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Localization of advanced glycation end-products and their receptor in tendinopathic lesions

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Summary. This study was designed to investigate the accumulation of advanced glycation end-products (AGEs) and the expression of the receptor of AGEs (RAGE) in tendinopathic tissues. In this study, tendinopathic posterior tibial tendons (PTT) were collected from patients (n=6). Redundant autografts of flexor digitorum longus tendon (FDL; n=3) were used for controls. The control and tendinopathic tendon tissues were used for extraction of proteins for western blot and sectioned for histology and immunohistochemistry. Tendinopathy of the PTT was confirmed histologically by the presentation of disorderly collagen fibers, high cellularity and increased vascularity. By immunohistochemistry, heterogeneous accumulation of AGEs was detected on the PTT sections and concentrated in areas, where collagen fibers were disorderly and tangled. In the PTT, roundish tenocytes were also AGEs-positive. In contrast, AGEs were diffuse, lightly stained in the FDL. A greater number of tenocytes within the tendinopathic lesions in the PTT were RAGE positive, compared to the tenocytes in the FDL. Western blot confirmed the presence of AGEs and RAGE in both tendinopathic PTT and control FDL but their band densities were not significantly different. The spatial relation of the accumulated AGEs and RAGE-positive tenocytes within the tendinopathic lesions indicates their involvement in the molecular pathology of tendinopathy.

Key words: Advanced glycation end-products, Receptor, Tendinopathy, Tendon

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Introduction

Relaying forces of muscle contraction to bone and moving the joint, tendon is pivotal for the physiological functions of musculoskeletal system. Tendinopathy is a common clinical condition presenting pain, swelling and functional impairment of the affected tendon. The incidence of tendinopathy is increasing, coinciding with an increasingly aging, more-than-ever active population (Maffulli et al., 2003; Teunis et al., 2014).

Aging significantly modifies the properties of tendon (Birch et al., 1999; Peffers et al., 2014). When the Achilles tendon was monitored for shear wave during walking, its peak wave speed was reduced in the aging group (Ebrahimi et al., 2020). Advanced glycation end-products (AGEs) are a complex class of proteins or lipids that are nonenzymatically modified by glycation and oxidation. The modification process, known as Maillard reaction, results in denaturation and cross-linking of the targeted proteins. Accumulation of AGEs happens during connective tissue aging and exerts pathogenic effects particularly on long-lived proteins, such as collagens. When rabbit Achilles tendons were treated with ribose for glycation *in vitro*, their stiffness was increased as high as 161% over the non-glycation tendons, as indicated by Young's modulus (Reddy, 2004). Subsequent studies discovered that accumulation of AGEs in tendon jeopardizes gliding between collagen fibers due to excessive collagen crosslinking (Li et al., 2013). The glycation-induced mechanical alteration makes tendon more susceptible to injury. The involvement of AGEs in tendinopathy, however, has not been specifically validated.

This study investigated the presence and localization of AGEs and receptor of AGEs (RAGE) in tendinopathic tissues, in comparison with control tendons, using immunohistochemistry and western blot.

Materials and methods

This study included six patients with tendinopathy of

the posterior tibial tendon (PTT), from 56 to 72 years of age (mean age: 65 years) and including 5 male and 1 female. The collection of tissue samples for research was approved by MedStar Health Institutional Review Broad (IRB; protocol # 2014-057). The IRB waived patient consent because this study "meets the criteria set forth in [45 CFR 46.101(b), Category (4)] and qualifies for exemption from the requirements of (45 CFR 46) federal regulation". According to the approved protocol, the authors had no access to the information that could identify individual participants during and after sample collection. The tendon samples used for this study were collected and used between June 25, 2014 and November 17, 2018. The clinical diagnosis of tendinopathy for each patient was made by a senior foot and ankle surgeon, supported by magnetic resonance imaging (MRI) and surgical inspection. The diseased tendon was debrided or excised and then repaired or reconstructed, depending on the extensiveness of tendinopathy. The surgically removed PTT tissues were collected for this study. Samples of healthy flexor digitorum longus tendon (FDL) were collected from three female donors, from 42 to 51 years of age (mean age: 46 years). During the procedure of FDL transfer, the tendon was reattached to the bone under a proper tension. Occasionally, an excessive portion of the tendon was available for collection to be the controls in this study. The color and tissue integrity of the excessive FDL autograft were inspected by the operating surgeons to rule out tear and degeneration. A portion of each tendinopathic or control tendon was fixed with 4% paraformaldehyde and sectioned with a cryostat. The tissue sections were stained with hematoxylin and eosin (H&E) and Picrosirius Red separately, and viewed under a light microscope or a polarizing microscope, respectively, for tendon histology and collagen fiber morphology.

