BJR

■ OSTEOARTHRITIS

Mechanical stress contributes to osteoarthritis development through the activation of transforming growth factor beta 1 (TGF-β1)

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Objectives

The role of mechanical stress and transforming growth factor beta 1 (TGF-β1) is important in the initiation and progression of osteoarthritis (OA). However, the underlying molecular mechanisms are not clearly known.

Methods

In this study, TGF-β1 from osteoclasts and knee joints were analyzed using a co-cultured cell model and an OA rat model, respectively. Five patients with a femoral neck fracture (four female and one male, mean 73.4 years (68 to 79)) were recruited between January 2015 and December 2015. Results showed that TGF-β1 was significantly upregulated in osteoclasts by cyclic loading in a time- and dose-dependent mode. The osteoclasts were subjected to cyclic loading before being co-cultured with chondrocytes for 24 hours.

Results

A significant decrease in the survival rate of co-cultured chondrocytes was found. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) assay demonstrated that mechanical stress-induced apoptosis occurred significantly in co-cultured chondrocytes but administration of the TGF-β1 receptor inhibitor, SB-505124, can significantly reverse these effects. Abdominal administration of SB-505124 can attenuate markedly articular cartilage degradation in OA rats.

Conclusion

Mechanical stress-induced overexpression of TGF-β1 from osteoclasts is responsible for chondrocyte apoptosis and cartilage degeneration in OA. Administration of a TGF-β1 inhibitor can inhibit articular cartilage degradation.

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Article focus

■ Transforming growth factor beta 1 (TGF-β1) and mechanical stress in progression of osteoarthritis (OA).

Key messages

■ TGF-β1 overexpression from osteoclasts under mechanical stress has detrimental effects on chondrocytes and articular cartilage in OA progression.

Strengths and limitations

■ The effects of TGF-β1, chondrocyte apoptosis, and articular degeneration were studied comprehensively.

■ However, how TGF-β1 is secreted from osteoclasts, and how it affects cartilage degeneration *in vivo*, should be studied further.

Introduction

Osteoarthritis (OA) is characterized by degradation of articular cartilage, thickening of subchondral bone, and formation of osteophytes.[1] In the early stages of OA, the possibility of accelerated subchondral bone remodelling has been confirmed.[2] In general, ageing, vitamin and mineral deficiencies, and drug abuse lead to an increased risk of OA development.[3] However, the pathogenic mechanisms of OA

Table I. The primer sequences used in this study

Gene	Forward	Reverse
TGF-β1	5'-GGTACCTGAACCCGTGTTGCT-3'	5'-TGTTGCTGTATTTCTGGTACAGCTC-3'
MMP-9	5'-GCATAAGGACGACGTGAATGGC-3'	5'-CGGTGTGGTGGTGGTTGGAG-3'
MMP-13	5'-CTTGATGCCATTACCAGTC-3'	5'-GGTTGGGAAGTTCTGGCCA-3'
Col-X	5'-ACAGGGGAGGTGATAGCATT-3'	5'-GACCAAAAGCCTTCATACATCTC-3'
Ctsk	5'-CTGAAGATGCTTTCCCATATGTGGG-3'	5'-GCAGGCGTTGTTCTTATTCCGAGC-3'
GAPDH	5'-ACAGGGGAGGTGATAGCATT-3'	5'-GACCAAAAGCCTTCATACATCTC-3'

TGF-β1, transforming growth factor beta 1; MMP, matrix metallopeptidase; Col-X, type X collagen; Ctsk, cathepsin K; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

have not been clearly identified. There is evidence that mechanical overload leads to the activation of anabolic processes in chondrocytes, including matrix proteins and matrix-degrading enzymes such as matrix metallopeptidases (MMPs).[4] Several *in vitro* and *in vivo* experiments suggest that mechanical stimulation contributes to OA development by altering specific signalling pathways in chondrocytes or joints.[5-7] In addition, mechanical stimulation-induced chondrocyte apoptosis has been identified as being involved in OA progression.[6] In ageing mice, disruption of Sirt1 in chondrocytes causes the accelerated progression of OA under mechanical stress.[8] Mechanical stress can also affect bone homeostasis. Load can lead to bone apposition,[9] whereas load removal can cause bone loss.[10] The direct effect of mechanical stress on osteoclasts has been studied and demonstrates that mechanical load can suppress osteoclast differentiation and fusion by the increase of nitric oxide (NO) via inducible nitrix oxide synthase (iNOS).[11] However, osteoclasts under mechanical stress and their contribution to chondrocyte metabolism remain unclear.

