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Advanced Glycation End Products Increase Transglutaminase Activity in Primary Porcine Tenocytes

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Abstract

Objectives: Tendon abnormalities, such as increased stiffness, thickness, and excess calcification, occur commonly in patients with diabetes mellitus and cause considerable disability. These changes are frequently attributed to increased cross-linking of extracellular matrix components by advanced glycation end-products (AGEs). However, cellular effects of AGEs, such as increased activity of the cross-linking transglutaminase (Tgase) enzymes, could also contribute to altered tissue biomechanics and calcification in diabetic tendons. We determined the effect of AGE-modified protein on tenocyte Tgase activity.

Research Design and Methods: Primary porcine tenocytes were exposed to N^ε-carboxymethyl-lysine (CML)-modified type I collagen in high or normal glucose media. Protein and mRNA levels of the Tgase enzymes and Tgase activity levels were measured, as were markers of apoptosis. We also determined the effect of antioxidants on CML-collagen mediated Tgase activity.

Results: Carboxymethyl-lysine-collagen increased Tgase activity in tenocytes 2.3- to 5.6-fold over unmodified collagen controls in both normal and high glucose media, without altering enzyme protein levels. Anti-oxidant treatment reduced the effect of CML-collagen on Tgase activity. Deoxyribonucleic acid laddering and annexin V protein levels were not altered by CML-collagen exposure.

Conclusions: Carboxymethyl-lysine-collagen increased Tgase activity in tenocytes, likely posttranslationally. Increased levels of Tgase-mediated cross-links may contribute to the excess calcification and biomechanical pathology seen in diabetic tendons.

Keywords

tendinopathy; diabetes mellitus; advanced glycation end-products; transglutaminases; factor XIIIa

Painful tendinopathies, including shoulder tendonitis, limited hand mobility, tendon ruptures, and adhesive capsulitis affect 30% to 60% of diabetic patients and cause

considerable disability among affected patients.¹ Diabetic tendons are thicker and stiffer than normal² and show increased rates of calcification.³ These changes often lead to other complications. For example, reduced length and increased thickness of the Achilles tendon may contribute to the development of Charcot arthropathy of the foot and its serious sequelae.⁴ Tendon calcifications increase risk of rupture.⁵ Diabetic tendinopathies are often chronic and typically respond poorly to the limited therapies currently available.⁶

Remarkably little is known about the pathogenesis of diabetic tendinopathy. Certainly, advanced glycation end-products (AGEs), resulting from exposure of proteins to high levels of sugar moieties, accumulate prematurely in the long-lived proteins of connective tissues, including tendons.⁷ Advanced glycation end-product-modified proteins affect extra-cellular matrix through several mechanisms. They directly modify extracellular matrix by cross-linking proteins.⁸ Advanced glycation end-product-modified proteins also act on extracellular matrix production at a cellular level. By signaling through cell surface receptors, such as receptor for advanced glycation end-products (RAGE), they can alter the extracellular matrix profile synthesized by resident connective tissue cells.^{9,10}

Although many different types of AGE-modified proteins exist, N^ε-carboxymethyl-lysine (CML)-modified proteins are thought to be particularly specific ligands for RAGE. In skin, activation of RAGE by AGE-modified protein altered extracellular matrix protein production and increased levels of profibrotic growth factors, such as transforming growth factor β .⁹ Reactive oxygen species are also stimulated by RAGE activation in many cell types and have been shown to mediate some of the cellular effects of AGE-modified proteins.¹⁰ In osteoblasts and some fibroblasts, AGE-modified proteins also modestly increased markers of apoptosis.^{11,12}

Transglutaminase (Tgase) enzymes, including type II Tgase and the activated form of factor XIIIa (factor XIIIa), catalyze the formation of highly protease-resistant intra- and intermolecular cross-links. Their substrates include many key extracellular matrix proteins such as collagen, fibronectin, osteopontin, and microfibrillar proteins.¹³ Transglutaminase-mediated modifications alter the structure and function of extra-cellular matrix and contribute to tissue fibrosis.¹⁴ They also play important roles in both normal and pathologic matrix calcification.^{15,16} Increased Tgase activity has been described with high glucose exposure in a diabetic kidney disease model,¹⁷ and Tgase inhibitors reduced the excess extracellular matrix elaboration induced by high glucose levels.¹⁸ Transglutaminases are also implicated in apoptosis, where they cross-link intracellular proteins to prevent leakage of inflammatory cell contents during cell death.¹⁹ To our knowledge, Tgases have not been directly implicated in the cellular effects of AGE-modified proteins in diabetic tendinopathy, and the Tgase enzymes remain poorly characterized in tendons.