Immunohistochemistry for AGEs and RAGE was performed on separate tissue sections. After heated antigen retrieval in sodium citrate buffer (pH 6), the randomly selected tissue sections were blocked with hydrogen peroxide and horse serum sequentially. The primary antibody of AGEs or RAGE (ab176173 and ab216329, respectively, rabbit anti-human; Abcam, Cambridge, Massachusetts, USA) was applied onto tissue sections at 1:100 dilution and incubated in a moisture chamber at 4°C overnight. After extensive washing in Tris buffered saline, the slides were applied with secondary biotinylated horse anti-rabbit antibody, followed with ABC reagents (VECTASTAIN Elite ABC system, Vector Laboratories, Burlingame, California, USA). Peroxidase substrate 3,3-diaminobenzidine was used for chromogenic detection of the targeted proteins. Cell nuclei were counterstained with hematoxylin. For the negative control tissue sections, the primary antibody was omitted.

To extract proteins from tendinopathic and control tendons, T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific), was added to the tissue sample at a ratio of 1:20 (w/v). Following homogenization, the sample was centrifuged at 16,000g for 20 minutes at 4°C. Protein concentration of the tissue lysates was measured with bicinchoninic acid (BCA) assay. Of the total proteins, 15μg was taken from individual tendon samples and loaded into 12% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad Laboratories, Hercules, California, USA) for western blot. After electrophoresis, proteins were transferred to Immun-Blot® PVDF Membrane (BioRad Laboratories) and separately incubated with AGEs or RAGE antibody specified previously (1:2000 dilution) at 4°C overnight. Primary antibody was omitted for the negative control lane. Protein bands were detected using horseradish peroxidase-conjugated secondary antibodies, and visualized with an ECL detection system, using a luminescent imager. After stripping antibodies, the membranes were incubated with the antibody for β-actin (sc-47778, Santa Cruz Biotechnology, Santa Cruz, California, USA) and western blotting was repeated. The gel images were inverted and the density of the western blotting bands was measured with ImageJ program (National Institutes of Health, Bethesda, Maryland, USA), for comparison between the tendinopathic and control groups.

Statistical analysis: data are presented as mean ± standard deviation. The blotting band densities of AGEs in the tendinopathic and control tendon groups were comparatively analyzed with unpaired t test (MedCalc 20.009, MedCalc Software Ltd., Ostend, Belgium). $P<0.05$ was set as significant.

Results

On histology, while FDL showed wavy tendon fibers and scattered elongated tenocytes, the lesion areas in the PTT were mesh-like fibers interposed with amorphous materials (Fig. 1A,B). Aside from the large tendinopathic lesions in the PTT, there were micro-sized lesions. Surrounded by spindle-shape tenocytes and tendon fibers, the small lesion was a cluster of round tenocytes scattered within an expanded extracellular matrix without tendon fibers (Fig. 1C). There was increased vascularity around the micro-lesions. Around isolated tendinopathic lesions, there were increased tenocytes, which were roundish and aligned circling the lesion (Fig. 1D).

Under a polarizing microscope, collagen fibers in the control FDL were aligned in parallel (Fig. 2A). The collagen fibers in the PTT tangled to a form of "ball". The collagen fibers were a heterogeneous mix of thick (type I collagen) and thin (type III collagen) fibers (Fig. 2B).