Transforming growth factor beta 1 (TGF-β1) is a cytokine and plays an important role in the induction of chondrogenesis.[12] The expression of TGF-β1 in healthy cartilage is significantly higher than in OA cartilage.[13] In the progression of chondrocytic phenotypic degeneration, including senescence and dedifferentiation, downregulation of TGF-β1 indirectly induces metabolic disorder in chondrocytes.[14] TGF-β1 promotes proliferation of chondrocytes through β-catenin signalling and maintains chondrocytic phenotype by enhancing collagen II synthesis.[14,15] In native mouse knee joints, injection of low-dose TGF-β1 increases the proteoglycan content of articular cartilage;[16] however, overexpression of TGF-β1 in the knee joint results in OA-like changes, including hyperplasia of the synovium and osteophyte formation in the knee joints of C57Bl/6 mice.[17] In co-cultured osteoclast precursors and OA synovial fibroblasts, TGF-β-induced osteoprotegerin (OPG) production in synovial fibroblasts inhibits osteoclast formation. Moreover, blocking TGF-β activity in OA joints promotes osteoclastogenesis and inhibits osteophyte formation.[13] In the early phases of OA, upregulation of TGF-β stimulates chondrocyte proliferation and attempts to repair injured cartilage.[18] In transgenic mice, overexpressed TGF-β1

leads to abnormal subchondral bone structure and articular cartilage degeneration.[19] More and more evidence shows that changes in subchondral bone in OA occurs rapidly. These changes occur at the same time as, if not earlier than, articular cartilage changes.[20] Furthermore, increased osteoclast activity is a main pathogenic factor in subchondral bone injury in OA, and dysregulation of TGF-β1 signalling is detected in subchondral bone by accelerating osteoclast bone resorption in OA.[18] To study the mechanical stress and TGF-β signalling in OA, an *in vitro* 3D collagen scaffold culture model was used. It is difficult to determine whether cells in the culture experience the same mechanical stimulus as in the skeleton. The mechanical environment that osteoclasts or chondrocytes experience *in vivo* is still under debate; however, cellular deformation caused by mechanical stress is believed to be essential in subsequent mechanotransduction. Different *in vitro* mechanical loading models were reviewed by Yang et al.[21] In our study, we investigated the role of mechanical overload in the development of OA, and studied the TGF-β1 signalling pathways in response to mechanical overload in the co-cultured cells of osteoclasts and chondrocytes, which was a valuable cell model for studying the pathogenesis of OA.

Patients and Methods

Five patients with a femoral neck fracture (four female and one male, mean 73.4 years (68 to 79)) were recruited from the Department of Orthopaedics, the Fifth Affiliated Hospital of Sun Yat-sen University (Zhuhai, China), between January 2015 and December 2015, and written informed consent was obtained. The study was approved by the Ethics Committee of the Department of Orthopaedics, the Fifth Affiliated Hospital of Sun Yat-sen University (Zhuhai, China). Femoral head was used to develop a cell culture of osteoclasts and chondrocyte.[6,22] The subchondral bone from the femoral head was ground into powder before being washed and incubated in Medium 199 (Sigma-Aldrich, St Louis, Missouri). The supernatant was incubated in the culture medium at 37°C, 5% CO_2 condition before use. Cartilage samples were cut from the femoral head and minced into small fragments, followed by digestion with 0.25% trypsin (Gibco/Invitrogen, Carlsbad, California) for 30 minutes at 37°C and then with 0.2 collagenase (Sigma-Aldrich)
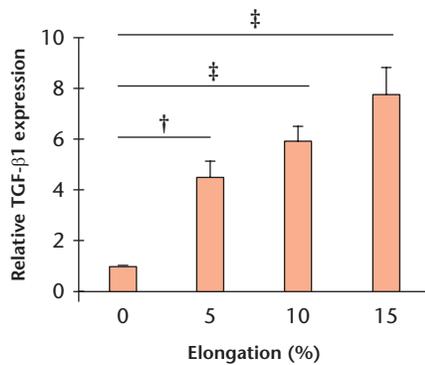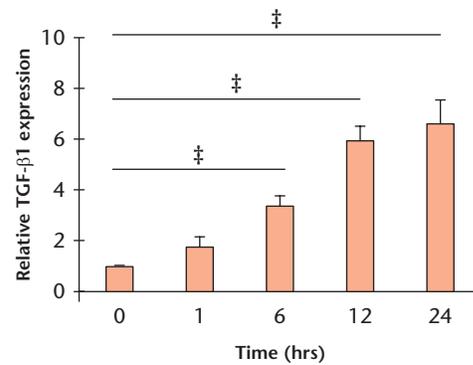
Fig. 1a

Fig. 1b

Graphs showing upregulated transforming growth factor beta 1 (TGF-β1) expression in osteoclasts under mechanical stress, in relation to a) elongation, and b) time. †p < 0.005; ‡p < 0.001.

for five hours at 37°C. The cell suspension was then filtered through a 40 μm cell strainer (BD Falcon, San Jose, California), and cells were collected by centrifugation at 800×g for ten minutes. The chondrocytes were then cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco/Invitrogen), supplemented with 10% foetal bovine serum (FBS; Gibco/Invitrogen)). The adherent cells were further incubated in a fresh medium after culturing overnight.

Ten-week-old male Sprague-Dawley (SD) rats (Guangzhou University of Traditional Chinese Medicine, Guangzhou, China) were allowed to acclimatize to the environment for one week. All experimental procedures were carried out in accordance with the guidelines of the Fifth Affiliated Hospital of Sun Yat-sen University on animal care. The anterior cruciate ligament (ACL) was transected to induce abnormal mechanical loading-associated OA of the left knee. Sham surgery was done by opening the joint capsule without ACL transection in the left knee in control rats. The animals were sacrificed at six weeks post-surgery, and samples of the knee joints were collected for further molecular and histological analyses. The rats were randomly divided into three groups: 1) a control group, in which rats underwent sham surgery (n=6); 2) a group, in which rats underwent ACL transection (ACLT) (n=6); and 3) a SB-505124 group, in which rats underwent ACLT and received SB-505124, administrated abdominally, once after surgery (n=6).