This study addresses the hypothesis that AGE-modified proteins increase Tgase activity in primary tenocytes, and this effect may contribute to the increased stiffness and excess calcification seen in diabetic tendinopathy. We show here that AGE-modified type I collagen markedly stimulates tenocyte Tgase activity, that this likely occurs posttranslationally, and is partially reversed by antioxidants.

MATERIALS AND METHODS

Materials

All materials were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Advanced glycation end-product-modified bovine serum albumin (AGE-BSA) was from BioVision Research Products (Mountainview, CA).

Tenocyte Cultures

Porcine patellar tendons were obtained from mature adult pigs and scraped free of any adherent synovial sheath or fat. Tenocytes were enzymatically released from surrounding matrix using 0.25% trypsin for 1 hour, followed by 1 mg/mL collagenase for 5 hours.²⁰ Cells were plated at a density of 3.0×10^5 cells/cm² and grown to near confluence (3–5 days) in 50% Ham's F-12/50% DMEM with 20% fetal calf serum and 1% Penicillin-Streptomycin-Fungizone (PSF; Invitrogen, Carlsbad, CA). Experiments were initiated within 5 days of cell plating. Scleraxis, a marker of the differentiated tenocyte phenotype,²¹ was demonstrated in tenocyte cultures by Western blotting (data not shown).

CML-Collagen and Control Collagen¹¹

Type I collagen (176 mg), the primary fibrillar collagen in native tendon, was dissolved in 1 mM HCl and incubated with 45 mM sodium cyanoborohydride in 0.2 M PBS(pH 7.8) and 155 mM glyoxylic acid for 24 hours at 37°C. Control collagen was treated with the same reaction mixture without glyoxylic acid. Modified residues in the CML-collagen preparations were quantified using the TNBS (trinitrobenzene sulfonic acid) assay.²² The preparation used here contained 7.3% modified residues. Carboxymethyl-lysine content was also directly measured in some preparations by gas chromatography mass spectrometry in acid hydrolyzed collagen as previously described.²³

These levels tended to be slightly higher than those measured with the TNBS assay, suggesting that not all lysyl residues were quantitatively derivatized by TNBS.

Tgase Activity Assay

Transglutaminase activity was measured in the cell layer with a standard radiometric assay based on the ability of Tgase to incorporate (³H) putrescine into dimethylcasein as previously described.²⁴ This assay measures activity of both type II Tgase and factor XIIIa and cannot distinguish between them. Briefly, media were removed and the cell layer was washed, scraped from the plate in 0.1% EDTA, and sonicated for 30 seconds. One hundred fifty microliters of cell suspension was added to 150 µL of 2× reaction mixture (250 mM Tris-HCl, 30 mM CaCl₂, 10 mg/mL *N,N* dimethyl casein, 200 µM putrescine, pH 8.3). One µCi ³H-putrescine was added to each sample and incubated for 30 minutes at 37°C. After precipitation of the proteins with 50% trichloroacetic acid, samples were blotted onto Whatman glass filters and counted in a Packard 1900TR scintillation counter. Results were expressed in Units (picomoles of (³H) putrescine incorporated into casein/30 minutes), corrected for the protein content of the sample as determined by the Lowry assay.²⁵

Western Blotting

Identical quantities of cell protein were loaded onto 10% NuPage (Invitrogen) Bis-Tris gels (30 µg protein/lane). After electrophoresis, proteins were blotted onto poly(vinylidene) difluoride membranes (Invitrogen). Membranes were blocked in a TBS-igepal-5% skim milk buffer for 1 hour at room temperature. They were then exposed to either antifactor XIIIa Ab-2 (1:1000, Abcam, Cambridge, MA) antitransglutaminase II Ab-4 (1:1000, NeoMarkers), or antiannexin V (1:1000, Abcam) for 1.5 hours. After washing, the membranes were exposed to peroxidase-goat antirabbit IgG (H+L) secondary antibody for 1 hour (1:2500, Invitrogen). Both the primary and secondary antibody exposures were performed in a TBS-igepal-0.5% skim milk buffer. SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) was used to visualize immunoreactive protein bands. Membranes were stripped and re-probed for tubulin (1:1000, Calbiochem) as a control for protein loading.

Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA (1 µg) was obtained from tenocytes with Trizol (Invitrogen). It was reverse transcribed using random hexamer primers and the Superscript First Strand Synthesis System for RT-PCR (Invitrogen) in a 20-µL reaction volume. The generated cDNA was amplified by PCR in an iCycler thermal cycler (BioRad, Hercules, CA) using PLATINUM Blue PCR Supermix (Invitrogen) with 0.4 µM primers and at the optimal hot start temperature of 94°C. Factor XIIIa and type II Tgase were individually multiplexed with 18S ribosomal RNA from the QuantumRNA 18S kit (Ambion, Austin, TX) which served as an internal standard and was used to correct for any differences in starting amounts of RNA. All gene specific primers were synthesized by Integrated DNA Technologies (Coralville, IA) from published primer pair sequences as follows for factor XIIIa: 5'-TCA ATG ACA TCA AGA CCA GAA G-3' and 5'-GAC ACC AGC AAA AAC CCA G-3'²⁶; and type II transglutaminase: 5'-CCG TTT TCC ACT AAG AGA TGC-3' and 5'-CCC AAA ATT CCA AGG TAT GTT-3'.²⁷ The number of amplification cycles was empirically determined to ensure PCR products were within the exponential phase of the amplification curve. PCRs were performed as follows: 2 minutes at 94°C, followed by 26 to 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by 5 minutes at 72°C. Ten microliters of PCR product were separated on a 2% agarose gel in Tris-borate EDTA buffer containing 1 µg/mL ethidium bromide. The gel was photographed under UV light and bands were quantified using densitometry with Kodak 1D Image Analysis software. Relative expression was calculated as the ratio of band intensity of PCR product/18S control band.

DNA Laddering

Deoxyribonucleic acid laddering was performed with a kit according to manufacturer's directions (TACS DNA laddering kit; R&D Systems, Minneapolis, MN).

Cell Viability

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) assay was used according to manufacturer's directions (BioAssay Systems, Hayward, CA) to assess cell

viability in tenocyte cultures. Healthy cells with functioning mitochondria turn the pale yellow chemical into a dark blue formazan product which can be quantified colorimetrically.

Cell injury was assessed by measuring levels of lactate dehydrogenase (LDH) released into the media (CytoScan; G-Biosciences, St. Louis, MO).

Statistics

All experiments were repeated 3 to 5 times. The Student t test was used to determine statistically significant differences between groups. Significance was set at $P < 0.05$.

RESULTS

CML-Collagen Stimulated Tgase Activity in Tenocytes in Both Normal and High Glucose Media

Baseline Tgase activity levels in cultured tenocytes were 0.04 to 0.10 U/mg protein. As shown in Figure 1, in *iso*-osmolar media with normal or high glucose levels, concentrations of 40 to 400 $\mu\text{g/mL}$ CML-modified collagen significantly increased tenocyte Tgase activity compared with controls exposed to 400 $\mu\text{g/mL}$ of unmodified type I collagen ($P < 0.001$). For example, 40 $\mu\text{g/mL}$ of CML-collagen increased Tgase activity from 0.049 ± 0.01 U/mg protein to 0.144 ± 0.03 U/mg protein. In tenocytes exposed to CML-collagen, Tgase levels increased 2.8- to 5.6-fold over control values at 72 hours, and peak increases were seen at concentrations of 160 $\mu\text{g/mL}$ CML-collagen. Carboxymethyl-lysine-collagen also increased Tgase activity after 4 and 24 hours of exposure in a similar dose-responsive pattern (data not shown). A commercial preparation of AGE-modified BSA produced a similar effect on Tgase activity at concentrations from 0.5 to 50 $\mu\text{g/mL}$ (data not shown). Doses of 10 to 400 $\mu\text{g/mL}$ of CML-modified proteins are typically used in the literature in cell culture models.¹² Unfortunately, many of these preparations are not standardized as to how much of the reference protein is actually CML-modified. Tissue levels of CML-modified proteins are in the 1 to 10 $\mu\text{g/mL}$ range.^{28,29} Thus, our effective dose range approximates those both used in the literature and measurable in human tissues.

