Inhibition of CD44 induces apoptosis, inflammation, and matrix metalloproteinase expression in tendinopathy

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Po-Ting Wu, a.b.c.d.e Wei-Ren Su, a.b Chia-Lung Li, b.a Jeng-Long Hsieh, f Ching-Hou Ma, g.h Chao-Liang Wu, i Li-Chieh Kuo, c J-I-Ming Jou, g.h and Shih-Yao Chen k.a

From the aDepartment of Orthopaedics, College of Medicine, National Cheng Kung University, Tainan, Taiwan, the bDepartment of Orthopaedics, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan, the cDepartment of Biomedical Engineering, College of Engineering, National Cheng Kung University, Tainan, Taiwan, the dDepartment of Orthopaedics, National Cheng Kung University Hospital Dou-Liou Branch, College of Medicine, National Cheng Kung University, Yulin, Taiwan, the eMedical Device Innovation Center, National Cheng Kung University, Tainan, Taiwan, the fDepartment of Nursing, College of Nursing, Chung Hwa University of Medical Technology, Tainan, Taiwan, the gDepartment of Orthopedics, E-DA Hospital, Kaoshiung, Taiwan, the hSchool of Medicine, College of Medicine, I-Shou University, Kaohsiung, Taiwan, the iDepartment of Biochemistry and Molecular Biology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, the jDepartment of Occupational Therapy, College of Medicine, National Cheng Kung University, Tainan, Taiwan, the kDepartment of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan, and the lDepartment of Internal Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan

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Apoptosis has emerged as a primary cause of tendinopathy. CD44 signaling pathways exert anti-apoptotic and -inflammatory effects on tumor cells, chondrocytes, and fibroblast-like synoviocytes. The aim of this study was to examine the association among CD44, apoptosis, and inflammation in tendinopathy. Expression of CD44 and apoptotic cell numbers in tendon tissue from patients with long head of biceps (LHB) tendinopathy were determined according to the histological grades of tendinopathy. Primary tenocytes from Achilles tendon of Sprague–Dawley rats 1 week after collagenase injection were cultured with an antagonizing antibody against CD44. Treatment responses were determined by evaluating cell viability and expression of tendon-related proliferation markers, inflammatory mediators, and apoptosis. The expression of CD44 and apoptosis were positively correlated with the severity of tendinopathy in the human LHB tendinopathy. Furthermore, CD44 expression and apoptotic cells were co-stained in tendinopathic tendon. Blocking the CD44 signaling pathways in rat primary tenocytes by OX-50 induced cell apoptosis and the elevated levels of cleaved caspase-3. Furthermore, they had decreased cell viability and expression of collagen type I, type III, tenomodulin, and phosphorylated AKT. In contrast, there were elevated levels of inflammatory mediators, including interleukin (IL)-1β, IL-6, tumor necrosis factor-α, cyclooxygenase-2, and phosphorylated NF-κB, as well as matrix metalloproteinase (MMP) family members including MMP-1, -3, -9, and -13 in tenocytes upon OX-50 treatment. This study is the first to demonstrate the association of CD44 and apoptosis in tendinopathy. Our data imply that CD44 may play a role in tendinopathy via regulating apoptosis, inflammation, and extracellular matrix homeostasis.

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1 To whom correspondence may be addressed: Dept. of Orthopedics, E-DA Hospital 1, Yida Rd., Jiao-Su Village, Yan-Chao District, Kaohsiung 82445, Taiwan. Tel.: 886-7-6150011; Fax: 886-7-6150913; E-mail: jming@mail.ncku.edu.tw.
2 To whom correspondence may be addressed: Dept. of Internal Medicine, College of Medicine, National Cheng Kung University 1, University Rd., Tainan 70101, Taiwan. Tel.: 886-6-2353535 (ext. 5535); E-mail: leonlai50@gmail.com.