Cell culture, cyclic loading, and co-cultured system. Osteoclasts and chondrocytes were maintained in α-minimal essential medium (α-MEM; Thermo Fisher Scientific, Waltham, Massachusetts), supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific) at 37°C in a humidified 5% CO_2 incubator (Thermo Fisher Scientific).[23] Osteoclasts were seeded into 3D collagen sponges.[24] The cyclic mechanical loading was induced by a computer-controlled bio-stretch device (Bio-Stretch; ICCT Technologies, Markham, Canada).[5] After 24 hours, cyclic loading-processed osteoclasts were co-cultured with chondrocytes.

Cell viability and TUNEL staining analysis. Chondrocyte proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation/viability assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the guidelines.

Quantitative assessment of apoptotic cells was assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) method, which examines Deoxyribose Nucleic Acid (DNA) strand breaks during apoptosis by using a BD ApoAlert DNA Fragmentation Assay Kit (Takara Bio, Inc., Mountain View, California). Cartilage were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodium citrate. After being washed, the cells were incubated with the reaction mixture for 60 minutes at 37°C. Cells were immediately analyzed using FACScan and the Cellquest program (both Becton Dickinson, Franklin Lakes, New Jersey).

After fixation in 75% alcohol for seven days, the tibia (n=6/group) specimens were decalcified in 0.5 M EDTA (pH=8.0) and then embedded in paraffin as per standard histological procedures. Sections measuring 5 μm were cut and stained with safranin O (Nanjing SenBeiJia Biological Technology Co., Ltd, Nanjing, China), and examined under a microscope (DM 2500; Leica, Wetzlar, Germany).

Enzyme-linked immunosorbent assay analyses (ELISA) were used to detect the expression levels of TGF-β1 in the *in vitro* culture cells and tissue homogenates of the rat knee joints. All procedures followed the ELISA kit instruction manual (Bio-Rad Laboratories, Marnes-La-Coquette, France). In brief, cell lysates and tissue homogenates were prepared using a Heidolph Diax 900 homogenizer (Elektro GmbH, Kelheim, Germany) in an ice-cold buffer containing 50 mM hydroxymethyl and 150 mM sodium chloride (NaCl) (pH 8.0), supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml aprotinin, leupeptin, and pepstatin. The solution was centrifuged for
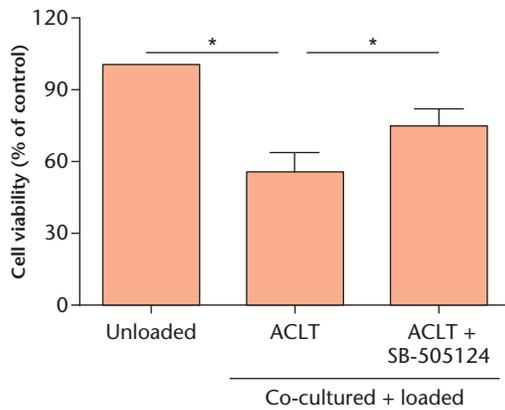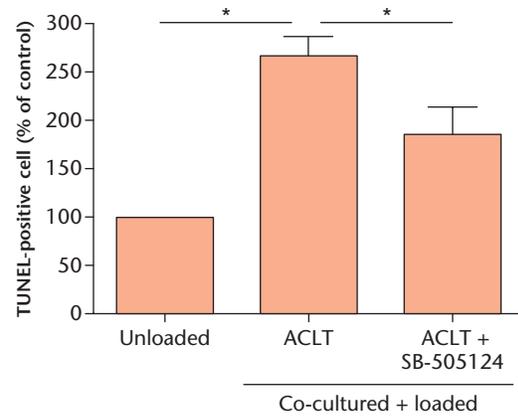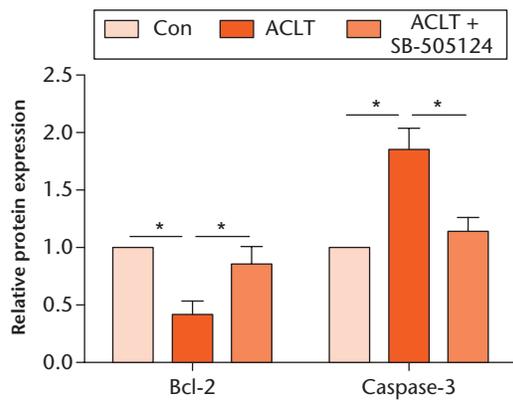
Fig. 2a

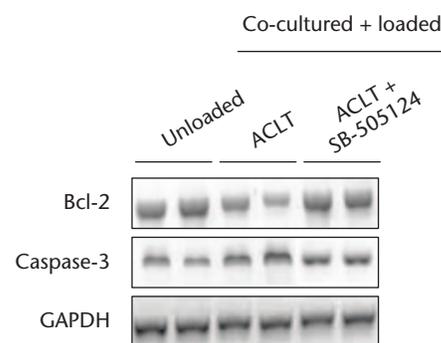
Fig. 2b

Fig. 2c

Fig. 2d

Cyclic loading-processed osteoclasts inhibit chondrocyte proliferation and induce apoptosis in osteoclast/chondrocyte co-cultured system. a) to c) Bar charts showing: a) cell viability; b) terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL)-positive cells; and c) B-cell lymphoma 2 (Bcl-2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression for each of the three groups. d) Western blotting analysis of Bcl-2, caspase-3, and GAPDH. ACLT, anterior cruciate ligament transection. *p < 0.05.