Glucose and Insulin Also Increased Tgase Levels

We observed a consistent difference in baseline Tgase levels between tenocytes incubated in high and normal glucose media. Transglutaminase activity was significantly higher in tenocytes incubated in 25 mM glucose compared with activity levels in 5.5 mM glucose ($P < 0.003$). As insulin levels may also be elevated in some diabetic patients, we also explored the effect of 20 to 100 ng/mL insulin on tenocyte Tgase activity in both high and normal glucose media. The 100 ng/mL dose of insulin significantly increased Tgase activity in high glucose media. Values were 0.049 ± 0.01 U/mg protein in control cultures and 0.079 ± 0.01 U/mg protein in cultures exposed to 100 ng/mL insulin. Insulin had no significant effects in normal glucose media or at 20 ng/mL doses ($P > 0.05$) (Fig. 2).

CML-Collagen Did Not Affect Protein Levels of Type II Tgase or Factor XIII A

Transglutaminase enzymes have not been well characterized in primary tenocytes. Both type II Tgase and factor XIII A proteins are easily detectable in primary tenocytes by Western

blotting (Fig. 3). These are the same Tgase enzymes found in other musculoskeletal cells, including osteoblasts³⁰ and chondrocytes.³¹ As shown in Figure 3, CML-collagen did not consistently affect protein levels of either enzyme, whereas small increases in factor XIIIa and type II Tgase mRNA were noted in both high glucose conditions and with exposure to CML-collagen.

CML-Collagen Does Not Induce Markers of Apoptosis or Alter Cell Viability

Alikhani et al¹¹ showed a modest increase in apoptosis after CML-collagen treatment in fibroblasts and osteoblasts. As Tgase enzymes may participate in apoptosis, we asked whether markers of apoptosis or cell death were also increased by CML-collagen. Annexin V, a commonly used marker of apoptosis, was not consistently increased by CML-collagen. No DNA laddering was observed with any treatment (data not shown). In addition, uptake and conversion of MTT, a measure of cell viability, was unchanged after 72 hours of CML-collagen treatment at all doses and conditions tested. Table 1 summarizes these data under high glucose conditions. Other measures of cell injury, including cell protein levels and release of lactate dehydrogenase, were also unchanged by CML-collagen.

Antioxidants Reduce the Effect of CML-Collagen on Tenocytes Tgase Activity

Prior studies suggest an important intermediary role for reactive oxygen species in some of the effects of AGEs.³² Interestingly, reactive oxygen species can also modulate Tgase activity by altering substrate susceptibility to cross-linking.³³ In order to investigate the role of reactive oxygen species in the action of CML-collagen on Tgase activity, we reduced reactive oxygen species with a variety of antioxidants and measured the effect of CML-collagen on Tgase activity. As shown in Figure 4, the antioxidants, N-acetyl cysteine, uric acid, and sodium selenite, significantly suppressed the ability of 160 μ L/mL of CML-collagen to stimulate Tgase activity in high glucose media ($P = 0.008$).

Reactive Oxygen Species Increase Tgase Activity

To further investigate the hypothesis that reactive oxygen species affect Tgase activity by altering substrate behavior, we incubated the standard reaction mixture from our Tgase assay, which contains the Tgase substrates N,N'-dimethyl casein and putrescine, with 1 mM hydrogen peroxide (H_2O_2) for 30 minutes. We then added 40 mU of purified guinea pig liver type II Tgase and measured enzyme activity. We used 20 U/mL of catalase to inactivate the H_2O_2 as an additional control. As shown in Figure 5, when the reaction mixture containing Tgase substrates was incubated with H_2O_2 , Tgase enzyme activity was significantly increased ($P < 0.05$). This effect was reduced in the presence of catalase. This observation supports the hypothesis that increased Tgase enzyme activity may occur when Tgase substrates are exposed to reactive oxygen species.

DISCUSSION

We show here that CML-collagen stimulates Tgase activity in tenocytes. High glucose and insulin levels also increased Tgase enzyme activity. Thus, several of the key metabolic abnormalities present in diabetic patients dramatically increase Tgase activity in tenocytes. This may represent an additional and under-recognized mechanism of extracellular matrix

modulation in diabetic tendons. Transglutaminase enzymes produce highly protease-resistant cross-links that alter the structure and function of affected extracellular matrices. Increased production of Tgase cross-links may impair the tendon's ability to remodel and repair and render them less able to respond to injury. Transglutaminase enzyme activity may also contribute to the excess thickness, stiffness, and calcification seen in diabetic tendinopathy.