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Tendinopathy is a chronic painful tendon disorder and accounts for 30–50% of musculoskeletal and sports-related problems (1, 2). The pathological features of tendinopathy comprise proliferation of tenocytes, intracellular abnormalities in tenocytes, disruption of collagen fibers, and a subsequent increase in noncollagenous matrix (3). The pathophysiology of tendinopathy remains largely unclear thus far. Nevertheless, the mechanisms that have been proposed include mechanical overload, inflammation, imbalance between matrix metalloproteinases (MMPs)3 and tissue inhibitors of metalloproteinases, and dysregulated apoptosis (1, 4–6).

Excessive apoptosis has long been linked to a spectrum of degenerative disorders, including osteoarthritis (OA) and tendinopathy (6–9). Hypoxia, mechanical loading, inflammation, and genetic predisposition are the risk factors that may lead to enhanced apoptotic tenocyte death (10–12). Tenocytes play a critical role in maintaining homeostasis of extracellular matrix (ECM). A potential association between changes in ECM composition and increased tenocyte apoptosis has been established (13, 14). Therefore, identification of the molecular mechanisms driving dysregulated tenocyte apoptosis is important in understanding the pathophysiology of tendinopathy.

3 The abbreviations used are: MMP, matrix metalloproteinase; OA, osteoarthritis; ECM, extracellular matrix; HA, hyaluronic acid; IL, interleukin; LHB, long head of biceps; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; α-SMA, α-smooth muscle actin; Tmdm, tenomodulin; qRT-PCR, quantitative RT-PCR; TNF, tumor necrosis factor; CCL, chronic lymphocytic leukemia; COX-2, cyclooxygenase-2; COL1A1 and COL3A1, collagen type I and III, respectively.
CD44 is a principal cell-surface receptor for hyaluronan (hyaluronic acid (HA)), which is a constituent of the ECM. We have previously demonstrated that CD44 is clearly located at the plasma membranes and cell-cell junctions of the cultured tenocytes and mediates the HA-induced down-regulation of MMP-1 and MMP-3 expression in interleukin (IL)-1β-stimulated tenocytes (15). Growing evidence indicates that signaling through CD44 can induce the anti-apoptotic effects on OA chondrocytes (16). Furthermore, RNAi or mAb targeting CD44 induces apoptosis in various kinds of cancer cells (17–19). However, the association between CD44 and apoptosis in tendinopathy has yet to be clarified. As such, this study was undertaken to address the role of CD44-mediated apoptotic process in tendinopathy. Our hypotheses are as follows: (i) that the expression of CD44 and apoptotic cell number are positively correlated with disease severity in human long head of biceps (LHB) tendinopathy and (ii) that blocking CD44 induces cell apoptosis and the expressions of MMPs, inflammatory mediators, and phosphorylated NF-κB in primary tenocytes from rats following collagenase injection, as well as the down-regulation of tendon-related proliferation marker expression. Our results implicate that CD44-mediated signaling pathways may not only inhibit apoptosis, but also mediate anti-inflammation and maintain ECM homeostasis during tendinopathy.

Results

Increased CD44 expression and enhanced apoptosis in tendon tissue from patients with LHB tendinopathy

The expression of CD44 was first correlated with apoptosis in the tendon of patients with LHB tendinopathy. Immunohistochemical and TUNEL stainings showed that CD44 and apoptotic cells were detected with increased expression levels and positively correlated with the histological grades of patients during progression of tendinopathy (Fig. 1A and C). Immunofluorescence and TUNEL stainings demonstrated co-staining of CD44 expression and apoptotic cells in tendon from patients with LHB tendinopathy (Fig. 1B).

Tendinopathic and myofibroblastic characteristics and increased CD44 expression in primary tenocytes from rats’ Achilles tendon treated with collagenase

Tenocytes from the collagenase-treated rat tendon at week 1 expressed higher levels of IL-1β, IL-6, cyclooxygenase-2 (COX-2), MMP-1, MMP-3, α-smooth muscle actin expression (α-SMA), and CD44 but lower levels of collagen type I (COL1A1) and type III COL3A1 than those from normal rat tendon (Fig. 2), indicating that these cells were under both tendinopathic and myofibroblastic conditions (p < 0.05 for all results; Fig. 2).

