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The disease modifying osteoarthritis drug diacerein is able to antagonize pro inflammatory state of chondrocytes under mild mechanical stimuli

B. Steinecker-Frohnwieser † * [a], L. Weigl ‡, W. Kullich †, B. Lohberger §

† *Ludwig Boltzmann Institute for Rehabilitation of Internal Diseases, Ludwig Boltzmann Cluster for Rheumatology, Balneology and Rehabilitation, Saalfelden, Austria*

‡ *Department of Special Anaesthesia and Pain Therapy, Medical University Vienna, Austria*

§ *Department of Orthopaedic Surgery, Medical University of Graz, Graz, Austria*

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SUMMARY

Objective: To investigate the combination of mild mechanical stimuli and a disease modifying osteoarthritis drug (DMOAD) in inflammatory activated chondrocytes and to study the combination of drug and mechanical tension on the cellular level as a model for an integrated biophysical approach for osteoarthritis (OA) treatments.

Methods: Interleukin-1beta (IL-1β) stimulated C28/I2 cells underwent mild mechanically treatment while cultured in the presence of the DMOAD diacerein. The pharmacological input of diacerein was evaluated by cell viability and cell proliferation measurements. Inflammation and treatment induced changes in key regulatory proteins and components of the extracellular matrix (ECM) were characterized by quantitative real-time PCR (qPCR). The effects on metalloproteinase-1 (MMP-1) activity and glycosaminoglycan (GAG) concentration in cell supernatants of treated cells were investigated.

Results: C28/I2 cells demonstrated significant changes in expression of inflammatory and cartilage destructive proteins in response to IL-1β stimulation. The chondroprotective action of diacerein in mechanically stimulated cells was mediated by a decrease in interleukin-8 (IL-8), fibronectin-1 (FN-1), collagen type I (Col 1) and MMP-1 expression levels, respectively. Augmented expression of interleukin-6 receptor (IL-6R) and the fibroblast growth factor receptors (FGFRs) by diacerein was not abolished by mechanical treatment. The observed effects were accompanied by a reduced cell proliferation rate, attenuated cell viability and extenuated MMP-1 activity.

Conclusion: Diacerein diversely regulates the expression of main regulatory proteins as well as components important to regenerate and set up ECM. Mechanical stimulation does not negatively influence the chondroprotective effect induced by diacerein treatment in immortalized human C28/I2 chondrocytes.

Introduction

Osteoarthritis (OA) is a multifactorial, complex disease affecting about 15% of the world's population. Worldwide the prevalence of OA increases steadily and generates an ever rising economic burden[1,2]. This joint disease, targets the structural and functional integrity of articular cartilage. The destruction of cartilage results in a gradual development of stiffness, limited motion and chronic pain, the major symptoms of OA[3]. The origin of pain and disability arises from the pathological imbalance of the dynamic state between destructive forces and repair mechanisms within the joints[4]. Medical treatment of OA mainly involves the application of non-steroidal drugs (NSAIDs), corticosteroids, or pain relievers like paracetamol[5,6], with deleterious side effects on the gastrointestinal tract, kidney and liver[7]; corticosteroid application leads to a progressive cartilage damage in weight bearing joints and has numerous other side effects (e.g., Cushing syndrome)[8].

Alternatively, current therapies for OA including disease modifying osteoarthritis drugs (DMOADs) try to preserve normal joint function, reduce disease's intensity and symptoms, and restrain the progression rate of OA[9]. The DMOAD diacerein, an anthraquinone, reduces the severity of OA and may be able to modify the course of

* Address correspondence and reprint requests to: B. Steinecker-Frohnwieser, Institute Branch, Gröbming, Austria. Tel.: 43-3685-23037; Fax: 43-3685-22323-142.
E-mail address: lbi.groebming@aon.at (B. Steinecker-Frohnwieser).
[a] SKA Rehabilitation Centre of the PVA, Hofmanning 214, A-8962 Gröbming, Austria.

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the disease[10]. The efficiency of diacerein is attributed to the down regulation of the interleukin-1beta (IL-1β) induced inflammatory pathways, mainly involved in cartilage destruction, thus, suppressing the cartilage-matrix breakdown[11–13]. At the gene expression level, diacerein decreases the production of pro-matrix metalloproteinases (MMPs) involved in cartilage degradation and augments tissue inhibitor of metalloproteinases-1 (TIMP-1) production[14,15]. Furthermore, studies revealed that diacerein antagonizes the IL-1β triggered mitogen-activated protein kinase (MAPKs) signaling cascades of articular chondrocytes[16]. Along with an observed regulation of transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1), diacerein seems to stimulate cartilage repair by up-regulating the expression of transforming growth factor-beta1 (TGF-β1) and β2 even in the absence of IL-1β[17].