Very little is known about the Tgase enzymes in tendons. We show here that tenocytes from normal mature tendon display measurable quantities of Tgase activity and contain the same 2 Tgase enzymes found in chondrocytes and osteoblasts. Nine members of the Tgase family have been characterized to date. Eight of these proteins are enzymes which catalyze the formation of unique N^ε (γ-glutamyl)lysine bonds. The Tgase enzymes have varied physiologic and pathologic functions. Type II Tgase is found in many cell types. It acts as a G-protein intracellularly. In its extracellular state, it is activated by high calcium levels to cross-link extracellular matrix protein substrates, such as collagen, osteopontin, and fibronectin. Less is known about factor XIIIa in connective tissues. It was first described as a circulating participant in the clotting cascade. In its cellular form, it is best characterized in platelets, chondrocytes, and bone cells.³⁴ It is likely secreted as a proenzyme (factor XIIIa), that requires proteolysis by thrombin or other proteases for activity. Its substrates include many of the same proteins as type II Tgase.

Transglutaminase enzymes have been implicated in diabetic kidney disease as well as in some genetic forms of diabetes. In kidneys of streptozotocin-treated rats, excess Tgase activity was noted in the peritubular interstitial space.³⁵ Fibrosis of proximal tubular endothelium was prevented by inhibition of Tgase-specific cross-link formation.¹⁸ Type II Tgase participates in the secretion of insulin by pancreatic islet cells. Type II Tgase knockout mice manifest a late-onset phenotype similar to that of maturity onset diabetes of the young which is attributed to this defect in insulin secretion.³⁶

Overactivity of the Tgase enzymes has been noted in many diseases of aging, including cataract formation, Alzheimer disease, and osteoarthritis.¹³ Interestingly, many of these processes also involve reactive oxygen species. The shared presence of pathologic calcification in degenerative diseases may further implicate Tgases in their pathogenesis. The Tgase enzymes play a key role in pathologic mineralization in many settings. They have been best studied in cartilage, where Tgase specific cross-links are found concentrated near calcium deposits in articular cartilage.¹⁶ In addition, inhibitors of Tgase activity dramatically decrease pathologic calcification in a cell culture model.¹⁶ A role for the Tgase enzymes in tendon calcification remains intriguing but unexplored. Certainly, the increased rate of tendon calcification in diabetic tendons indirectly supports a possible association.³

Our data suggest a possible role for reactive oxygen species in the effects of CML-collagen on Tgase activity. Antioxidants suppressed the effect of CML-collagen on Tgase activity. Although we did not directly measure reactive oxygen species, this effect was very consistent with multiple antioxidants. A role for reactive oxygen species in modulating Tgase activity has been described in other systems. The commonly invoked mechanism is

through free radical-mediated alterations in the susceptibility of substrates to cross-linking.
33,37

Although the mechanisms of the effect of CML-collagen on tenocyte Tgase activity are not fully apparent, they appear to be largely posttranslational. We could document no consistent increases in protein levels for the Tgase enzymes after CML-collagen treatment and only modest changes in mRNA. The rapid onset of this effect also suggests that transcription and translation are not required. Transglutaminase enzymes are often posttranslationally regulated; factor XIIIa by proteases such as thrombin as well as calcium, pH and ionic strength, and type II Tgase by levels of GTP and calcium. Our data suggest that free radical-mediated increased susceptibility to cross-linking explains at least some of the increased Tgase activity with CML-collagen exposure. A similar effect has been noted with other substrates of Tgase. We believe that in this system, free radicals induced by AGE-modified proteins alter the enzyme or substrate to increase Tgase activity.

These studies are not without limitations. Our activity assay cannot distinguish between the enzymes responsible for Tgase activity in tenocytes. It is also possible that tendons contain other types of Tgase enzymes that we did not detect. We did not explore the effect of other individual AGEs in this system, although a similar effect was seen with a commercial preparation of AGE-modified BSA. Further work to validate this effect in human tendons from diabetic and nondiabetic patients and to further delineate the mechanism of this effect is currently underway in our laboratory.

In summary, we show here that CML-collagen increases Tgase activity in primary tenocytes. This may be an additional, novel pathway mediating pathologic changes in diabetic tendons. Although the mechanism of this effect is not fully elucidated, it appears to occur posttranslationally and to be at least partially mediated by reactive oxygen species. Further investigation into the role of the Tgase enzymes in diabetic tendinopathy is clearly warranted and may prove helpful in designing effective therapies for this common and disabling condition.

