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Extracellular HMGB-1 activates inflammatory signaling in tendon cells and tissues

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Abstract

Background: Increasing evidence indicates that secretion of high mobility group box 1 protein (HMGB-1) is functionally associated with tendinopathy development. However, the underlying effect and mechanism of extracellular HMGB-1 on tendon cells are unclear.

Methods: We tested the effect of exogenous HMGB-1 on cell growth, migration, and inflammatory signaling responses with isolated rat Achilles tendon cells. Also, we studied the role of extracellular HMGB-1, when administrated alone or in combination with mechanical overloading induced by intensive treadmill running (ITR), in stimulating inflammatory effects in tendon tissues.

Results: By using *in vitro* and *in vivo* models, we show for the first time that exogenous HMGB-1 dose-dependently induces inflammatory reactions in tendon cells and tendon tissue. Extracellular HMGB-1 promoted redistribution of HMGB-1 from the nucleus to the cytoplasm, and activated canonical nuclear factor kappa B (NF-κB) signaling and mitogen-activated protein kinase (MAPK) signaling. Short-term administration of HMGB-1 induced hyper-cellularity of rat Achilles tendon tissues, accompanied with enhanced immune cell infiltration. Additional ITR to HMGB-1 treatment worsens these responses, and application of HMGB-1 specific inhibitor glycyrrhizin (GL) completely abolishes such inflammatory effects in tendon tissues.

Conclusion: Collectively, these results confirm that HMGB-1 plays key roles in the induction of tendinopathy. Our findings improve the understanding of the molecular and cellular mechanisms during tendinopathy development, and provide essential information for potential targeted treatments of tendinopathy.

Keywords: HMGB1, tendinopathy

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Introduction

The main function of tendons is to transfer the strength between muscles and bones, and to promote joint activity.[1] Therefore, tendons often bear huge mechanical loads in daily life, which often results in tendon disorders over time.[2,3] With the increase in life expectancy and activities, the incidences of tendon injury may continue to rise, and chronic tendon diseases account for the vast majority. Chronic tendon diseases, such as Achilles tendinitis, plantar fasciitis, tennis elbow, rotator cuff injury, etc., are referred to as tendinopathy, which can rise with the tendon being subjected to long-term and persistent high-intensity mechanical loads.[2–4]

In the early stages of tendinopathy, the presence of inflammation caused by excessive mechanical stimulation often results in persistent pain in the affected area.[5] During prolonged inflammatory state, the tendon tissue structure will be irreversibly altered, cartilage or ossification and lipid deposition will occur in the tendon tissue.[5] Such aberrations will lead to a decrease in the

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mechanical strength of the affected tendon and ligament, which is prone to rupture.[4,5] Therefore, the occurrence of chronic tendon disease can seriously affect the quality of a patient's life.

It is widely recognized that abnormal environmental factors rise from long-term inflammatory conditions and overload mechanical stimulation induce tendon-derived stem cell osteogenesis, chondrogenesis, and adipogenesis during tendinopathy.[2,4–9] Nevertheless, the mechanism of tenocyte reacting to early inflammation is still unclear. High mobility group box 1 (HMGB-1) is a DNA-binding protein that has diverse, location-specific, roles.[6,7,9–12] Nucleic HMGB-1 is responsible for regulating gene expression and DNA stability.[6,7,9] Upon inflammatory trigger, HMGB-1 is re-distributed towards the cytosol and actively released into the extracellular environment.[7,9] Exocrine of HMGB-1 mediates inflammation responses locally and triggers the production of a variety of cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), matrix remodeling mediator Tenascin-C, and catabolic responder matrix metalloproteinases (MMPs).[12–14] Our recent findings have confirmed that mechanical overloading placed on the tendon tissues can induces HMGB-1 release and consequent pro-inflammatory phenotype with the aberrant tendon remodeling that is responsible for the induction of tendinopathy.[9]

Considering the structure of tendon tissue, which contains low levels of vascularization and neuronization, the interactive effects between secreted HMGB-1 and tendon cells may be essential for the development of tendinopathy.[6,9,12] Previous studies have shown that extracellular HMGB-1 mediated inflammation may be related to the activation of the nuclear factor kappa B (NF-κB) pathway.[6,13,14] NF-κB target genes are pleiotropic, ranging from cytokines/chemokines and their modulators, to stress response genes.[15] Among these target genes, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) were reported to further activate NF-κB signals, generating positive feedback loops to prolong the inflammatory state.[14,16,17] Many inflammation responding signals that serve upstream or downstream of NF-κB activation, such as mitogen-activated protein kinase (MAPK) pathways, may collectively intensify the regional inflammation, and consequently worsen the extent of tendinopathy. However, such molecular mechanisms remain largely unknown in tendon tissues.

In order to address the effect of extracellular HMGB-1 in tendon cells and the corresponding molecular mechanisms, we studied the responses of rat tendon-derived cells to exogenous HMGB-1 treatment. We also analyzed the responses of rat Achilles tendon tissues following short period of HMGB-1 local injection, alone, or in combination of intensive treadmill running (ITR). We report that exogenous HMGB-1 promotes the relocation of endogenous HMGB-1 protein from the nucleus to the cytosol, and elicits inflammatory and catabolic responses as marked by enhanced production of cytokines and MMPs in tendon cells. Exogenous HMGB-1 activates intracellular NF-κB and MAPK signaling pathways, which is accompanied with increased mobility but not proliferation of tenocytes. Furthermore, both exogenous HMGB-1 and mechanical overloading in the form of short-term ITR can induce tendinopathy-like phenotypes in tendon tissues, and administration of GL can effectively block such early development of tendinopathy.