1)AGEs: By immunohistochemistry, AGEs presented in the FDL in a diffuse fashion without an identifiable structural pattern (Fig. 3A). In the lesion areas of the PTT, the high-cell-density tenocytes were

positive for AGEs. The intracellular stain of AGEs was mostly in the roundish tenocytes (Fig. 3B). However, not all roundish tenocytes were AGEs-positive, especially when they were scattered around a lesion (Fig. 3D,E). Remarkably, there was enhanced accumulation of AGEs in the matrix. AGEs stain appeared as localized patches,

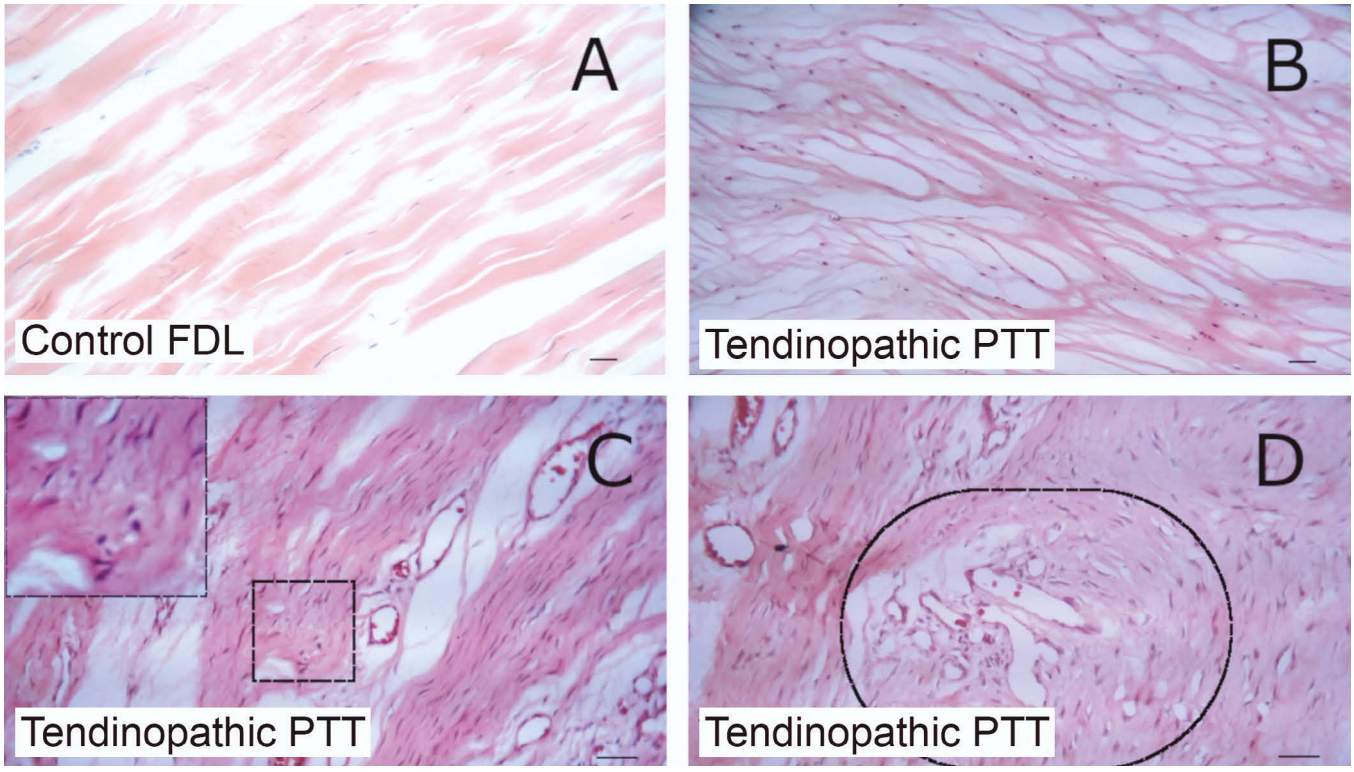

Fig. 1. Histology of tendinopathic PTT and control FDL. **A.** In FDL, fibers are aligned in parallel; tenocytes elongated and vascular structures scarce. **B.** Tendinopathic PTT appears "mesh-like". Tendon fibers are separated by matrix "voids". The density of tenocytes is moderately increased. **C.** A focal tendinopathic lesion shows roundish tenocytes, expanded extracellular matrix and disappearance of fibril structures, surrounded by vascularization (inset is the enlarged section area). **D.** In PTT tendon, there are micro-matrix "voids" and vascularization within the tendinopathic area. Tenocytes are increased locally and arranged surrounding the lesion (H&E staining). Scale bars: 100 μm.

