area of the nuclei (%) were quantified in each image using the image analysis software Image J (version 1.44p, National Institutes of Health, Bethesda, MD, USA), and the average area of 3 high-power fields was calculated. Then, the pentosidine-positive area of the extracellular matrix (ECM; %) was calculated by subtracting the pentosidine-positive area of the nuclei from the total pentosidine-positive area.

Statistical analyses

Statistical analyses were performed using commercial software (StatMate III, Atms Co., Ltd., Tokyo, Japan). The data from the water-soluble tetrazolium-1 assay, β-galactosidase assay, and real-time RT-PCR were analyzed using one-way analysis of variance between groups with and without various concentrations of pentosidine. The pentosidine-positive area and the biomechanical properties of OLETF rats were compared with those of LETO rats using Mann-Whitney’s U-test. Pearson’s correlation coefficients were also assessed to identify correlations between the pentosidine-positive area of the ECM and the biomechanical properties of the Achilles tendons. Statistical significance was defined as a P value less than 0.05.

Results

In vitro study

Pentosidine administration had no effect on the cell viability of NIH3T3 fibroblasts (Figure 2A). In the cell senescence assay, pentosidine increased the senescence-associated β-galactosidase activity of NIH3T3 fibroblasts in a dose-dependent manner (Figure 2B). Fluorescence observed in cells treated with 0.5 and 1.0 μmol/L of pentosidinewas significantly greater than that in control cells.

Real-time PCR analysis showed that pentosidine reduced the expression of Col1a1 and Col3a1 in a dose-dependent manner (Figures 3A and B). Gene expression of Col1a1 and Col3a1 in cells treated with 0.5 and 1.0 μmol/L of pentosidine were suppressed compared with that in cells cultured in the standard medium.

In vivo study

Plasma glucose levels of OLETF rats were greater than 200 mg/dL; however, those of LETO rats were less than 100 mg/dL (p<0.001, Table 1). Body weight in OLETF rats was significantly increased compared with that in LETO rats (p<0.001, Table 1). The cross-sectional area of the Achilles tendons of OLETF rats did not differ from that of LETO rats (Table 1). In the biomechanical analysis, the ultimate stress of the Achilles tendons in OLETF rats was lower than that of LETO rats (Table 1). However, no statistically significant difference was observed between these groups (p=0.08). Young’s elastic modulus and strain to failure in OLETF rats were similar to those in LETO rats (Table 1). Although statistical significance was not observed, toughness in OLETF rats was relatively lower than that in LETO rats (Table 1).
Table 1. Biomechanical properties of Achilles tendons in OLETF and LETO rats

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
<th>Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toughness (N/mm²)</td>
<td>121.8 (76.9)</td>
<td>169.4 (102.7)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Strain to Failure (%)</td>
<td>22.7 (11.7)</td>
<td>28.7 (5.4)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD). Significance is set at p<0.05.

Discussion

The results of this study showed that the Achilles tendons of OLETF rats, which are animal models of type II diabetes, had a greater level of pentosidine deposition compared with that in the Achilles tendons of LETO rats.

Furthermore, the ultimate stress of the Achilles tendon decreased as pentosidine deposition in the tendon ECM increased. Our results suggest that the presence of diabetes mellitus may deteriorate tendon biomechanical properties by increasing pentosidine deposition.

Pentosidine in tendon tissue increases with aging and a hyperglycemic condition [19,23]. AGEs have cytotoxicity [9] and induce inflammation and apoptosis in fibroblasts [10,12]. In addition, AGEs deteriorate the mechanical properties of tendons [16]. The current study showed that pentosidine induced cell senescence in NIH3T3 fibroblasts in a dose-dependent manner without affecting cell viability. Moreover, the expression of Col1a1 and Col3a1 was decreased in fibroblasts treated with pentosidine, indicating that pentosidine may deteriorate cell activity and impede tendon tissue turnover and remodeling.

In this study, we used OLETF rats as models of type II diabetes mellitus. In type I diabetic rats, the hyperglycemic condition negatively affects the mechanical properties of the patella tendon [16]. Fox et al. [16] have shown that Young’s modulus is significantly decreased in STZ-induced diabetic male Lewis rats compared with that in control rats. However, no significant differences in mean load-to-failure or stiffness were observed between the groups. In our study, the ultimate stress of the Achilles tendons decreased as the level of pentosidine deposition in the tendon ECM increased. These differences may be related to the type of diabetes mellitus. STZ induces a loss of body weight in rats, which decreases the cross-sectional area of the tendon tissue [16], in turn altering its mechanical properties. In OLETF rats, the ultimate stress of the Achilles tendon was lower than that in LETO rats even though body weight in OLETF rats was higher. Moreover, the STZ-induced diabetic rat is an acute-onset model of diabetes, whereas the OLETF rat model represents chronic diabetes.
mellitus. These findings suggest that the glycation of the tendon tissue may alter the biomechanical properties of the tendons.

Pentosidine forms cross-links between lysine and arginine residues and is not expected to be deposited in the nuclei. However, our study showed that the nuclei were stained by the anti-pentosidine antibody. A previous study has shown that the distribution of pentosidine and other AGEs is stronger in the cells than in the ECM of arthritic cartilage [24]. The mechanical properties of tendon tissue might be related to ECM condition; therefore, we calculated the area of the ECM stained by an anti-pentosidine antibody excluding the nuclei. Our results showed that the pentosidine-positive area in the ECM was negatively correlated with the ultimate stress of rat Achilles tendons. Pentosidine, which deposits in collagen fibers, may modify the biomechanical properties of tendon tissue. In this study, we assessed the effect of pentosidine on fibroblasts in vitro and demonstrated that pentosidine deposits in the collagen fibrils and affects the biomechanical properties of the tendon collagen [15]. Moreover, pentosidine can affect the biology of satellite cells and stem cells around collagen bundles. The deposited pentosidine might induce cellular senescence of fibroblast precursors and inhibit collagen gene expression in fibroblasts, in turn causing a decline in collagen turnover and inducing the aging of tendon tissue.

The potential clinical relevance of this study is that greater pentosidine deposition on tendon tissue might be observed in patients with type II diabetes mellitus and that this deposition deteriorates the biomechanical properties of human tendon tissue. Preventing pentosidine deposition may be a potential therapeutic approach for tendinopathy and tendon rupture in diabetic patients.

This study has several limitations. First, we used 20-week-old OLETF and LETO rats. We intended to assess the biomechanical properties of the tendon tissue without degenerative change. OLETF rats display type II diabetes mellitus. These findings suggest that the glycation of the tendon tissue may alter the biomechanical properties of the tendons of diabetic patients.

Acknowledgements

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