Materials and methods

Ethics statement

All animal procedures in the present study were conducted with approval of the Committee on Ethics of Second Military Medical University, and in compliance with the Shanghai Changzheng Hospital Institutional Animal Care and Use Committee [IRB#: CZEC(2017)-13], following international guidelines for animal treatment. All animals were obtained from the Experimental Animal House of Second Military Medical University (Shanghai, China). They were maintained with free access to food and water in plastic cages, and kept with unlimited cage activities and on a 12 h light/dark cycle. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Cell isolation and culture

We obtained rat Achilles tendon from 3- to 4-month-old female Sprague Dowley (SD) rats weighing 200–250 g. Briefly, the tendon sheath and the surrounding paratenon were stripped, and the tendon tissues were cut into small pieces and digested with 3 mg/ml collagenase type I (Worthington, Lakewood, NJ, USA) and 4 mg/ml dispase (Roche, Shanghai, China) in PBS at 37°C for 6 h. After being filtered through a 70 μm filter

and centrifuged at 700 g for 10 min, cell pellets were re-suspended in 20% fetal bovine serum (Gibco, Shanghai, China) in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Shanghai, China) with 100 unit/ml penicillin and streptomycin, and cultured in T-25 flasks at 5% CO_2, 37°C. Subculture was performed when 80% confluence was reached. For all experiments, cells in passages three to five were used.

FACS analysis

We immunolabeled 5×10^5 cells with 1 mg of phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies for 1 h at 4°C. Antibodies against CD29 (1:100, 11-0291-82), CD44 (1:160, 11-0441-82), CD90 (1:100, 11-0902-82), CD105 (1:160, 12-1051-82), CD45 (1:100, 11-0451-82) or CD106 (1:100, 12-1061-82) were purchased from Invitrogen (Shanghai, China), isotype-matched IgGs were purchased from BD Biosciences (San Jose, CA, USA). After three washes with PBS containing 1% FBS and 0.01% azide, stained cells were subject to fluorescence-activated cell sorting (FACS) analysis with an Epics-XL-MCL flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The percentage of the cell population in each quadrant was calculated using the FACSCAN program (BD Biosciences).

Immunostaining of tendon tissue

For immunohistochemical analysis of isolated tendon cells, fixed sections were treated with 0.05% trypsin for 20 min at 37°C and washed with PBS three times, then immunolabeled using primary antibodies against antibodies to type I collagen (1 μg/ml, ab90395, Abcam, Shanghai, China) or type III collagen (1 μg/ml, ab34712, Abcam) at 25°C for 1 h. Next, sections were washed three times with PBS and incubated with Cy3-conjugated goat anti-rabbit IgG antibody (1 μg/ml, ab6939, Abcam) at room temperature for 2 h. Slides were then counterstained with Hoechst 33342 (228551, Abcam). For immunostaining of tendon tissue, dissected rat Achilles tendons were processed immediately, fixed in 4% paraformaldehyde for 15 min, and blocked with universal blocking solution (ThermoFisher Scientific, Shanghai, China). Paraffin-embedded tendon tissue was stained histochemically with hematoxylin and eosin (H&E; ab245880, Abcam), and CD68 (1 μg/ml, ab955, Abcam). The sections were counterstained with hematoxylin.

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was isolated from the heart apical region, the skeletal muscle, and the tendon tissues of different treatment groups, or from confluent rat Achilles tendon cells, using TRIzol Reagent (Invitrogen, Shanghai, China) as described in the manufacturer's protocol. RNA integrity was confirmed by agarose gel electrophoresis. cDNAs were obtained by reverse transcribing total RNAs with 50 U of SuperScript II RT using random hexamer primers (Invitrogen, Shanghai, China). Real-time quantitative PCR was performed on the QuantStudio three Real-Time PCR system using SYBR green master mix (Applied Biosystems, Shanghai, China). The primers were designed with Primer three software and are listed in the Supplemental Material.

Cell growth and motility

To study cell growth, tenocytes (5×10^3 cells/well) were plated in 96-well plates and cultured from day 1 to day 6. Cell counting kit 8 (CCK-8) solution (10 μl; Dojindo, Shanghai, China) was added to each well at testing time points in each day. Plates were incubated at 37°C for 1 h in 5% CO_2, after which absorbance readings were obtained at 450 nm. To address the effect of extracellular HMGB-1 on tenocyte motility, a transwell assay was performed (Corning, Shanghai, China). Briefly, cells were serum-starved for 24 h, then a total of 1×10^5 cells in 200 μl serum-free DMEM were placed in the upper chamber, and 800 μl serum-free DMEM medium containing saline or 1 and 10 μg/ml rat recombinant HMGB-1 (1690-HMB-050, R&D Systems, Minneapolis, MN, USA) was added into the lower chamber. After 24 h incubation at 37°C, cells that migrated through to the bottom surface of the membrane were washed with PBS, fixed, stained with 0.2% crystal violet (G1062, Solarbio, Beijing, China), photographed, and counted under a microscope (×200, Olympus Corporation, Tokyo, Japan).

Isolation of tenocyte cytoplasmic and nuclear extracts

Isolation of cytoplasmic and nuclear extracts was performed as previously described.[9] Briefly, tenocytes were trypsinized and washed twice in 1 ml of ice-cold PBS. The cell pellets were

re-suspended in 400 µl lysis buffer containing protease inhibitors and 10% Nonidet P-40 (ST2045, Beyotime Biotech Inc., Shanghai, China), and incubated on ice for 15 min. The cell suspension was mixed vigorously for 15 s then centrifuged for 1.5 min at 14,000 × *g*. The supernatants were harvested as the cytoplasmic extracts. The pellets were then re-suspended in 25 µl ice-cold nuclear extraction buffer (P0027, Beyotime Biotech Inc, Shanghai, China), incubated for 30 min with intermittent mixing, then centrifuged. The supernatant were harvested as described for the nuclear extracts.