15 minutes at $16000 \times g$, 4°C, and a total of 50 μl of supernatant was added into the wells coated with specific TGF-β1 detection antibodies. After the addition of 50 μl of biotin-conjugated secondary antibody, horseradish peroxidase (HRP)-conjugated avidin, and substrate, sample absorbance values were recorded using a Wallac Victor2 microplate reader (Perkin Elmer, Gaithersburg, Maryland) at a wavelength of 650 nm. Standard curves were drawn based on the sample concentrations and absorbance values in order to calculate the concentration of TGF-β1 in the specimens.

Ribonucleic Acid (RNA) was extracted from chondrocytes using TRIzol (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions. Synthesis of cDNA was performed on 2 μg total RNA using Moloney Murine Leukemia Virus reverse transcriptase (Promega Corporation, Madison, Wisconsin) and oligo (dT) 15 primers (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative Polymerase Chain Reaction (qPCR) was performed using the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific). SDS software version 2.1 (Applied Biosystems; Thermo Fisher Scientific) was used to calculate the

quantification cycle fluorescence value (Cq), and the $2^{-\Delta\Delta Cq}$ method for the relative expression levels of RANK[19] normalizing to the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences that were used are given in Table I.

Cells and tibia were homogenized and lysed in NP-40 buffer (Beyotime Institute of Biotechnology). Following five to ten minute at boiling temperature, cells were centrifuged at $10000 \times g$, 4°C, for ten minutes to obtain the supernatant. Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, Massachusetts). Membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline and 0.1% (w/v) Tween 20 (TBST), and incubated at 4°C overnight with the following primary antibodies: TGF-β1; B-cell lymphoma 2 (Bcl-2); caspase-3; MMP-9; cathepsin K (Ctsk); and GAPDH (all from Santa Cruz Biotechnology, Inc., Dallas, Texas). After being washed, the membranes were incubated with HRP-conjugated anti-immunoglobulin G (IgG) at room temperature for two hours. Signal detection was carried out with an enhanced chemiluminescence
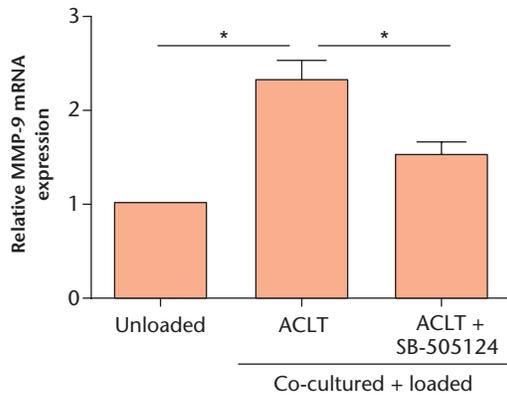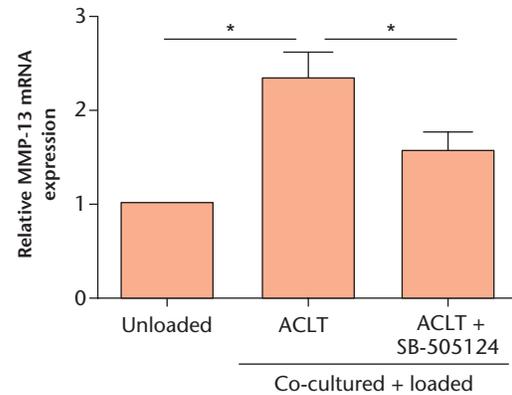
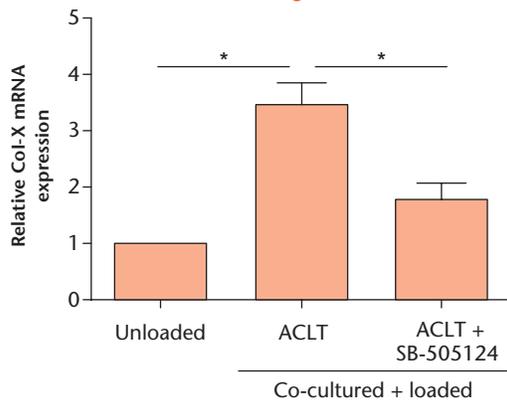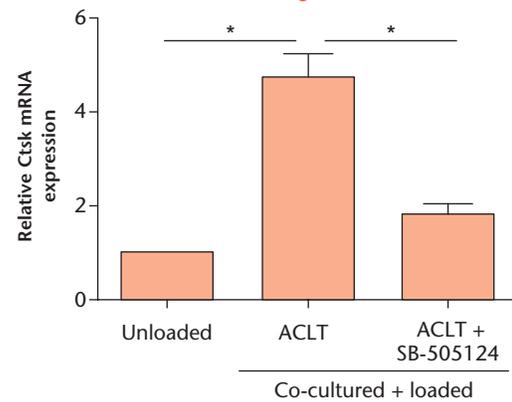Bar charts showing how cyclic loading-processed osteoclasts regulate cartilage degeneration-related markers in osteoclast/chondrocyte co-cultured system: a) matrix metallopeptidase (MMP)-9; b) MMP-13; c) type X collagen (Col-X); and d) cathepsin K (Ctsk). ACLT, anterior cruciate ligament transection. *p < 0.05.