Blocking CD44 induces cell apoptosis of primary tenocytes from rats following collagenase injection

To determine and clarify the role of CD44-associated apoptosis in tendinopathy, OX-50, a CD44-antagonizing antibody, was added to the cell culture of primary tenocytes from rats following collagenase injection. After treatment with OX-50 (10 μg/ml) for 24 h, cell viability was significantly reduced, as determined by WST-8 analysis (p < 0.001; Fig. 3A). Furthermore, TUNEL-positive cells were observed in the OX-50–treated tenocytes for 24 h (Fig. 3B). Furthermore, a reduction in phosphorylated AKT levels and, in contrast, induction in the ratios of cleaved caspase-3 to pro-caspase-3 and phosphorylated NF-κB to NF-κB in the OX-50–treated tenocytes were simultaneously detected, as determined by immunoblotting (Fig. 3C).

Blocking CD44 regulates expression of tendon-related markers, MMPs, and inflammatory mediators

We further investigated the expression of COL1A1, COL3A1, and tenomodulin (tnmd), which are related molecules produced by proliferative tenocytes in OX-50–treated cells. Their mRNA and protein expression levels were significantly down-regulated, as determined by quantitative RT-PCR (qRT-PCR) (p < 0.05 for all results; Fig. 3D) and immunoblotting (see Fig. 5A). In contrast, the mRNA expression of MMP family members, including MMP-1, -3, -9, and -13, and inflammation-related genes, including IL-1beta, IL-6, TNFalpha, and COX-2, was concomitantly up-regulated in OX-50–treated tenocytes, as measured by qRT-PCR (p < 0.05 for all results; Fig. 4). Furthermore, the protein expression levels of MMP-1 and -3, COX-2, and IL-6 were increased in OX-50–treated tenocytes compared with those in the control cells, as determined by immunoblotting and ELISA analyses (Fig. 5B). However, TNFα and IL-1β levels were undetectable between the control and OX-50–treated cells by ELISA tests.

Discussion

This is the first study to demonstrate the association of CD44 and apoptosis in tendinopathy. As the severity of tendinopathy progressed, the expression of CD44 and apoptosis correlated positively in patients with LHB tendinopathy. CD44 expression and TUNEL-positive cells were co-stained in tendinopathic tendon. Blocking the CD44 signaling pathways using OX-50 significantly induced cell apoptosis and the levels of cleaved caspase-3 in tenocytes from rat Achilles tendons following collagenase injection. Moreover, OX-50 decreased tenocyte viability and the expression of COL1A1, COL3A1, tnmd, and phosphorylated AKT. On the other hand, there were elevated levels of inflammatory mediator (including IL-1β, IL-6, TNFα, COX-2, and phosphorylated NF-κB) as well as MMP family member (including MMP-1, -3, -9, and -13) expression in tenocytes upon OX-50 treatment.

HA is known to regulate various cellular mechanisms through interacting with CD44, including cell adhesion, growth, survival, migration, and differentiation. It has been documented that enrichment of HA to the chondrocyte surface by binding to the CD44 receptor induces cell proliferation and matrix synthesis. Similarly, it has been found that HA reduces IL-1β–stimulated MMP-13 expression and attenuates oxidative stress-induced apoptosis in human OA chondrocytes through engaging with CD44 (20). The anti-apoptotic effects of HA on anti-FAS–induced apoptosis of chondrocytes from OA patients are abrogated by anti-CD44 antibodies (16). Short-hairpin RNA targeting CD44 inhibits cell proliferation and promotes apoptosis of colon carcinoma cells, whereas anti-CD44
small interference RNA impairs chronic lymphocytic leukemia (CLL) cell viability, whereas engagement of CD44 by HA protects CLL cells from apoptosis (17, 18). Moreover, anti-CD44 antibody could induce apoptosis in chondrosarcoma cell line SW1353 (19). Various cancer-related studies also demonstrated that CD44 promoted cell survival and suppressed apoptosis through AKT signaling and HA synthesis (21–23). These observations correlate with our findings that increased expression of CD44 is related to enhanced apoptosis during progression of LHB tendinopathy (Fig. 1). Furthermore, the OX-50–treated rat tenocytes from collagenase-injected Achilles tendons showed higher cleaved caspase-3 level and apoptotic cell number than control counterparts. In addition, they had down-regulated phosphorylated AKT expression and reduced
CD44-related apoptosis in tendinopathy