Western blot analysis

Cells isolated from rat Achilles tendon were treated with 1 or 10 µg/ml rat recombinant HMGB-1 (1690-HMB-050, R&D Systems) for 24 h; saline was used as a control. Cells were placed in a radioimmunoprecipitation assay (RIPA) lysis buffer solution with protein inhibitors for 30 min. The digested cells were then sonicated and centrifuged at 13,000 rpm for 15 min at 4°C. Extracted total proteins or compartmentalized proteins were quantified *via* the BCA method (ThermoFisher Scientific, Shanghai, China). A total of 30 µg proteins from each group were separated on a 10% or 12% SDS-PAGE, then transferred electrophoretically onto a nitrocellulose membrane. After blocking for 2 h with 5% milk in Tris-buffered saline with Tween (TBST) buffer solution, membranes were incubated with primary antibodies at 1:1000, followed by goat anti-rabbit (ab6721, Abcam) or goat anti-mouse (ab97023, Abcam) horseradish peroxidase (HRP)-conjugated secondary antibody at 1:5000, with proper washing in between antibody incubations. HMGB-1 (1690P) antibody was purchased from R&D systems. Antibodies against TNF-α (ab1793), MMP-13 (ab75606), Col-1 (ab21286), Tenascin-C (ab108930) and p-IKKαβ (ab55341) were purchased from Abcam. Antibodies against IL-6 (3833), MMP-3 (14351), p-ERK (4370T), ERK1/2 (4695S), p-JNK (9251S), JNK (9252P), p-p38 (4511P), p38 (9212S), IKKα (2682), IKKβ (2678), p-IKBα (2859T), IKBα (4812S), p-p65 (3033S) and p65 (8242S) were purchased from Cell Signaling Technology (Shanghai, China). Actin antibody was obtained from Solarbio (Beijing, China) and applied at a 1:5000 ratio. Positive signals were detected using an ECL chemiluminescent reagent (Beyotime Biotechnology, Shanghai, China), and the ChemiDoc imaging system (Bio-Rad, Beijing, China).

In vivo *experiments*

For short-term HMGB-1 or GL administration, we used a total of 24 SD rats (female, 6 months) with three rats in each of the four groups: (i) normal control group where rats received saline injection around Achilles tendon tissue, (ii) HMGB-1 group where rats received daily injection of HMGB-1 around Achilles tendon tissue (1 µg/kg body weight, Sigma-Aldrich, Shanghai, China) for 2 weeks or (iii) 4 weeks, and (iv) rats received daily injection of HMGB-1 around Achilles tendon tissue followed by intraperitoneal (IP) injection of GL (50 mg/kg body weight, Sigma-Aldrich) daily, for 4 weeks. All rats remained in cages and were allowed cage activities.

Mechanical overload stimulation was achieved using a treadmill, with a running speed set as 17 m/min. In the first week, rats were trained for 15 min to accommodate them to the treadmill running protocol and environment. In the following 2–4 weeks, rats ran for 3 h a day, 5 days a week (ITR). A total of nine rats (female, 6 months) were divided into three groups with three rats in each group: (i) ITR with saline injection group, where rats received daily saline injection around Achilles tendon tissue 15 min before the beginning of ITR regimen; (ii) ITR with GL injection group, where rats received daily IP injection of GL 15 min before the beginning of ITR regimen; and (iii) ITR with HMGB-1 injection group, where rats received daily injection of HMGB-1 (1 µg/kg body weight, Sigma-Aldrich) around Achilles tendon tissue 15 min before the beginning of the ITR regimen. Performance of the rats was recorded to recommend inclusive/exclusive criteria. Immediately after the end of all running regimens, the Achilles tendons were harvested from all groups of rats and used for immunostaining.

Statistical analyses

Each experiment was performed at least three times, and representative data were reported. All statistical analyses were performed *via* student's *t* test. Differences with a probability of less than 0.05 were considered statistically significant. All

statistical analyses were done by SPSS 17.0 (SPSS, Chicago, IL, USA).

Results

Extraction and identification of rat tendon cells

In order to determine the effect of secreted HMGB-1 on tendon cells, we extracted cells from rat Achilles tendon tissues. These cells are expanded and analyzed *in vitro* as previously described.[8,9,18] To confirm the purification of isolated tenocytes, first, we used flow cytometry analysis to examine the presence of surface mesenchymal stromal cell markers on these cells.[8,18] Over 92.7% of isolated cells were positive for the mesenchymal stromal cell markers (CD29, CD44, and CD105), and over 98.3% of these cells were positive for the fibroblast marker CD90 (Figure 1a). They were all negative for the leukocyte marker CD45 and the endothelial cell markers CD106, thus verifying the lack of contaminating leukocytes and endothelial cells (Figure 1a). Immunocytochemistry staining further confirmed the homologous expression of type I collagen (Col-I) in isolated tendon cells, while type III collagen (Col-III) expression was not detected (Figure 1b). Assessment of mRNA levels revealed that these rat tenocytes expressed significantly higher amount of tendon marker Scleraxis as compared with the cardiac muscles. Their Tenomodulin and Tenascin-C gene expression levels were also significantly higher than the skeletal muscles (Figure 1c). The above isolated cells demonstrated typical tendon markers and thus were used to study the cellular effects and mechanisms of extracellular HMGB-1.

Exogenous HMGB-1 increased tenocytes motility

To clarify the cellular effect of HMGB-1 in tendon inflammation, tendon cells were stimulated by extracellular HMGB-1, and cell growth and motility were studied by CCK-8 and transwell assay, respectively. We found that tenocytes exhibited a dose-dependent increase in their cell motility, when exposed to different concentrations of HMGB-1, and the differences were significant relative to control (Figure 2a). However, 6 days of rat recombinant HMGB-1 treatment did not affect tendon cell proliferation, even when we increased the HMGB-1 concentration to 10 μg/ml (Figure 2b), indicating that a loss of nuclear HMGB-1 function may be an early event in tendon cells induced by extracellular HMGB-1.