(ECL) system (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey).

Statistical analysis. Mean and standard error of the mean (SEM) were used for data description. All statistical analyses were performed using GraphPad Prism software, version 5.0 (GraphPad Software Inc., La Jolla, California). Groups were compared using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test as a *post hoc* test to compare the mean values of each group. A statistically significant difference was considered to be p<0.05.

Results

Upregulated TGF-β1 expression in osteoclasts under mechanical stress. Human osteoclasts were isolated from the femoral head of patients with a fracture of the femoral neck and seeded into collagen sponges. After being subjected to a cyclic mechanical loading at 1 Hz for 12 hours, the expression of TGF-β1 from the *in vitro* cultured osteoclasts was significantly upregulated with the increase of the elongation (0% to 15%) (Fig. 1a). Moreover, at 10% elongation and 1 Hz cyclic loading, the expressions of TGF-β1 were increased in a time-dependent manner (0 to 24 hours) (Fig. 1b).

Cyclic loading-processed osteoclasts inhibit chondrocyte proliferation and induce apoptosis in osteoclast/

chondrocyte co-cultured system. Osteoclasts under cyclic loading, 10% elongation, and 1 Hz for 12 hours were co-cultured with chondrocytes. The chondrocyte viability was determined at 24 hours after the co-cultured system by MTT assay. Results showed that chondrocytes in this co-cultured system demonstrated cell growth inhibition as compared with unloaded co-cultured cells (Fig. 2a). Next, we examined apoptosis in co-cultured cells by flow cytometry. TUNEL staining results demonstrated that cyclic loading-processed osteoclasts co-cultured with chondrocytes induced cell apoptosis compared with the control group (Fig. 2b). However, SB-505124 treatment could significantly reverse the inhibition of chondrocyte proliferation caused by cyclic loading-processed osteoclasts, and induced apoptosis in the osteoclast/chondrocyte co-cultured system (Figs 2a and 2b). Furthermore, we found that mechanical pressure injury increased the caspase-3 protein levels and inhibited the Bcl-2 protein levels in loaded co-cultured cells as compared with unloaded co-cultured cells. However, administration of SB-505124 reversed the upregulation of caspase-3 and the downregulation of Bcl-2 in loaded co-cultured cells (Figs 2c and 2d).

Cyclic loading-processed osteoclasts regulate cartilage degeneration-related markers in osteoclast/chondrocyte co-cultured system. We examined the mRNA levels of MMP-9 and MMP-13 in an osteoclast/chondrocyte

Fig. 4a   Fig. 4b   Fig. 4c

Fig. 4d   Fig. 4e   Fig. 4f   Fig. 4g

Fig. 4h   Fig. 4i   Fig. 4j   Fig. 4k
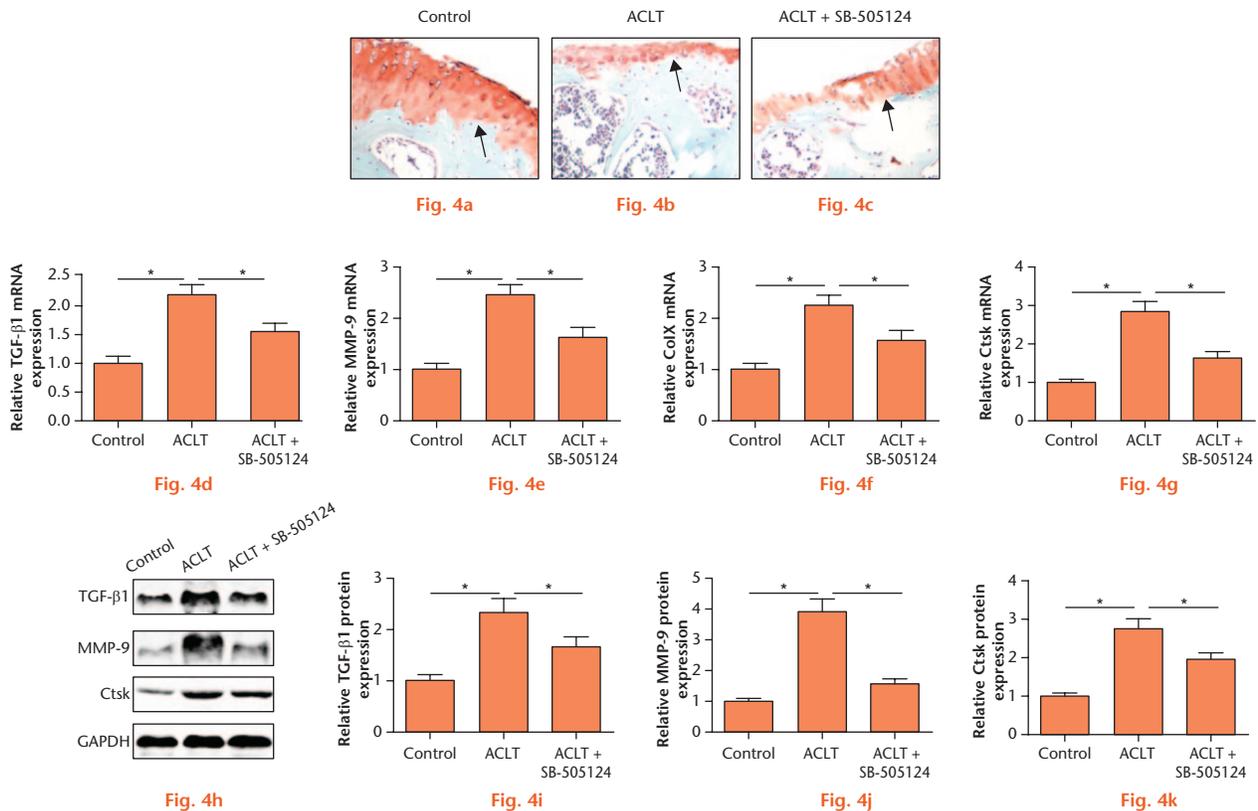
Transforming growth factor-beta 1 (TGF-β1) is involved in anterior cruciate ligament (ACL)-induced osteoarthritis (OA) in a rat model. a) to c) Histology showing the results of Safranin O staining for: a) the control group; b) the ACL transection (ACLT) group; and c) the ACLT + SB-505124 group. d) to g) Bar charts showing relative mRNA expression for: d) TGF-β1; e) matrix metallopeptidase (MMP)-9; f) type X collagen (Col-X); and g) cathepsin K (Ctsk). h) Western blotting analysis of TGF-β1, MMP-9, Ctsk, and GAPDH. i) to k) Bar charts showing relative protein expression for: i) TGF-β1; j) MMP-9; and k) Ctsk. *$p < 0.05$.