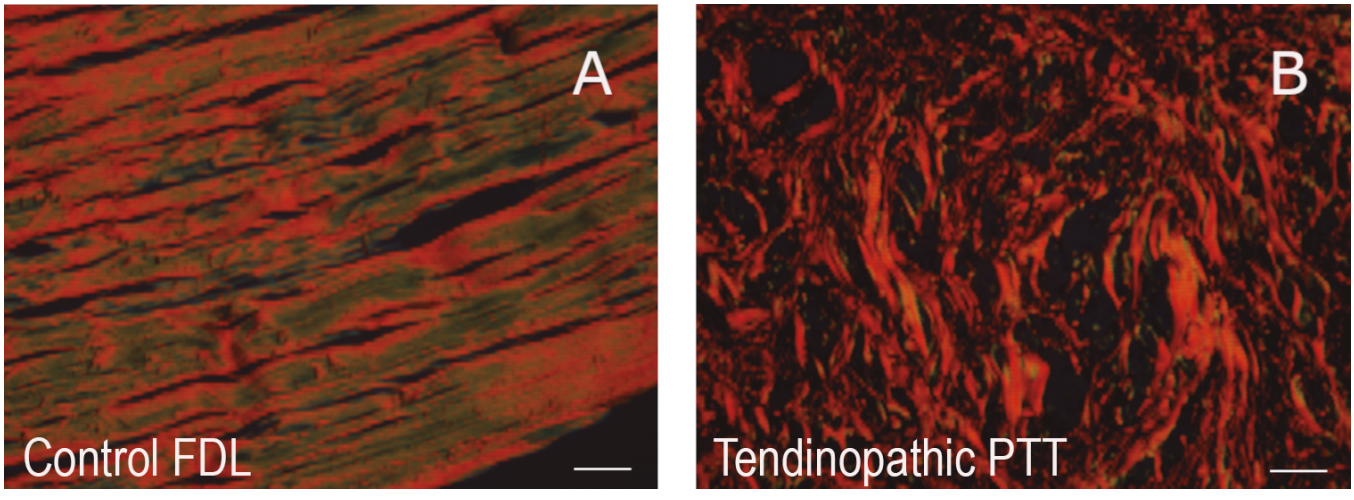

Fig. 2. Collagen fibers in the tendinopathic PTT and control FDL by polarizing microscopy. **A.** Collagen fibers in the FDL are bundled together and aligned in parallel. **B.** Collagen fibers in the lesion of tendinopathic PTT are tangled into a "ball" of fibers, without clear orientations. The composition of the collagen fibers is a mixture of type I (red) and type III (green) collagens (Picrosirius Red staining). Scale bars: 100 μm.

where collagen fibers were tangled, in a larger lesion (Fig. 3C). The patches of AGEs-stained matrix also appeared in the areas with minimal tendinopathic changes in the PTT (Fig. 3D). On the margin of tendinopathic lesions, intense accumulation of AGEs was localized in the matrix where voids of collagen fibers presented (Fig. 3E). By western blot, AGEs presented in all the examined FDL and PTT (Fig 3F). The densities of the AGE bands were not significantly different between the control and tendinopathic groups (172±16 vs. 179±18; p=0.57).