Exogenous HMGB-1 induces inflammatory signaling activation in tendon cells

To understand the above findings that extracellular HMGB-1 did not affect cell growth, we investigated the compartmental expression of endogenous HMGB-1 in isolated tenocytes. Western blot analysis was performed to detect subcellular HMGB-1 protein expression after exposure to different amounts of HMGB-1 in the medium for 24 h. We found that the amount of HMGB-1 protein in the nucleus was significantly reduced, with a corresponding increase in cytoplasmic HMGB-1 protein levels, when tendon cells were treated with exogenous HMGB-1 (Figure 3a). These findings indicated that, as an early event in tendon cells, rather than sustaining the cell proliferation that is associated primarily with nuclear HMGB-1 functions, extracellular HMGB-1 might promote intracellular redistribution and exocrine export of HMGB-1 towards a pro-inflammatory effect, thus generating a positive feedback loop. Subsequent work confirmed the elevated expression of inflammatory mediators TNF-α and IL-6 concurrent with the subcellular redistribution of HMGB-1, which were correlated positively with the concentration of HMGB-1 treatment (Figure 3a).

In our previous work, we found that tendon inflammation caused by increased HMGB-1 secretion during mechanical overloading is associated with an increased production of catabolic genes involved in remodeling.[9] In line with this finding, we found that tendon cells demonstrated a dose-dependent elevation of MMP-3 and MMP-13 expressions *in vitro* when exposed to exogenous HMGB-1 (Figure 3a). In addition, HMGB-1 drastically reduced expression of tendon markers that were normally abundant in the tenocytes, such as Col-I and Tenascin-C proteins (Figure 3a). In fact, 10 μg/ml HMGB-1 treatments almost abolished Col-I expression in these tendon cells, indicating a possible degenerative change induced by extracellular HMGB-1.

To identify possible molecular mechanisms of extracellular HMGB-1 in regulating the initiation of tendon inflammation, we verified the activation of NF-κB and MAPK signaling upon HMGB-1

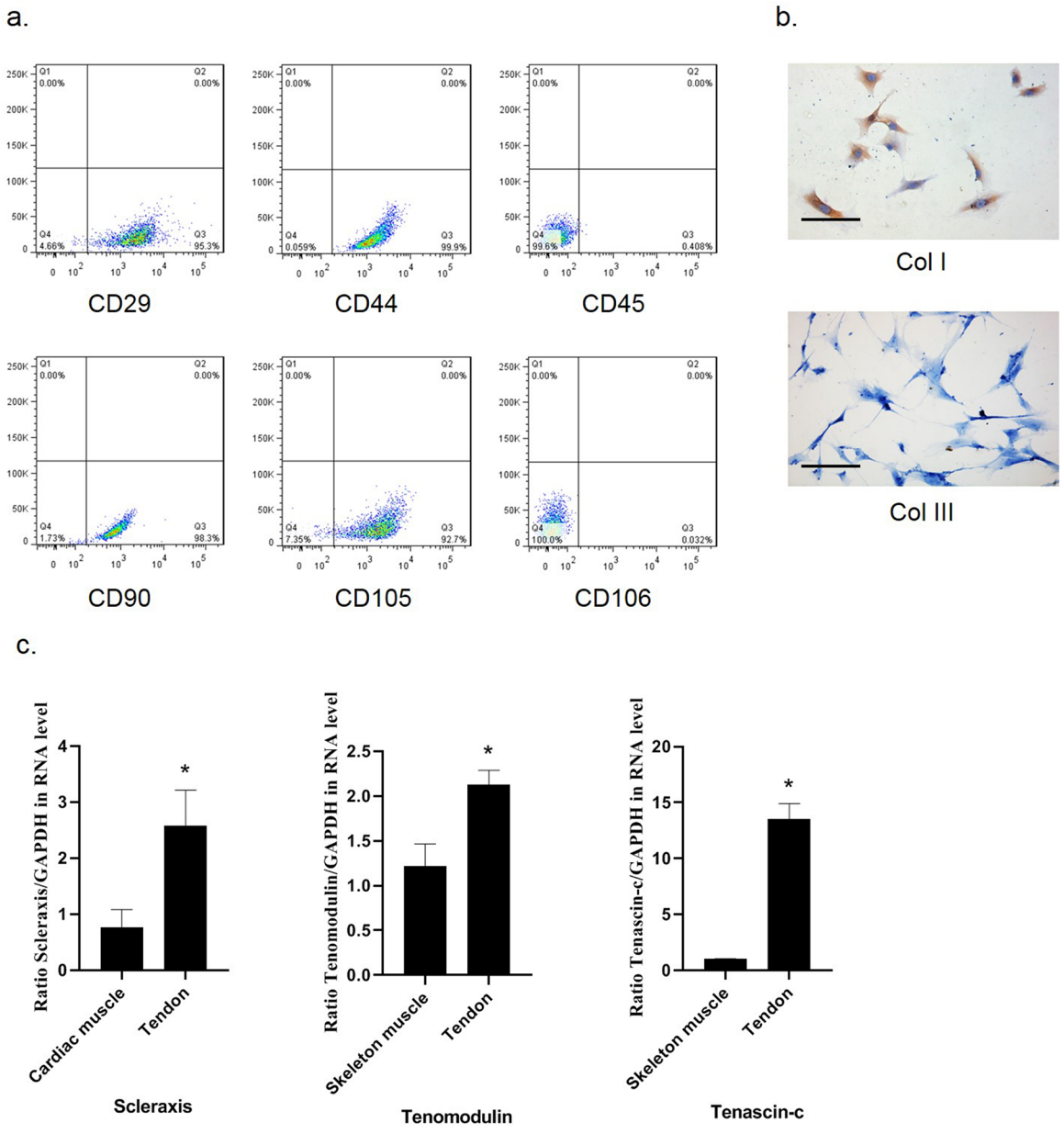

Figure 1. Isolation and characterization of rat tendon cells. (a) Flow cytometry analysis of the expression of cell surface markers related to mesenchymal stromal cells, leukocytes, and endothelial cells on isolated rat Achilles tendon cells. (b) Immunocytochemistry staining of collagen proteins related to tendon in isolated cells. Bars, 50 mm. (c) qRT-PCR analysis of gene expression profiles related to muscle cells and tendon cells (mean ± SEM; $n = 3$; $^*p < 0.05$).
GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean.