co-cultured system. Indeed, mechanical stress significantly upregulated the mRNA levels of MMP-9 and MMP-13 in osteoclast/chondrocyte co-cultured cells compared with the control group. The addition of SB-505124 to the co-cultured cells with mechanical stress inhibited MMP-9 and MMP-13 expression significantly when compared with the non-treatment group (Figs 3a and 3b). Moreover, the mRNA levels of type X collagen (Col-X) were significantly higher in the co-cultured cells with mechanical stress than in the unloaded group. However, in the presence of SB-505124, upregulation of Col-X caused by mechanical stress was reversed (Fig. 3c). Furthermore, we found that administration of SB-505124 reversed the upregulation of Ctsk in loaded co-cultured cells (Fig. 3d), which is a lysosomal cysteine protease and is involved in matrix remodelling.

TGF-β1 is involved in ACL-induced OA in a rat model. First, Safranin O staining demonstrated that the articular cartilage in ACLT rats exhibited accelerated proteoglycan loss as compared with that of the rats in the control group. However, SB-505124 treatment markedly attenuated articular cartilage degradation in the surgical rats (Fig. 4a). The mRNA and protein levels of TGF-β1 were significantly upregulated in the articular cartilage of ACLT rats as compared with the normal group. The upregulation

of TGF-β1 mRNA and protein expression in the proximal tibia from ACLT rats was reversed by the administration of SB-505124 (Figs 4b, 4f, and 4g). The expression of matrix-degrading enzyme MMP-9 was significantly increased in surgically induced OA cartilage compared with the control group as shown by real-time PCR (Fig. 4c) and Western blotting (Figs 4f and 4h). Moreover, Col-X expression is affected following ACLT operation. This upregulation of Col-X could be completely blocked by SB-505124 in ACLT rats (Fig. 4d). We found that the mRNA and protein expression of Ctsk were significantly upregulated in the articular cartilage of ACLT rats as compared with the normal group. The upregulation of Ctsk mRNA and protein expression was reversed by administration of SB-505124 in ACLT rats (Figs 4e, 4f and 4i).

Discussion

Cartilage degeneration is a prominent feature of OA. In this process, mechanical stress plays a crucial role in promoting metabolic disorder in chondrocytes.[25] Evidence suggests that mechanical stress can directly contribute to matrix remodelling by inducing MMP expression in a variety of tissues.[26] Previous studies indicate that chondrocytes are highly responsive to mechanical stress, which can induce TGFs, matrix-degrading enzymes, and

collagenase to mediate biochemical signals in articular cartilage remodelling.[3,18,25] In SW1353 chondrocyte-like cells, mechanical stress induces MMP-13 expression by runt-related transcription factor 2 (RUNX2).[25]

Our study demonstrates that mechanical stress can be a trigger for the upregulation of TGF-β1 in osteoclasts. Mechanical stress-processed osteoclasts inhibit chondrocyte proliferation and induce apoptosis in an osteoclast/chondrocyte co-cultured system. However, interruptive TGF-β1 signalling by SB-505124 can inhibit chondrocyte dysfunction and OA-like changes *in vitro*. In an ACLT rat model, we also show that overexpressed TGF-β1 is involved in articular cartilage degeneration and OA-like changes. Our results are in line with those of Yang et al[5] who showed that the expression of MMP-13 and Col-X were significantly increased in ACLT-induced OA rats.