Figure 2. Expression of inflammation-related mediators, MMPs, α-SMA, CD44, and tendon-related markers in rat primary tenocytes. Tenocytes were isolated from Achilles tendons of Sprague–Dawley rats treated (T) or untreated (Un) with intratendinous injection of collagenase I (10 lambda/rat, 1.5 mg/lambdapa) for 1 week. Shown is expression of IL-1β, IL-6, COX-2, MMP-1, MMP-3, α-SMA, CD44, COL1A1, and COL3A1 in primary tenocytes, as determined by qRT-PCR. Values are the mean ± S.E. (error bars) (n = 5). Results are representative of at least two independent experiments.

In conclusion, by using tendon specimens from patients with LHB tendinopathy and tenocytes from rats following collagenase injection, we demonstrated that blocking the CD44 signal pathway induces tenocyte apoptosis, decreases tenocyte viability and proliferation, and increases inflammatory cytokines and MMP expression. CD44 might play a role in tendinopathy via regulating apoptosis, inflammation, and ECM homeostasis. These findings provide a new approach to treating patients with tendinopathy.

Materials and methods

Ethics statement

All of the experimental rats were purchased from the Animal Center at National Cheng Kung University, and the following animal experiments were done strictly in accordance with protocols approved by the Institutional Animal Care and Use Committee of National Cheng Kung University (approval 105-156). The human study was approved by the Institutional Review Board of National Cheng Kung University Hospital (approval A-ER-106-063) and was done strictly in accordance with the approved guidelines. Informed consent was obtained.

4. P.-T. Wu, W.-R. Su, C.-L. Li, J.-L. Hsieh, C.-H. Ma, C.-L. Wu, L.-C. Kuo, I.-M. Jou, and S.-Y. Chen, unpublished data.
from all patients. The human study abides by the Declaration of Helsinki principles.

**LHB specimens and histopathological grades**

Fourteen consecutive patients (6 men, 8 women; mean age, 62.1 years; age range, 52–76 years) undergoing arthroscopic treatment for a rotator cuff tear and LHB tendinopathy at our university hospital were recruited. The tenodesis or tenotomy of LHB was done, and the pathological tendon area was harvested for the following histological examinations. The specimens were fixed in fresh 4% paraformaldehyde for 16–24 h at 4 °C and then subsequently dehydrated, paraffin-embedded, and longitudinally sectioned. Sequential 4-μm sections were stained with hematoxylin and eosin and examined under a light microscope.

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**Figure 3. Cell viability, apoptosis, and expression of tendon-related markers in rat primary tenocytes via CD44 blockage.** Tenocytes were isolated from Achilles tendons of Sprague–Dawley rats treated with ultrasound-guided intratendinous injection of collagenase I (20 lambda/rat, 1.5 mg/lambda) for 1 week. A, cell viability was determined by WST-8 assay after OX-50 treatment (10 μg/ml) for 24 h and expressed as the percentages of surviving cells relative to those in control cells. Each value shown represents the mean ± S.E. (error bars) (n = 4). B, TUNEL staining in tenocytes after OX-50 treatment (10 μg/ml) for 24 h. Scale bars, 100 μm in ×200 magnifications. C, expression of phospho-AKT, AKT, cleaved caspase-3 (c-Cas 3), pro-caspase-3 (p-Cas 3), and β-actin in tenocytes after OX-50 treatment (10 μg/ml) for 30 min (p-AKT and AKT), 8 h (cleaved caspase-3) and 60 min (p-NF-κB and NF-κB), as determined by immunoblotting. D, expression of COL1A1, COL3A1, and tnmD in tenocytes after OX-50 treatment (10 μg/ml) for 24 h, as determined by qRT-PCR. Values are the mean ± S.E. (error bars) (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001. Results are representative of at least three independent experiments.