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REFERENCES

1. Sukenik S, Weitzman S, Buskila D, et al. Limited joint mobility and other rheumatological manifestations in diabetic patients. *Diabete Metab.* 1987;13:187–192. [PubMed: 3609420]
2. Akturk M, Karaahmetoglu S, Kacar M, et al. Thickness of the supraspinatus and biceps tendons in diabetic patients. *Diabetes Care.* 2002;25:408.
3. Mavrikakis M, Drimis S, Kontoyannis D, et al. Calcific shoulder periarthritis (tendinitis) in adult onset diabetes mellitus: a controlled study. *Ann Rheum Dis.* 1989;48:211–214. [PubMed: 2930276]
4. Grant W, Sullivan R, Sonenshine D, et al. Electron microscopic investigation of the effects of diabetes mellitus on the Achilles tendon. *J Foot Ankle Surg.* 1997;36:272–278. [PubMed: 9298442]
5. Jim Y, Hsu H, Chang C, et al. Coexistence of calcific tendonitis and rotator cuff tear: an arthrographic study. *Skeletal Radiol.* 1993;22: 183–185. [PubMed: 8480205]

6. Moren-Hybbinette I, Moritz U, Schersten B. The clinical picture of the painful diabetic shoulder-natural history, social consequences and analysis of the concomitant hand syndrome. *Acta Med Scand.* 1987;221:73–82. [PubMed: 2436441]
7. Vogt B, Schleicher E, Wieland O.e -amino-lysine-bound glucose in human tissues obtained at autopsy: Increase in diabetes mellitus. *Diabetes.* 1982;31:1123–1127. [PubMed: 6816647]
8. Lancaster R, Haut R, DeCamp C. Changes in the mechanical properties of patellar tendon preparations of spontaneously diabetic dogs under long term insulin therapy. *J Biomech.* 1994;27:1105–1108. [PubMed: 8089166]
9. Nah S-S, Choi I-Y, Yoo B, et al. Advanced glycation end products increases matrix metalloproteinase-1 –3, and –13, and TNF-alpha in human osteoarthritic chondrocytes. *FEBS Lett.* 2007;581:1928–1932. [PubMed: 17434489]
10. Kislinger T, Fu C, Huber B, et al. N(epsilon)-carboxymethyl)lysine adducts of proteins are ligands for receptors for advanced glycation end products that activate cell signaling pathways and modulate gene expression. *J Biol Chem.* 1999;274:31740–31749. [PubMed: 10531386]
11. Alikhani M, Alikhani Z, Boyd C, et al. Advanced glycation end products stimulate osteoblast apoptosis via the MAP kinase and cystolic apoptotic pathways. *Bone.* 2007;40:345–353. [PubMed: 17064973]
12. Alikhani Z, Alikhani M, Boyd C, et al. Advanced glycation end products enhance expression of pro-apoptotic genes and stimulate fibroblast apoptosis through cytoplasmic and mitochondrial pathways. *J Biol Chem.* 2005;280:12087–12095. [PubMed: 15590648]
13. Lorand L, Graham R. Transglutaminases: crosslinking enzymes with pleiotrophic functions. *Nature.* 2003;4:140–157.
14. Douthwaite J, Johnson T, Haylor J, et al. Effects of transforming growth factor-beta1 on renal extracellular matrix components and their regulating factors. *J Am Soc Nephrol.* 1999;10:2109–2119. [PubMed: 10505687]
15. Aeschlimann D, Mosher D, Paulsson M. Tissue transglutaminase and Factor XIII in cartilage and bone remodeling. *Semin Thromb Hemost.* 1996;22(5):437–443. [PubMed: 8989828]
16. Heinkel D, Gohr C, Uzuki M, et al. Transglutaminase contributes to CPPD crystal formation in osteoarthritis. *Front Biosci.* 2004;9:3257–3261. [PubMed: 15353354]
17. El Nahas A, Abo-Zenah H, Skill N, et al. Elevated (F-Glutamyl)lysine in human diabetic nephropathy results from increased expression and cellular release of tissue transglutaminase. *Nephron Clin Pract.* 2004;97:c108–17. [PubMed: 15292688]
18. Skill N, Johnson T, Coutts I, et al. Inhibition of transglutaminase activity reduces extracellular matrix accumulation induced by high glucose levels in proximal tubular epithelial cells. *J Biol Chem.* 2004; 279:47754–47762. [PubMed: 15326185]
19. Nagy L, Thomazy V, Saydak M, et al. The promoter of the mouse tissue transglutaminase gene directs tissue-specific, retinoid-regulated and apoptosis-linked expression. *Cell Death Differ.* 1997;4:534–547. [PubMed: 14555966]
20. Lanberti P, Wezeman F. Biologic behavior of an InVitro hydrated collagen-human tenocyte tendon model. *Clin Orthop Rel Res.* 2002; 397:414–423.
21. Schulze-Tanzil G, Mobasheri A, Clegg P, et al. Cultivation of human tenocytes in high-density culture. *Histochem Cell Biol.* 2004;122: 219–228. [PubMed: 15338228]
22. Kertesz G, Bourcier B, Cailla H, et al. Immunoradiometric assay of succinylated corticotropin: an improved method for quantification of ACTH. *Clin Chem.* 1998;44:78–85. [PubMed: 9550562]
23. Monnier V, Bautista O, Kenny D, et al. Skin collagen glycation, glyoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type I diabetes. *Diabetes.* 1999;48:870–880. [PubMed: 10102706]
24. Rosenthal AK, Derfus BA, Henry LA. Transglutaminase activity in aging articular chondrocytes and articular cartilage vesicles. *Arthritis Rheum.* 1997;40(5):966–970. [PubMed: 9153560]
25. Lowry O, Rosebrough N, Farr A, et al. Protein measurement with Folin-phenol reagent. *J Biol Chem.* 1951;193:265–275. [PubMed: 14907713]
26. D'Argenio G, Calvani M, Della Valle N, et al. Differential expression of multiple transglutaminases in human colon: impaired keratinocyte transglutaminase expression in ulcerative colitis. *Gut.* 2005;54: 496–502. [PubMed: 15753534]