2) RAGE: By immunohistochemistry, very few tenocytes in the FDL were RAGE positive (Fig. 4A). On the sections of PTT, clusters of tenocytes were positive for RAGE (Fig. 4B). RAGE was dotted intracellularly (Fig. 4C). Western blot confirmed expression of RAGE in both PTT and FDL but the quantity (the intensity of bands) of RAGE expressed in the PTT and FDL groups was inconsistent (Fig. 4D).

Discussion

This study revealed the accumulation of AGEs and the protein expression of RAGE in human tendinopathic tissues. The pathogenesis of tendinopathy has been studied with animal models, induced by running on treadmills, and local injections of collagenase and cytokines. Those animal models, however, only partially simulate the molecular and cellular pathology of tendinopathy. Despite inherent variables of host genetics and pathological stages, human tendinopathic samples present the original pathology. This study used tendinopathic PTT, which is a common clinical condition in foot and ankle clinics. The histological appearance of the PTT samples in this study was in line with the major pathologies reported in the literature: disorganized collagen fibers, high cellularity and increased vascular bundles (Khan et al., 1999).

Besides confirming the clinical diagnosis of tendinopathy, the histology of PTT in this study demonstrated that the tendinopathic lesions were highly heterogeneous in their sizes, shapes and local pathological features. There were isolated tendinopathic lesions within regular, normal-looking tendon structures. The lesions were recognizable by 1) microscopic matrix voids among dense collagen fibers; 2) clusters of roundish tenocytes and 3) tangled collagen fibers. It is likely that these lesions, though small, contribute to the symptoms and dysfunction of the tendinopathic tendons. When mechanical forces are applied to a tendon, the fascicles and fibers within the tendon are stretched. At a microstructure level, the force translates to inter-fibrillar