**Figure 4. mRNA expression of inflammation-related mediators and MMPs in rat primary tenocytes via CD44 blockage.** A, expression of TNFα, IL-1β, IL-6, and COX-2 in OX-50–treated rat primary tenocytes for 6 h (TNFα and IL-1β) and 24 h (IL-6 and COX-2), as determined by qRT-PCR. Values are the mean ± S.E. (error bars) (n = 3). B, expression of MMP-1, -3, -9, and -13 in OX-50–treated rat primary tenocytes for 24 h, as determined by qRT-PCR. Values are the mean ± S.E. (n = 3). Results are representative of at least three independent experiments. See the legend to Fig. 3 for other definitions.
microscope for changes in tenocyte morphology and collagen bundle characteristics. Based on our previous study (35), a modified semiquantitative method scored each factor (tenocyte morphology and collagen bundle characteristics) on a four-point scale. According to the sum of scores, the tendinopathy was graded as 0–3 (0, ≤ 2, 3–4, or ≥ 5 points). The histological grading was assessed by two observers unaware of the clinical and arthroscopic conditions of the patients. If an inconsistency existed, the field was reassessed, and a final score was decided upon.

**Immunohistochemical, immunoblotting, and immunofluorescence assessments**

The sections were deparaffinized in xylene, dehydrated in alcohol, epitope-unmasked by heating, washed with H2O2 in PBS, and stained with antibodies against CD44 (1:100; #37259, Cell Signaling Technology), in combination with the chromogen 3-amino-9-ethylcarbazole (Zymed Laboratories Inc.) or subjected to the DeadEnd™ colorimetric TUNEL system (#G7360, Promega). Cell lysates of OX-50 (10 µg/ml; #GTX76383, GeneTex)–treated tenocytes were subjected to immunoblot analyses with antibodies against phosphorylated AKT (1:1000; #9271, Cell Signaling), total AKT (1:1000; #9272, Cell Signaling), phosphorylated NF-κB (1:1000; #3033, Cell Signaling), total NF-κB (1:200; #SC-109, Santa Cruz Biotechnology, Inc.), cleaved caspase-3 (1:500; #9664, Cell Signaling), caspase-3 (1:1000; #9662, Cell Signaling), MMP-1 (1:1000; #GTX00674, GeneTex), MMP-3 (1:1000; #14351, Cell Signaling), COL1A1 (1:1000; #GTX112731, GeneTex), COL3A1 (1:1000; #GTX76383, GeneTex), tenomodulin (1:1000; #bs-7525R, Bioss), and COX-2 (1:1000; #sc-1747, Santa Cruz Biotechnology) in combination with a horseradish peroxidase–conjugated secondary antibody (1:10,000; Jackson ImmunoResearch) and quantitative control anti-β-actin antibodies (1:10,000; #A3854, Sigma-Aldrich). The signal intensity in immunoblot analyses was further quantitated using ImageJ software (National Institutes of Health). Cells with immunohistochemical stainings of TUNEL and CD44 were identified and counted in five high-power fields (×400) to determine the average percentages of TUNEL and CD44-positive cells corresponding to hematoxylin-stained total cells. For CD44 and TUNEL dual-immunofluorescence staining, paraffin-embedded sections receiving similar pretreatment were subjected to the DeadEnd™ fluorometric TUNEL system (#G3250, Promega) and incubated with antibodies against CD44 (1:100; #37259, Cell Signaling), followed by Alexa Fluor 594–conjugated secondary antibodies (1:200; #A-11012, Thermo Fisher Scientific).