27. Sun Y, Kandel R. Deep zone articular chondrocytes in vitro express genes that show specific changes with mineralization. *J Bone Miner Res.* 1999;14:1916–1925. [PubMed: 10571692]
28. van de Merbel N, Mentink C, Wolfenbuttel B. Liquid chromatographic method for the quantitative determination of N-carboxymethyllysine in human plasma proteins. *J Chromatogr.* 2004;808:163–168.
29. Schleicher E, Wagner E, Nerlich A. Increased accumulation of the glycoxidation product Ne-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest.* 1997;99:457–468. [PubMed: 9022079]
30. Nakano Y, Al-jallad H, Mousa A, et al. Expression and localization of plasma transglutaminase factor XIIIa in bone. *J Histochem Cytochem.* 2007;55:675–685. [PubMed: 17341477]
31. Rosenthal A, Masuda I, Gohr C, et al. The transglutaminase, Factor XIIIa, is present in articular chondrocytes. *Osteoarthritis Cartilage.* 2001;9:578–581. [PubMed: 11520172]
32. Lin C-L, Wang F-S, Kuo Y, et al. Ras modulation of superoxide activates ERK-dependent fibronectin expression in diabetes-induced renal injuries. *Kidney Int.* 2006;69:1593–1600. [PubMed: 16572112]
33. Groenen P, Seccia M, Smulders R, et al. Exposure of AH-crystallin to hydroxyl radicals enhances the transglutaminase susceptibility of its existing amine-donor and amine acceptor sites. *Biochem J.* 1993; 293:399–404. [PubMed: 8343121]
34. Muszbek L, Yee V, Hevessy Z. Blood coagulation factor XIII: structure and function. *Thromb Res.* 1999;94:271–305. [PubMed: 10379818]
35. Skill N, Griffin M, El Nahas A, et al. Increases in renal α -(F-glutamyl)lysine crosslinks results from compartment-specific changes in tissue transglutaminase in early experimental diabetic nephropathy; Pathologic implications. *Lab Invest.* 2001;81:705–716. [PubMed: 11351042]
36. Bernassola F, Federici M, Corazzari M, et al. Role of transglutaminase 2 in glucose tolerance: knockout mice studies and a putative mutation in a MODY patient. *FASEB J.* 2002;16:1371–1378. [PubMed: 12205028]
37. Holmes F, Haynes L. Superactivation of transglutaminase type 2 without change in enzyme level occurs during progressive neurodegeneration in the mnd mouse. *Neurosci Lett.* 1996;213:185–188. [PubMed: 8873145]

FIGURE 1.