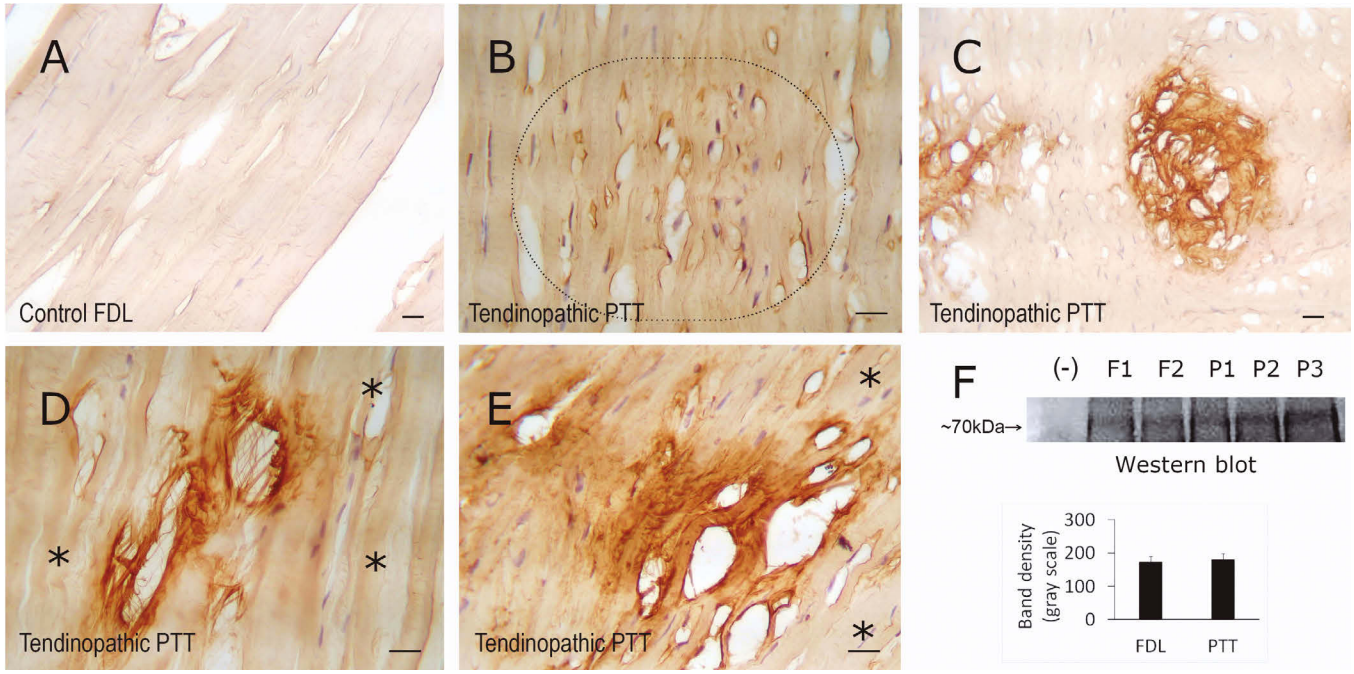

Fig. 3. Identification and localization of AGEs in the tendinopathic PTT and control FDL. **A.** There is only light stain of AGEs on the FDL sections. **B.** In tendinopathic PTT, clustered tenocytes are positively stained with AGEs. The majority of AGEs-positive tenocytes are roundish. **C.** Within a larger lesion (note the disappearance of fibril structures and increased tenocytes) in PTT, accumulation of AGEs appears in isolated areas where collagen fibers are tangled. The AGEs-stained area has a clear margin (on the right) or a blurry one (left). **D.** Patched stain of AGEs is surrounded by regular tendon structures (noted with *) in PTT. There are a few roundish tenocytes in the AGEs-stained area. **E.** Between regular tendon structures (noted with *) and a tendinopathic lesion, there is focal accumulation of AGEs. Note: Counterstain with hematoxylin. **F.** Western blot shows AGEs in both PTT and FDL protein samples. Note: F1 and F2 are protein samples of the FDL and P1, P2 and P3 are protein samples of the tendinopathic PTT; (-) represents the negative control. Scale bars: 50 μm.

shear load (Szczesny and Elliott, 2014). In this context, the presentation of micro-lesions, small yet obstructive to inter-fibrillar movement, could impair the functions of the tendon. By microscopy, it is indistinguishable whether these lesions were at an early stage of development or on the perimeter of a large lesion. Nevertheless, the pathologies of roundish tenocytes, tenocyte clusters and matrix voids add more details to the cellular pathology of tendinopathy. From the viewpoint of experimental pathology, it is challenging to quantitatively investigate the micro-sized diverse lesions as their biochemistry could be easily masked by the large volume of tissues sampled and the abundance of matrix proteins in the tendon. This probably explains the inconclusive results of western blot on AGEs and RAGE in tendinopathy, although these molecules were localized in and around the tendinopathic lesions at a high density by immunohistochemistry.

It is noteworthy that the phenotype of tenocytes is largely defined by their signature morphology and location in the tendon (Kannus, 2000). The roundish shape of the tenocytes within or surrounding the lesions was reminiscent of tenoblasts, which have a much wider cellular body than tenocytes (Luesma et al., 2021). It has been suggested that tenoblasts mainly present in the immature tendon and recess to tenocytes as the tendon matures (Moore and De Beaux, 1987). But they do reappear in tendinopathy and become proliferative (Rolf et al., 2001). Their role in tendinopathy pathology has not been determined, since tenoblasts also produce the protein-lysing matrix metalloproteinases (Chuen et al., 2004). At present, there are no specific biochemical markers that label the phenotype transition from tenoblasts to tenocytes. This study did not characterize the phenotype and trace the origin of these roundish, presumably, tenocytes but, nevertheless, suggests that the transformation of tenocyte phenotypes could be a significant pathology of tendinopathy.