**Primary culture of tenocytes from collagenase-injected rat Achilles tendon**

Male Sprague–Dawley rats (4–6 weeks old) were purchased from LASCO (Taipei, Taiwan) and housed in the specific pathogen-free condition with a light/dark cycle of 12 h in the Animal Center of National Cheng Kung University (Tainan, Taiwan). Twenty-four rats were treated with ultrasound-guided intratendinous injection of collagenase I (1.5 mg/λmbda, 10 lambda injection/rat, Sigma-Aldrich). One week after the index procedure, Achilles tendons were harvested after the rats had been killed with an overdose of isoflurane. The preparation of tendon samples and tenocyte culture methods are the same as in our previous study (15). Well-characterized second-to-fourth passage cells were used in this experiment; they showed no phenotypic drift of major tenocyte markers, such as cell shape (elongated and spindle-shaped with apposition), and tenomodulin expression identified using anti-tenomodulin antibody (Santa Cruz Biotechnology) as described previously (15). Because low-density plating and subsequent colony formation were the standard methods to isolate the tendon progenitor cells (36) and not used in our primary culture, the cell culture system we utilized contained mostly the differentiated tenocytes but not the tendon progenitor cells. Furthermore, tenocytes were identified by their tendinopathic and myofibroblastic natures identified by detecting proliferative markers (COL1A1 and COL1A3), inflammatory mediators (IL-1β, IL-6, and COX-2), MMP-1, MMP-3, CD44, and α-SMA with qRT-PCR (Fig. 2).

**Cell viability and apoptosis analyses**

After treatment with OX-50 for 24 h, tenocytes were analyzed using WST-8 assay with measurement of absorbance at 450 nm. Cell viability was represented as the percentages normalized with the average WST-8 values of the control group. The DeadEnd™ colorimetric TUNEL system (#G7360, Promega) was used for detecting apoptotic cells in OX-50–treated tenocytes according to the standard protocol.

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**Figure 5. Protein expression of inflammation-related mediators, MMPs, and tendon-related markers in rat primary tenocytes via CD44 blockage.** A, expression of COL1A1, COL3A1, tnm/d, COX-2, MMP-1, and MMP-3 in OX-50–treated rat primary tenocytes for 72 h, as determined by immunoblotting. B, expression of IL-6 in the supernatant of OX-50–treated rat primary tenocytes for 72 h, as determined by ELISA. Results are representative of at least two independent experiments. See the legend to Fig. 3 for other definitions. Error bars, S.E.
qRT-PCR and ELISA

Total RNA from tenocytes was isolated with TRIzol reagents (#15596018, Invitrogen), and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (#4368813, Applied Biosystems) for qRT-PCR by SYBR® Green PCR kit (#208054, Qiagen) with primer pairs specific to COL1A1 (forward, 5'-ATCTACCCCAACCCCATAGGA-3'; reverse, 5'-GCGAGGAAGTCTGCTTGGATAG-3'), COL3A1 (forward, 5'-TGGTGATTCAATGGAGGAGGA-3'; reverse, 5'-GATCTCTAGTGGCTTGGTGT-3'), and MMP-1 (forward, 5'-CACTAATGACTGGAGGTACCACAGCA-3'; reverse, 5'-CACTAATGACTGGAGGTACCACAGCA-3'). 35, 328–333 CrossRef Medline

Statistical analysis

Data are expressed as mean ± S.E. Statistical significance between two groups was analyzed using Student’s t test because of the normal distribution of our data that was assessed using the SigmaPlot software. The correlation among CD44 expression, TUNEL-positive cell number, and the different histological grades of tendinopathy were analyzed using the Spearman correlation rank test. p < 0.05 was considered significant.

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