stimulation. NF-κB signals were found to be activated when we analyzed the total and phosphorylated proteins involved the canonical NF-κB pathway. We found that 1 μg/ml HMGB-1 markedly increased IKKα/β and IKBα phosphorylation, and that this was accompanied by a reduction in the corresponding total kinase proteins (Figure 3b). Treatment with HMGB-1 resulted in the accumulation of phosphorylated p65 in tendon cells in a dose-dependent manner (Figure 3b). In addition to NF-κB signaling, the expression levels of phosphorylated ERK and JNK showed a 0.5- and 2-fold

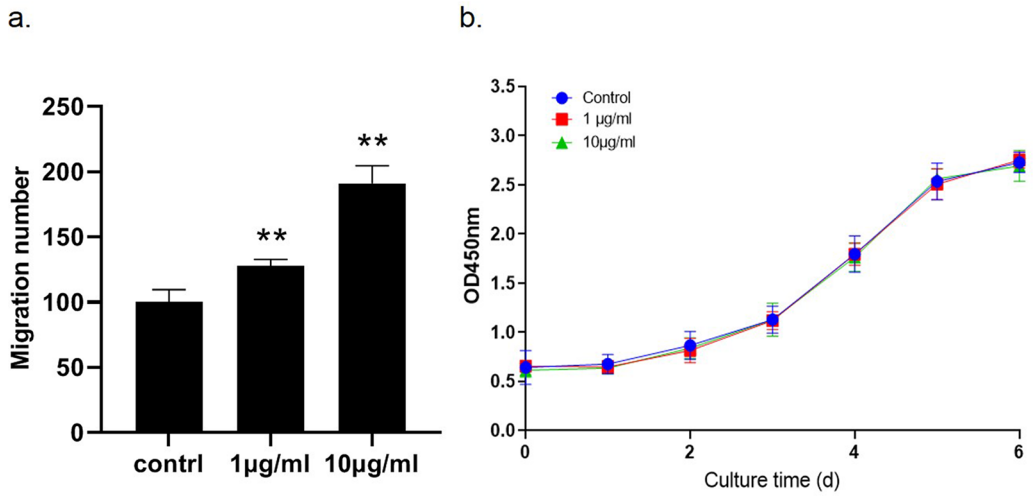

Figure 2. Cellular effect of exogenous HMGB-1 treatment on tendon cells. (a) Transwell chamber was used to analyze tendon cell migration. Cells (5×10^5) in suspension were added to the top chamber with serum-free medium containing 1 μg/ml or 10 μg/ml recombinant HMGB-1, medium with 20% serum were added to the bottom chamber. Quantification of migrated cells was performed after incubation for 24 h. Medium with added saline was used as a control (mean ± SEM; $n = 3$; $^{**}p < 0.001$). (b) CCK-8 test was used to detect cell proliferation with recombinant HMGB-1 treatment up to 6 days, saline was used as control. No statistical significant differences were found between HMGB-1 treated and control group (mean ± SEM; $n = 3$).
CCK-8, cell counting kit 8; HMGB-1, high mobility group box 1 protein; SEM, standard error of the mean.

increase, respectively, when treated by 10 μg/ml exogenous HMGB-1, whereas p38 was only moderately activated in the HMGB-1 treated groups compared with the control group (Figure 3b). These findings indicate that extracellular HMGB-1 can potentially activate inflammatory responses through the MAPK and canonical NF-kB signaling pathway in tendon cells.

Both exogenous HMGB-1 and short-term ITR induce tendinopathy, and administration of GL prevents tendinopathy development

Our previous study in a mouse model has shown that HMGB-1 can be released to the tendon extracellular matrix (ECM) and initiates an inflammatory cascade in response to mechanical overloading.[9] Such an effect was inhibited by the HMGB-1 antagonist GL.[9] In order to study if the tendinopathy induced by mechanical overloading is directly associated with the quantity of HMGB-1 in the extracellular milieu, we performed separated *in vivo* studies focusing on the rat Achilles tendon.

First, we tested the ability of exogenous HMGB-1 to induce inflammatory responses in regular tendon tissues. We found that tendon sections administrated with recombinant rat HMGB-1 exhibited hyper-cellularity after 2 weeks, whereas saline caused no tendon cell proliferation (Figure 4a). In 4 weeks, tissues at the HMGB-1 injection site showed more extensive proliferation, and no such activity was observed when HMGB-1 was inhibited by GL administration (Figure 4a). This time-dependent increase in cell growth was accompanied by the presence of vessel-like structure formation, and an elevated infiltration of immune cells, mainly monocytes ($CD68^+$) (Figure 4a, b). Accordingly, HMGB-1 reduced collagen I RNA expression, whereas the RNA levels of IL-6 and MMP-13 were both significantly induced by exogenous HMGB-1 treatment (Figure 4c). Such observed alterations in tendon morphology, tissue structures, and molecular inflammatory responses were unanimously reverted with GL treatment, indicating an essential involvement of HMGB-1 in promoting early development of tendon inflammation (Figure 4a–c).