The summary of several clinical studies performed over the last 20 years implicate that oral intake of diacerein improves symptoms of patients with hip and knee OA and allows the reduction of NSAID consumption[18–20].

An alternative therapeutic approach, the dynamic loading of joints in OA patients, demonstrates the effectiveness of non-drug treatment modalities[21,22] such as an increase in cartilage thickness as a consequence of physiological mechanical loading[23]. In joints, cartilage function depends on composition and structural integrity of the cartilage matrix. Mechanical signals like stretch and flow-induced dynamic compression are utilized by chondrocytes to maintain the balance between degradation and synthesis of matrix macromolecules[24]. Therefore chondrocytes transmit mechanical signals into a physiological response via different signaling pathways including matrix receptors, calcium and/or ion-channels[25,26]. Under excessive loading or injury, however, degradation exceeds synthesis, causing joint degeneration and, eventually, OA[27]. It has been shown that physiological mechanical stimulation is able to inhibit IL-1β induced matrix degradation[28,29], whereas increased peak stress and strain rates provoke cartilage damage[30] and worsens the risk of injury[24].

Regeneration of the ECM based on moderate mechanical stimulation combined with the DMOAD diacerein might be beneficial to slow the progression of the disease. Therefore our study was designed to investigate the influence of diacerein combined with mechanical stimulation on regulatory parameters of the chondrocyte cell line C28/I2.

Material & methods

Cell culture

Cells from an immortalized human chondrocyte cell line (C28/I2, kindly provided by Prof. M.B. Goldring, Harvard Institute of Medicine, Boston, MA) were cultured in Dulbeco's modified eagle's medium (DMEM high glucose; GIBCO, Invitrogen, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; GIBCO, Invitrogen), 1% L-glutamine (GIBCO, Invitrogen), 100 units/ml penicillin (GIBCO, Invitrogen), 100 μg/ml streptomycin (GIBCO, Invitrogen) and 0.25 μg amphotericin B (PAA Laboratories, Pasching, Austria). Cells were kept at 37°C in a humidified atmosphere of 5% CO_2 and passaged by trypsinisation upon reaching confluence. IL-1β (10 ng/ml, Sigma–Aldrich, US) was added 24 h after seeding. It has been shown that C28/I2 cells are suitable for the study of mechano-signaling of chondrocytes[31,32].

Cell proliferation and viability assay

MTS assay (Brand, Voerde-Friedrichsfeld, Germany) was used to measure the metabolic activity of cells: 5×10^3 C28/I2 cells per well were seeded into 96 well plates and the CellTiter 96 AQueous Assay (Promega, Mannheim, Germany) was performed following the manufacturers' instructions after 1, 6, and 24 h. Untreated cells were used as negative controls.

The xCELLigence DP device from Roche Diagnostics (Mannheim, Germany) was used to monitor the proliferation of cells seeded on electronic microtiter plates (E-Plate; Roche Diagnostic) in real-time[33]. Cells were treated with different concentrations of diacerein as specified and measured for 24 h. Cell density measurements were performed in triplicates with signal detection every 20 min. The cell index (CI) is a measure for the cell density of cells and was normalized to the time point of addition of diacerein/IL-1β. Acquisition and analysis was performed with the RTCA software (Version 1.2, Roche Diagnostics).

Determination of MMPs

Cells were plated in six well plates and treated with ±IL-1β for 3 days after which the supernatants were collected. Duplicate supernatants were pooled and diluted 2.5 fold in dilution buffer. Detection of MMP-1, -3, and -13 in the supernatants was done according to the Fluorokine MAP Human MMP Kit manufacturer's instruction (R&D Systems Europe, Abington, UK). The micro particles were detected using the Luminex200System (Luminex Corporation, Austin, US). The reader was set to read a minimum of 100 beads with identical unique detection signal and the results were expressed as median fluorescent intensity (MFI).

The levels of both, the endogenous active MMP-1 and the MMP-1 in these samples that can be activated by p-Aminophenylmercuric Acetate (APMA) were measured quantitatively by the Human Active MMP-1 Fluorescent Assay (R&D, Minneapolis, US). The active MMP-1 of cell culture supernatants from mechanical stimulated and diacerein/IL-1β treated cells (50 μl) were measured according to the manufacturer's manual. The measured fluorescent signal (320 nm/405 nm) is proportional to the amount of enzyme activity in the sample.