Accumulation of AGEs in connective tissues is a biomarker of advanced aging. In aging tendons, the

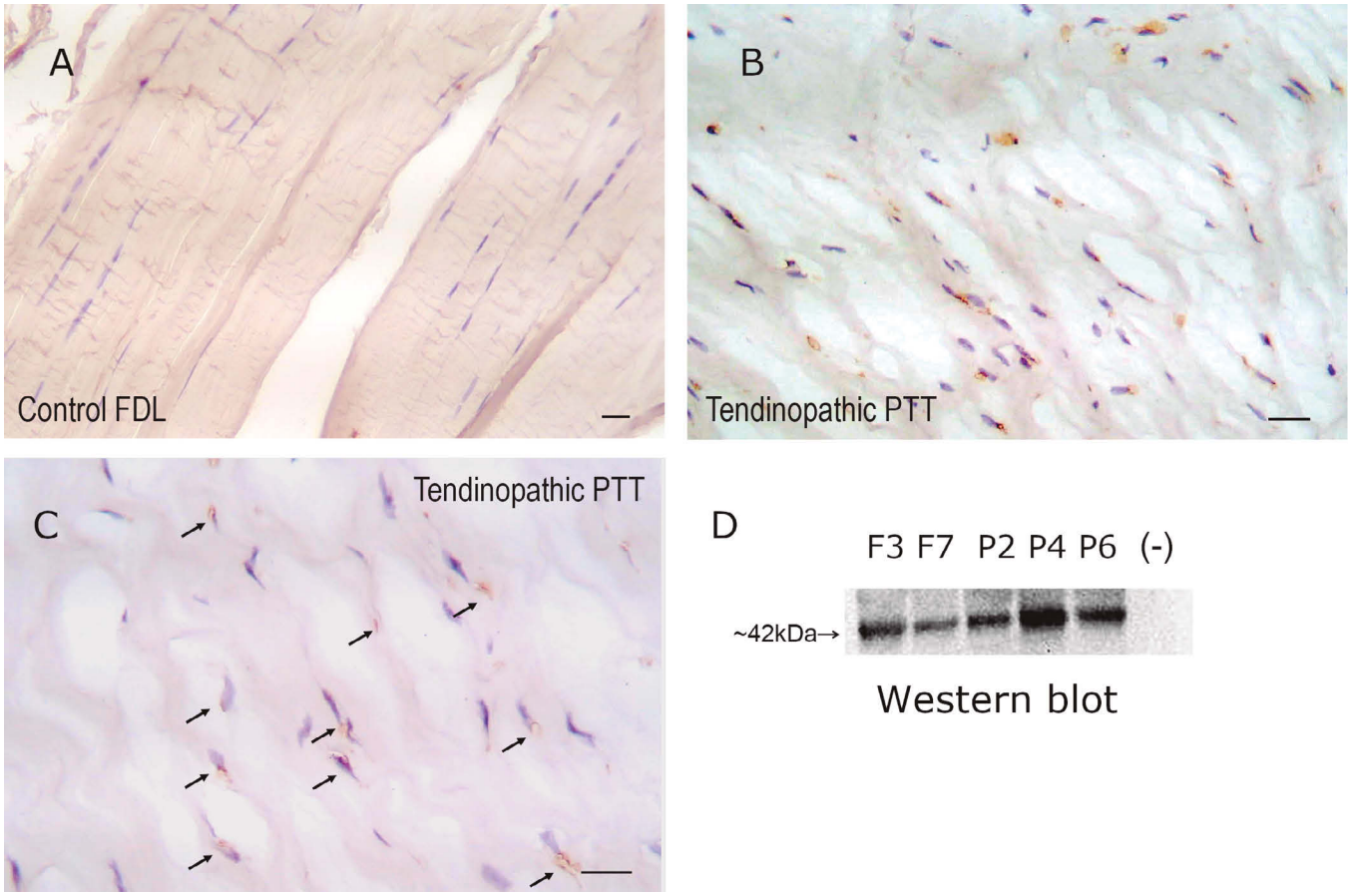

Fig. 4. Identification and localization of RAGE in the tendinopathic PTT and control FDL. **A, B.** While RAGE-positive tenocytes are inconspicuous in the FDL, there are a large number of tenocytes that are RAGE-positive in the tendinopathic lesion of PTT. The RAGE is located intracellularly. **C.** In another tendinopathic area, at a higher magnification, RAGE is located in some of the tenocytes in the lesion (RAGE-positive tenocytes are marked with arrows; Counterstain with hematoxylin). **D.** Western blot identifies RAGE expression in the control FDL and tendinopathic PTT. But the quantity of RAGE among the FDL and PTT samples varies considerably. Note: F3 and F7 are protein samples of the FDL, and P2, P4 and P6 are protein samples of the tendinopathic PTT; (-) represents the negative control. Scale bars: 50 μm.