Given the significance of HMGB-1 for tendinopathy, we hypothesize that exposure to extracellular HMGB-1 could render tendon tissues more prone to continuous inflammatory stress associated with mechanical overloading. In order to test this theory,

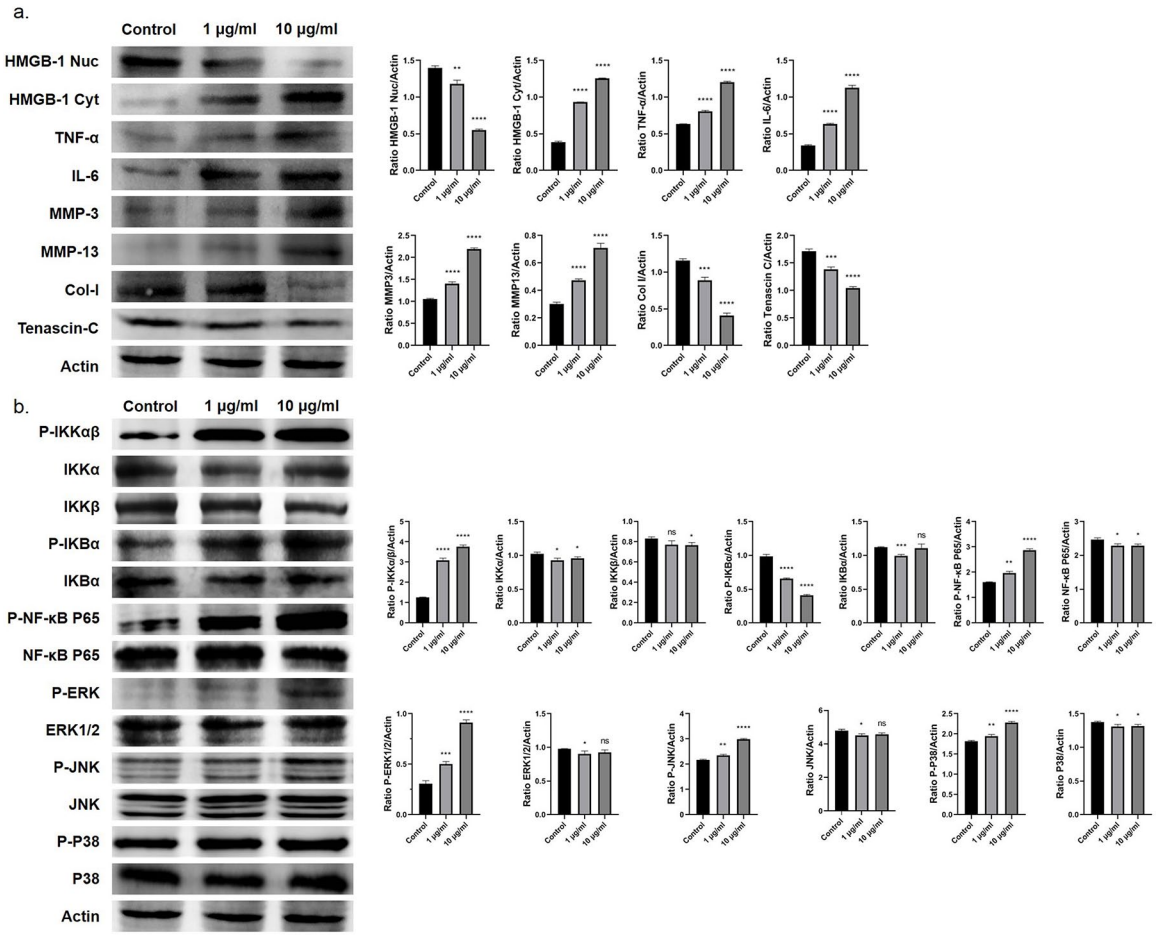

Figure 3. Intracellular signaling activities with exogenous HMGB-1 treatment in tendon cells. Recombinant HMGB-1 was added to cell medium at 1 μg/ml or 10 μg/ml, and incubated for 24 h. Cells were harvested and western blot was performed. Representative images of saline-treated *versus* HMGB-1-treated rat tendon cells were shown and quantified (mean ± SD; $n = 3$). Corresponding densitometric data were all normalized to those of Actin within the same group. For detection of subcellular HMGB-1 distribution, compartmentalized lysates were used (a). For detection of intracellular signaling activities, whole cell lysates were used. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$; ****$p < 0.00001$; ns, $p > 0.05$.
HMGB-1, high mobility group box 1 protein; SD, standard deviation.

we performed another *in vivo* experiment focusing on the response of the rat Achilles tendon tissue to HMGB-1 pretreatment followed by treadmill running. Consistent with our previous finding,[9] H&E staining revealed that short-term ITR markedly increased tendon cell proliferation and vessel structure formation (Figure 4d), similar to those from HMGB-1 treatment alone (Figure 4a). Moreover, comparable levels of $CD68^+$ cell infiltration were found in the tendon tissues from the ITR group as in those from the HMGB-1 group (Figure 4a, d). Interestingly, administration of GL fully restored these inflammatory effects induced by accumulated HMGB-1 from both exogenous and endogenous sources in tendon tissues (Figure 4d–f).[9] Taken together, these data demonstrate that the tendon tissue exhibits increased susceptibility to inflammation brought by ITR, with predisposition to HMGB-1 treatment.