IL-6 and IL-8 determination

Ready-to-use Sandwich ELISAs (human IL-6 and human IL-8 Platinum ELISA, eBioscience, San Diego USA) were used to quantify IL-6 and IL-8. Supernatants were used undiluted or diluted as required from 1:5 to 1:100 and proceeded according to the manufacturer's instruction. All measurements were performed in duplicates at 450 nm with micro plate reader SpectraMax Plus 384 (Molecular Devices, Sunnyvale, US) or Anthos 2010 (Anthos Labtec Instruments GesmbH., Wals, Austria).

Reverse transcription polymerase chain reaction (RT-PCR)

Total ribonucleic acid (RNA) was isolated from treated and untreated cells with the RNeasy Mini Kit and DNase-I treatment according to the manufacturer's manual (Qiagen, Hilden, Germany). Quantification and quality control of the isolated RNA was accomplished by determining the optical density at A_{260} as well as the A_{260}/A_{280} ratio by a NanoPhotometer (Implen, Munich, Germany) and by denaturing agarose gel electrophoresis.

One microgram RNA was reverse transcribed for 30 min at 37°C (iScriptcDNA Synthesis Kit, BioRad, Hercules, USA). The sequences of the PCR primers and the sizes of the amplicons are given in Table I. Col-II quantitative RT-PCR (qPCR) was performed with the QuantiTect primer assay (Qiagen). All other primer sequences were derived from the Primerbank database (http://pga.mgh.harvard.edu/primerbank). Reactions were performed in duplicates. Amplification was achieved with the RealMasterMix SYBR ROX (5 Prime,

Table I
Sequences of primer-pairs used for quantitative RT-PCR are given; the amplicon sizes to be expected are listed in the very right column

Gene (accession nr.)	Forward primer	Reverse primer	Amplicon size (bp)
MMP-1	5′CTGTTCAGGGACAGAATGTGCT3′	5′TCGATATGCTTCACAGTTCTAGGG3′	85
MMP-13	5′TCCTCTTCTTGAGCTGGACTCATT3	5′CGCTCTGCAAACTGGAGGTC3′	97
IL-6	5′AAATTCGGTACATCCTCGACGG3′	5′GGAAGGTTCAGGTTGTTTTCTGC3′	112
IL-8	5′ACTGAGAGTGATTGAGAGTGGAC3′	5′AACCCTCTGCACCCAGTTTTC3′	112
Col I (NM_000088)	5′GTTCAGCTTTGTGGACCTCCG3′	5′CTTGGTCTCGTCACAGATCAC3′	235
Col-XII (NM_001852)	5′CCTCCCTGGTGAGATTGGAAT3′	5′TTGGTTGGACACAGGAAATCC3′	147
FN1	5′CCCCATTCCAGGACACTTCTG3′	5′GCCCACGGTAACAACCTCTT3′	203
ITGβ1 (NM_002211)	5′CAAGCAGGGCCAAATTGTGG3′	5′CCTTTGCTACGGTTGGTTACATT3′	185
NF-κB	5′GCCTGACTTTGAGGGACTGTA3′	5′CTAGATGCAAGGCTGTTCGTC3′	137
TGF-β (NM_000660)	5′CAAGCAGAGTACACACAGCAT3′	5′TGCTCCACTTTTAACTTGAGCC3′	129
JNK1 (NM_002750)	5′AGAAGCTAAGCCGACCATTTC3′	5′TCTAGGGATTTCTGTGGTGTGA3′	133
IL-6R (NM_181359)	5′CCCCTCAGCAATGTTGTTTGT3′	5′CTCCGGGACTGCTAACTGG3′	171
EGFR (NM_005228)	5′AAGGAAATCCTCGATGAAGCCT3′	5′TGTCTTTGTGTTCCCGGACATA3′	154
FGFR (NM_000142)	5′TCCTTGCACAACGTCACCTTT3′	5′GCAGAGTGATGAGAAAACCCAA3′	83
VEGFA (NM_001171627)	5′CGCAGCTACTGCCATCCAAT3′	5′GTGAGGTTTGATCCGCATAATCT3′	192
GAPDH (NM_002046)	5′TGATGACATCAAGAAGGTGGTGAAG3′	5′TCCTTGGAGGCCATGTGGGCCAT3′	102
Aldolase (NM_000034)	5′ATGAGTCCACTGGGAGCATTG3′	5′ACCGCCCTTGGATTTGATAAC3′	209
ETIF (NM_003753)	5′CTACCAGCCGTTCAGCAAAG3′	5′CACCACCAAACTGAGAGGAGT3′	110