TGF β1 is considered a central regulator of articular cartilage remodelling under mechanical stress, which is a crucial indirect mediator of OA via induction of TGF-β1 production.[3] Blockage of TGF-β1 signalling attenuates articular cartilage degeneration in the ACLT rodent model of OA.[27] It has been previously reported that excessive TGF-β 1 action in subchondral bone can induce temporo-mandibular joint OA.[28] In the progression of osteoclast bone resorption, dysregulation of TGF-β 1 alters the fate of bone marrow mesenchymal stem cells (MSCs), uncoupling bone remodelling, and causing skeletal disorders.[12] A previous study indicates that TGF-β1 is essential in the mediation of osteoclast differentiation in a co-cultured system with rheumatoid arthritis (RA) synovial fibroblasts.[13] However, a complete understanding of the TGF-β1 signalling in an osteoclast/chondrocyte co-cultured system remains elusive. In the present study, we demonstrated that not only magnitudes of elongation to osteoclasts but also the duration of time increased the mRNA and protein expression of TGF-β1. Our study has shown osteoclasts co-cultured with chondrocytes as a valuable cell model for studying the pathogenesis of OA under mechanical stress. It also showed that chondrocyte growth was repressed by cyclic loading-processed osteoclasts. Moreover, chondrocyte apoptosis was induced in a co-cultured system. However, the TGF-β1 receptor inhibitor, SB-505124, could significantly reverse the suppression of chondrocyte growth and the induction of apoptosis. Interestingly, transgenic expression of active TGF-β1 in osteoblastic cells is sufficient to induce osteoarthritis, whereas direct inhibition of TGF-β1 activity in subchondral bone attenuated the degeneration of articular cartilage.[12] These observations suggest that high levels of active TGF-β in bone remodelling are associated with the progression of OA.

Various proteins are involved in the phenotypic change of chondrocytes by regulating the expression of chondrocyte-specific proteins such as collagen type II (Col-II), glycoproteins, and proteoglycans (PGs).[14,29] In senescence stages, chondrocytes exhibit high expression of Col-II and low expression of Col-X. Moreover, the cartilage-degrading enzyme, MMP-13, is actively produced in senescent chondrocytes.[14] In our study, we found that cyclic loading-processed osteoclasts regulated cartilage degeneration-related markers in an osteoclast/chondrocyte co-cultured system, including the upregulation of Col-X, MMP-9, and MMP-13 in co-cultured cells. Importantly, the TGF-β1 receptor inhibitor, SB-505124, could inhibit levels of Col-X, MMP-9, and MMP-13 in co-cultured cells. Based on these observations, we suggest that the direct suppression of TGF-β1 signalling in chondrocytes could alleviate mechanical stress-induced chondrocyte dysfunction. Intriguingly, TGF-β1 has varying roles in chondrogenesis *in vivo*; introducing TGF-β1 into the periosteum of a femur causes chondrocyte differentiation and cartilage formation,[30] whereas inhibition of the TGF-β1 signalling pathway delays the development of osteoarthritis in both mouse and rat models.[12] In our work, elevation of TGF-β1 levels had been observed in ACLT-induced OA rats, and articular cartilage degradation was accompanied by the progression of OA in ACLT surgical rats. However, SB-505124 treatment markedly reduced articular cartilage degradation and inhibited the expression of TGF-β1 in the surgical rats.

In conclusion, TGF-β1 is one of the key cytokines responsible for chondrocyte dysfunction in a cyclic loading-processed osteoclast/chondrocyte co-cultured system. We also found that overexpressed TGF-β1 was proven to have a detrimental effect in response to mechanical stress-induced articular cartilage degradation in a rat model. These results suggest that TGF-β1 can be used as a potential pathway, worthy of further study, in the regulation of mechanical stress-induced OA.

References

1. **Varady NH, Grodzinsky AJ.** Osteoarthritis year in review 2015: mechanics. *Osteoarthritis Cartilage* 2016;24:27–35.

2. **Hirata Y, Inaba Y, Kobayashi N, et al.** Correlation between mechanical stress by finite element analysis and 18F-fluoride PET uptake in hip osteoarthritis patients. *J Orthop Res* 2015;33:78–83.

3. **Madej W, van Caam A, Blaney Davidson EN, van der Kraan PM, Buma P.** Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P signaling. *Osteoarthritis Cartilage* 2014;22:1018–1025.

4. **Loeser RF, Goldring SR, Scanzello CR, Goldring MB.** Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum* 2012;64:1697–1707.

5. **Yang X, Guan Y, Tian S, et al.** Mechanical and IL-1β responsive miR-365 contributes to osteoarthritis development by targeting histone deacetylase 4. *Int J Mol Sci* 2016;17:436.

6. **Jin L, Zhao J, Jing W, et al.** Role of miR-146a in human chondrocyte apoptosis in response to mechanical pressure injury in vitro. *Int J Mol Med* 2014;34:451–463.

7. **Camacho A, Simão M, Ea HK, et al.** Iron overload in a murine model of hereditary hemochromatosis is associated with accelerated progression of osteoarthritis under mechanical stress. *Osteoarthritis Cartilage* 2016;24:494–502.