Discussion

At present, the main treatment for chronic tendon diseases is to take nonsteroidal anti-inflammatory drugs (NSAIDS) or local injections of hormones and local anesthetics to achieve a temporary break from symptoms. These treatment methods limit the patient's daily activity and affect their quality of

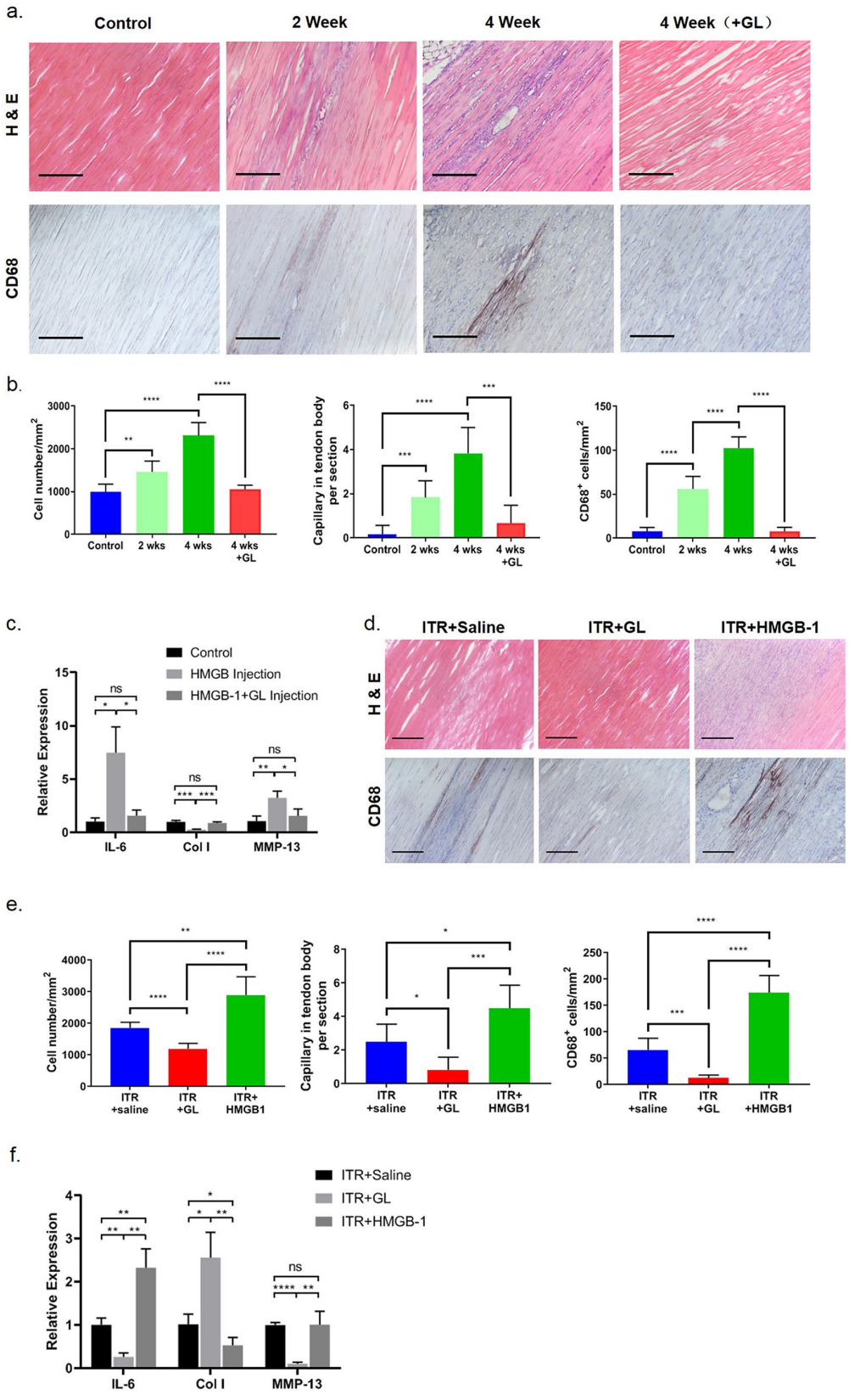

Figure 4. *(Continued)*

Figure 4. Rat Achilles tendon tissue responses to short-term ITR alone or in combination of HMGB-1 administration. H&E staining was used to study the development of tendinopathy, and IHC of CD68 was used to study inflammatory cells like macrophages and monocytes. (a) Para-Achilles tendon tissues were injected daily with 1 μg/kg recombinant HMGB-1 or saline for 2 or 4 weeks. In parallel, IP injections of GL following HMGB-1 administration were performed for 4 weeks (far right panels). (b) and (e) The total tendon cell numbers (top left), total vessel counts (top right) and total CD68 positively stained cell numbers (bottom) in two independent fields of three different rats from each group were manually calculated and summarized (mean ± SD; $n = 6$). (c) and (f) Collagen I, IL-6, and MMP-13 RNA levels from the tendon tissues of each group were analyzed by RT-qPCR (mean ± SD; $n = 3$). (d) Figures show representative results of at least three samples from the group of short-term ITR in addition to HMGB-1 administration. Bar: 100 μm. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$; ****$p < 0.00001$; ns, $p > 0.05$.
H&E, hematoxylin and eosin; GL, glycyrrhizin; HMGB-1, high mobility group box 1 protein; IHC, immunohistochemistry; IL, interleukin; IP, intraperitoneal; ITR, intensive treadmill running; MMP, matrix metalloproteinase; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation.

life, as adverse reactions emerge from long-term or repeated use.[1,4] In recent years, the use of platelet-rich plasma (PRP) in the treatment of tendinopathy has gradually prevailed, but recent clinical randomized experiments have showed no profound advantages of this method.[19–21] In view of the current treatment status of chronic tendinopathy in clinics, there is no specific treatment for chronic tendon diseases.[1,4,19,20]

Such lack of desired effect in current treatment, and the heavy social and economic burdens it brings, have urged research into the mechanism of chronic tendon disease development and the corresponding targeted treatments for specific molecules involved. During chronic inflammation, high levels of HMGB-1 are released into the extracellular milieu, where it promotes release pro-inflammatory cytokines, including TNF-α and IL-6, by macrophages.[6,7,9] Prolonged presence of inflammatory mediators can stimulate continuous secretion of HMGB-1 *via* lysosomal exocytosis and passive diffusion.[7,22] The persistence of HMGB-1 in the microenvironment is a key factor that drives inflammation and worsens associated symptoms.[2,7,9] Our previous studies have confirmed the presence of HMGB-1 in tendon cells and tissues, while the ECM of normal tendon tissues contains almost no HMGB-1.[9,23,24] We also found that HMGB-1 can be secreted from tendon cells to the extracellular space when stimulating the tendon tissues with mechanical overloading.[9]