concentration of AGE adduct was increased 60% over the controls in mice (Wood et al., 2011). AGEs have long been speculated about playing a role in tendinopathy as they could impact tendon mechanics fundamentally. In the extracellular domain, AGEs cross-link collagens and make tendons stiffer biomechanically (Li et al., 2013; Lee and Veres, 2019). The current study detected accumulation of AGEs in tendinopathic PTT on tissue sections and in total proteins, although its quantification was no different from FDL. Moreover, this study demonstrated that, rather than spreading across the entire tendon, AGEs were accumulated in patched areas whether they were inside or on the margin of a larger lesion. Locally accumulated AGEs were also present in small lesions surrounded by regular tendon structure. By its spatial relation with the tendinopathic lesions, accumulation of AGEs may act in several ways to influence the pathology. It is possible that, locally, the AGEs-crosslinked collagens form spots of "stress concentration" within the tendon and become the molecular foundation of "intrinsic risk factors" of tendinopathy. Additionally, AGEs-crosslinking reduces the turnover rate of collagens and their associated proteins. Consequently, the tendon becomes less adaptive to the dynamics of mechanical loading and prone to injury. Since the PTT samples were collected from the surgical cases that had advanced tendinopathy, it can't be ruled out that the patched accumulation of AGEs was the remnants of extensive proteolysis and matrix degradation in the late stage of tendinopathy.

Besides showing that accumulation of AGEs might alter the tendon's mechanical properties, immunohistochemistry revealed that the accumulated AGEs interfere with tendon biology in tendinopathy. In the tendinopathic areas of the PTT, tenocytes were intracellularly stained with AGEs. Intracellular accumulation of AGEs modifies molecular chaperones, induces endoplasmic reticulum stress, and even causes apoptosis (Yamabe et al., 2013). Indeed, AGEs influence the biology of tendon broadly, including inhibiting the mitochondrial function and proliferation of tenocytes (Patel et al., 2019). The morphological evidence provided by this study warrants further investigation of AGEs in the pathology of tendinopathy.

In addition to crosslinking the intracellular and extracellular molecules, AGEs propagate signals through binding with RAGE, which is a multiligand receptor on of many cell types. The AGEs/RAGE signaling pathway increases the production of oxygen radicals and pro-inflammatory cytokines (Prasad and Mishra, 2018). In this study, RAGE was expressed by the roundish tenocytes. It has been recognized that tenocytes often change their phenotypes in diseases and injury but the tenocyte phenotype itself is not well defined. The expression of RAGE by the roundish tenocytes might partially mark the changing phenotype of the tenocytes.

A limitation of this study is its small sample size, although the revealed pathological features were consistent throughout. Due to limited options of clinical sample selection, there was a mean age discrepancy between the tendinopathy and control groups. The control tendons in this study were taken from FDL when healthy FDL was transferred to another location surgically and sometimes an excessive portion of the FDL was resected. Healthy PTT, however, is not commonly transferred due to its importance to stabilize hindfoot during weight bearing. FDL and PTT are similar in shape, function, and histology, and the pair was similarly used in another PTT tendinopathy study (Bridgeman et al., 2010).

In conclusion, this study demonstrated "patched" accumulation of AGEs and enhanced expression of RAGE by clustered tenocytes within tendinopathic lesions. The spatial relations of AGEs and RAGE with tendinopathic lesions warrants further investigation of their roles in tendon biology and degeneration.

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