8. **Matsuzaki T, Matsushita T, Takayama K, et al.** Disruption of Sirt1 in chondrocytes causes accelerated progression of osteoarthritis under mechanical stress and during ageing in mice. *Ann Rheum Dis* 2014;73:1397–1404.

9. **Srinivasan S, Weimer DA, Agans SC, Bain SD, Gross TS.** Low-magnitude mechanical loading becomes osteogenic when rest is inserted between each load cycle. *J Bone Miner Res* 2002;17:1613–1620.

10. **Schneider V, Oganov V, LeBlanc A, et al.** Bone and body mass changes during space flight. *Acta Astronaut* 1995;36:463–466.

11. **Shibata K, Yoshimura Y, Kikuiri T, et al.** Effect of the release from mechanical stress on osteoclastogenesis in RAW264.7 cells. *Int J Mol Med* 2011;28:73–79.

12. **Crane JL, Cao X.** Bone marrow mesenchymal stem cells and TGF-β signaling in bone remodeling. *J Clin Invest* 2014;124:466–472.

13. **Hase H, Kanno Y, Kojima H, Sakurai D, Kobata T.** Coculture of osteoclast precursors with rheumatoid synovial fibroblasts induces osteoclastogenesis via transforming growth factor beta-mediated down-regulation of osteoprotegerin. *Arthritis Rheum* 2008;58:3356–3365.

14. **Ashraf S, Cha B-H, Kim JS, et al.** Regulation of senescence associated signaling mechanisms in chondrocytes for cartilage tissue regeneration. *Osteoarthritis Cartilage* 2016;24:196–205.

15. **Li T-F, Chen D, Wu Q, et al.** Transforming growth factor-beta stimulates cyclin D1 expression through activation of beta-catenin signaling in chondrocytes. *J Biol Chem* 2006;281:21296–21304.

16. **van Beuningen HM, van der Kraan PM, Arntz OJ, van den Berg WB.** Transforming growth factor-beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. *Lab Invest* 1994;71:279–290.

17. **Bakker AC, van de, Loo FA, van Beuningen HM, et al.** Overexpression of active TGF-beta-1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation. *Osteoarthritis Cartilage* 2001;9:128–136.

18. **Zhen G, Cao X.** Targeting TGFβ signaling in subchondral bone and articular cartilage homeostasis. *Trends Pharmacol Sci* 2014;35:227–236.

19. **Tang Y, Wu X, Lei W, et al.** TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. *Nat Med* 2009;15:757–765.

20. **Fang H, Huang L, Welch I, et al.** Early changes of articular cartilage and subchondral bone in the DMM mouse model of osteoarthritis. *Sci Rep* 2018;8:2855.

21. **Yang L, Yang Y, Wang S, Li Y, Zhao Z.** In vitro mechanical loading models for periodontal ligament cells: from two-dimensional to three-dimensional models. *Arch Oral Biol* 2015;60:416–424.

22. **Atkins GJ, Kostakis P, Vincent C, et al.** RANK Expression as a cell surface marker of human osteoclast precursors in peripheral blood, bone marrow, and giant cell tumors of bone. *J Bone Miner Res* 2006;21:1339–1349.

23. **Shang W, Zhao LJ, Dong XL, et al.** Curcumin inhibits osteoclastogenic potential in PBMCs from rheumatoid arthritis patients via the suppression of MAPK/RANK/c-Fos/NFATc1 signaling pathways. *Mol Med Rep* 2016;14:3620-3626.

24. **Mizuno S, Glowacki J.** Three-dimensional composite of demineralized bone powder and collagen for in vitro analysis of chondroinduction of human dermal fibroblasts. *Biomaterials* 1996;17:1819–1825.

25. **Tetsunaga T, Nishida K, Furumatsu T, et al.** Regulation of mechanical stress-induced MMP-13 and ADAMTS-5 expression by RUNX-2 transcriptional factor in SW1353 chondrocyte-like cells. *Osteoarthritis Cartilage* 2011;19:222–232.

26. **Ruddy JM, Jones JA, Stroud RE, et al.** Differential effects of mechanical and biological stimuli on matrix metalloproteinase promoter activation in the thoracic aorta. *Circulation* 2009;120(Suppl):S262–S268.

27. **Zhen G, Wen C, Jia X, et al.** Inhibition of TGF-β signaling in mesenchymal stem cells of subchondral bone attenuates osteoarthritis. *Nat Med* 2013;19:704–712.

28. **Jiao K, Zhang M, Niu L, et al.** Overexpressed TGF-β in subchondral bone leads to mandibular condyle degradation. *J Dent Res* 2014;93:140–147.

29. **Papaioannou G, Mirzamohammadi F, Lisse TS, et al.** MicroRNA-140 provides robustness to the regulation of hypertrophic chondrocyte differentiation by the PTHrP-HDAC4 pathway. *J Bone Miner Res* 2015;30:1044–1052.

30. **Blaney Davidson EN, van der Kraan PM, van den Berg WB.** TGF-beta and osteoarthritis. *Osteoarthritis Cartilage* 2007;15:597–604.

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- S-Y. Feng: Designing the study, Editing the manuscript.
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Conflict of Interest Statement
- None declared