In the current study, we have shown that exogenous HMGB-1 actively promotes the induction of inflammation-related gene expressions, such as TNF-α and IL-6, and catabolic responders involved in tissue remodeling, such as MMP-3 and MMP-13. In addition, we have found that continuous administration of HMGB-1 to the rat Achilles tendon tissue enhances regional angiogenesis, tenocyte proliferation and immune cell infiltration after 4 weeks. These observations were reverted when the HMGB-1 specific inhibitor GL was administrated. Similar HMGB-1 induced inflammatory responses in tendon tissues were observed with mechanical overloading stimulation. After 4 weeks with ITR – a previously defined trigger for endogenous HMGB-1 release in tendon tissue – Achilles tendons displayed levels of hyper-cellularity and inflammatory phenotypes comparable with those from 4 weeks of HMGB-1 treatment alone.[9] GL treatment reversed both of these tendinopathy-like effects. Interestingly, a combination treatment of exogenous HMGB-1 and ITR for 4 weeks led to an increase in the intensity of tendinopathy-like phenotypes in the tendon tissues, compared with either treatment alone. Such dose-dependent correlation of extracellular HMGB-1 levels with the pro-inflammatory effects further confirmed the essential role of HMGB-1 in the early development of tendon sterile inflammation and the severity of tendinopathy.

HMGB-1 exerts various biological effects depending on its subcellular locations, partnering molecules, and redox states. In the nucleus, HMGB-1 can induce chromatin conformation change, mediating DNA replication and repair, gene transcription, and overall homeostasis.[7,25–27] Upon stress, HMGB-1 is actively translocated from the nucleus to the cytosol, where it interacts with Beclin-1 and promotes autophagy.[7,28] Here, we have shown that the exogenous HMGB-1 actively promotes the redistribution of endogenous HMGB-1 from the nucleus towards the

cytosol of isolated tendon cells, which is correlated with an induction of tenocyte migration. Meanwhile, unlike the observed hyper-cellularity induced by HMGB-1 *in vivo*, cell proliferative activity is not altered by HMGB-1 treatment *in vitro*, at least for 6 days. Therefore, in addition to the obvious difference in manageable concentrations of HMGB-1 that tendon cells are exposed to, we suspected that both extended time and additional environmental components from the ECM may contribute to the HMGB-1-induced hyper-cellularity observed *in vivo*.

Extensive research has been applied to the mechanism whereby HMGB-1 regulates the cells of the immune system, but reports on how HMGB-1 regulates tenocytes are rare. Extracellular HMGB-1 modulates cellular inflammatory responses by signal transduction through several previously identified receptors, such as TLRs and RAGE.[13,29–31] These signals are largely receptor type- and cell content-dependent, mediating or inhibiting chemotaxis, cell growth, and migration.[25] Examples of responding signals downstream of HMGB-1 include activation of MAPK pathways and phosphorylation of both IKK-β and IKK-α.[32–34] Phosphorylated IKK-α/β promote the nuclear translocation of activated NF-κB, which up-regulates the expression of a panel of pro-inflammatory genes, including cell surface receptors such as RAGE and TLR4.[13,29] Collectively, they create positive feedback and feed-forward network systems to enhance overall inflammatory effects. Therefore, in search of mechanisms addressing how HMGB-1 exocrine pathways stimulate tendon inflammation, we speculate that the NF-κB signaling pathway and MAPK signaling activation may be involved.[35] Indeed, we found that tendon cells exhibit up-regulation of canonical NF-κB signaling activities with exogenous HMGB-1 treatment. Also, all three MAPK subfamilies were dose-dependently activated by HMGB-1 treatment, indicated by enhanced levels of p-ERK, p-JNK, and p-p38 expression. As there is pleiotropic signaling transduction among these inflammatory pathways, the influence of NF-κB and these MAPK kinases can be mutual. Detailed studies addressing the mechanism of expression and secretion of inflammatory cytokines associated with HMGB-1 secretion from tendon cells and the molecular responses of tendon cells to autocrine/paracrine HMGB-1 are needed, as this is one limitation of the current study.

Our findings have associated exogenous HMGB-1 with a continuous loop of inflammatory responses from within tendon cells and tissues. When prolonged, such effects may drive tendon cells towards an overall catabolic state, with abnormal signals for cell differentiation and blood vessel formation, which may lead collectively to a compromised mechanical property of the tendon. Nonetheless, another limitation of this study is that, because GL has been shown to be efficiently protective over tendinopathy development, in findings of our previous and current research, the effect of GL in preventing tendon degeneration should be investigated.

Taken together, our findings of intracellular mechanisms downstream of HMGB-1 stimulation in tenocytes provide vital information to identify the main players and potential targets during tendon tissue inflammation development. Given the physiologically relevant feature of the ITR model with repetitive human activities as labor or sport exercises, our findings elucidate additional targets involved in the development of chronic tendon disease, and potential mechanisms for combination therapy against tendinopathy.

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Author contributions

CZ, XG and GZ performed the research and analyzed the data. CZ and WW wrote the manuscript. JS, DN and YZ designed the experiments and revised the manuscript. JS, DN and YZ analyzed and interpreted the data. WW contributed to statistical analyses. JZ and TY collected the experimental data. CZ, XG and GZ are co-first authors and contributed equally to this study. JS, DN and YZ are co-corresponding authors and contributed equally to this study. All authors reviewed and approved the final version of the manuscript. JS, DN and YZ take full responsibility for the work and approved the final version to be published.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Supplemental material

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