The effect of CML-collagen on Tgase activity in tenocytes. Tenocytes were incubated in media containing normal (5.5 mM, grey bars) or high (25 mM, black bars) glucose. Various doses of CML-collagen or 400 µg/mL control collagen (control) were added to the media and the cells were incubated for 72 hours. Transglutaminase activity was quantified in the cell layer using a standard radiometric assay corrected for cell protein. Values represent means $\pm$ SD ($n = 12$). Carboxymethyl-lysine-collagen increased Tgase activity over control values at all concentrations tested (* $P < 0.001$).

FIGURE 2.

The effect of insulin on Tgase activity in tenocytes. Tenocytes were incubated in media containing normal (5.5 mM, grey bars) or high (25 mM, black bars) glucose with or without insulin. After 72 hours of incubation, Tgase activity was measured in the celllayer using a standard radiometric assay corrected for cell protein. Values represent means $\pm$ SD (n = 6). Insulin at 100 ng/mL increased Tgase activity in high glucose media (* $P < 0.01$)

FIGURE 3.

The effects of CML-collagen on Tgase mRNA and protein levels. Tenocytes maintained in media with either normal (5.5 mM, grey bars) or high (25 mM, black bars) glucose were treated with various doses of CML-collagen or 400 µg/mL control collagen. After 72 hours of incubation, mRNA and protein fractions were isolated from the cell layer. Specific primers for type II transglutaminase (panel A) or factor XIIIa (panel C) were used in RT-PCR to estimate mRNA levels for these enzymes. Results are expressed as a ratio of the band of interest to that of 18S. Western blots were also performed using specific antibodies for type II transglutaminase (panel B) and factor XIIIa (panel D). Blots were stripped and re-probed with antitubulin antibodies. Lane 1, low glucose collagen control; lane 2, low glucose 40 µg/mL CML-collagen; lane 3, low glucose 160 µg/mL CML-collagen; lane 4, low glucose 400 µg/mL CML-collagen; lane 5, high glucose collagen control; lane 6, high glucose 40 µg/mL CML-collagen; lane 7, high glucose 160 µg/mL CML-collagen; lane 8, high glucose 400 µg/mL CML-collagen. No significant differences in protein levels were noted on densitometry.

FIGURE 4.

The effects of the antioxidants on Tgase activity in tenocytes. Tenocytes in high glucose media were exposed to 400 $\mu\text{g/mL}$ control collagen (black bars) or 160 $\mu\text{g/mL}$ CML-collagen (grey bars) with or without the antioxidants 500 μM uric acid, 1 mM sodium selenite, or 10 mM N-acetyl cysteine. Transglutaminase activity was measured after 72 hours. All of the antioxidants significantly decreased CML-mediated stimulation of Tgase activity ($n = 12$; $*P = 0.008$).

FIGURE 5.

The effect of hydrogen peroxide on Tgase activity. Transglutaminase reaction mixture containing *N,N'*-dimethylcasein and (³H) putrescine was incubated with or without 1 mM H₂O₂ and/or 20 U/mL catalase for 30 minutes. Forty mU of purified guinea pig liver type II Tgase was added and the reaction was allowed to proceed for 30 minutes. Quantities of ³H putrescine incorporated into *N,N'*-dimethylcasein were quantified by liquid scintigraphy (n = 6; **P* < 0.05).

TABLE 1.

The Effect of CML-Collagen on Markers of Cell Injury and Apoptosis *

	Control	CML-Collagen
Protein (mg/well)	8.7 ± 0.3	8.4 ± 0.7
LDH (U/well)	1.4 ± 0.2	1.4 ± 0.3
Annexin V (IU)	13.7 ± 0.9	16.1 ± 3.1
MTT (mU/mg protein)	2.1 ± 0.1	3.0 ± 0.1

* Tenocytes were exposed to control collagen (control, 400 µg/ml) or CML-collagen (160 µg/ml) for 72 hours in high glucose media. Cell injury was assessed by measuring cell protein levels, release of LDH into the media, annexin V levels, and the MTT assay. There were no significant differences in any of these parameters between groups ($P > .05$, $n = 6$).