Hamburg, Germany) on a realplex mastercycler (Eppendorf, Hamburg, Germany). Each qPCR run consisted of a standard 3-step PCR temperature protocol (annealing temperature of 60°C) followed by a melting curve protocol to confirm a single gene-specific peak and to detect primer dimerization. Relative quantification of expression levels were obtained by the ΔΔCt method based on the geometric mean of the internal controls glyceraldehyde 3-phosphate dehydrogenase (GAPDH), aldolase, and eukaryotic translation initiation factor 3 (ETIF), respectively. Assessment of the ratio values is based on the calculation of changes in the relative expression for all genes of interest (control vs treated groups) by the ΔΔCt method. For the IL-1β stimulation [Fig. 1] ΔCt values of the unstimulated cells functioned as control. For all other experiments the control group was from cells stimulated with IL-1β. The ratio values for treated and untreated groups were calculated according to the formula: $r = 2^{-\Delta\Delta Ct}$ from which the percent change was derived.

Mechanical stimulation of C28/I2 cells

The Flexercell FX-5000 Tension System (FX5K; Flexcell International Corp, Hillsborough, USA) was used to apply mechanical cyclic tensile stretch on chondrocytes. This system uses flexible silicon membranes (BioFlex plates) in six well-plates. The deformation of the flexible bottom causes the attached cells to deform. Programming of the magnitude, duration, and frequency of the negative pressure in the Flexercell apparatus creates desired strain profiles[34].

Chondrocytes were seeded (5×10^4 cells/well) onto the pronectin-coated BioFlex plates, using six wells per treatment group. After the cells reached 70–80% confluence, chondrocytes were subjected to a continuous cyclic mechanical strain profile with tension cycles lasting 2 s each (0.5 Hz). The elongation was applied as a sinusoidal curve with a maximum displacement of 10%. The stimulation was repeatedly applied for 8 h and followed by 4 h rest. While stretching, the cultures were kept in an incubator for cell culture (37°C, 5% CO_2). Control cultures were grown under the same conditions but without the strain protocol.

Assay for total collagen and glycosaminoglycan (GAG) synthesis

After mechanical stimulation for 24 h, total collagen and GAG synthesis were measured in cell culture supernatants in triplicates by the Sircol dye assay (Biocolor, Newtownabbey, UK) according to the manufacturer's instructions. Absorbance was measured at 555 nm. The calibration curve was set up on the basis of a collagen standard provided by the manufacturer.

GAG amount was measured using the Blyscan 1,9-dimethylmethylene blue (DMMB) assay kit (Biocolor) according to the manufacturer's instructions. Absorbance was measured at 656 nm. The calibration curve was set up on the basis of a collagen standard provided by the manufacturer.

Statistical analysis

RT-qPCR as well as ELISA experiments were performed in duplicates. Data were analyzed by the Shapiro–Wilk test for normality and presented as mean (±standard deviation) or as median and the 25th and 75th percentile. Comparison of two groups was accomplished by use of a two-sample *t*-test (parametric data) or with Mann–Whitney rank sum test (non-parametric data). For multiple comparisons the one way analysis of variance (ANOVA) followed by the Holm–Sidak test was used. Probability values smaller than 0.05 were considered significant. Data analysis was performed with the SigmaPlot software (Systat Software Inc., Erkrath, Germany).

Results

IL-1β changed the expression of IL-6, IL-8 and MMP-13 in C28/I2 cells

To characterize their inflammatory state, C28/I2 cells were treated with IL-1β over a period of 3 days and the influence on biomarkers involved in inflammation was measured by qPCR.

The expression of MMP-13 was increased 8.7 (4.9, 9.8) fold (median (25th, 75th percentile)), IL-6 3.2 (2.4, 4.7) fold and the expression of IL-8 by a factor of 15.6 (14.0, 22.9), whereas MMP-1 was reduced to 52% (21%, 72%) and MMP-3 to 39% (30%, 56%) of their original values [Fig. 1(A)]. IL-1β induced the augmentation of IL-6 (46.2 ± 2.9 ng/ml vs control 5.1 ± 0.5 ng/ml) and IL-8 (150.5 ± 22.8 ng/ml vs control 4.2 ± 0.5 ng/ml) [Fig. 1(B)]. The increase of IL-8 by IL-1β stimulation was 5-fold more pronounced than for IL-6. The high basal expression of MMP-1 (22.9 ± 6.4 ng/ml) at the protein level explains the small relative change of MMP-1 messengerRNA (mRNA) (0.5 fold) when cells were exposed to IL-1β [Fig. 1(C)]. Protein concentrations for MMP-3 and MMP-13 differed only slightly from control values.

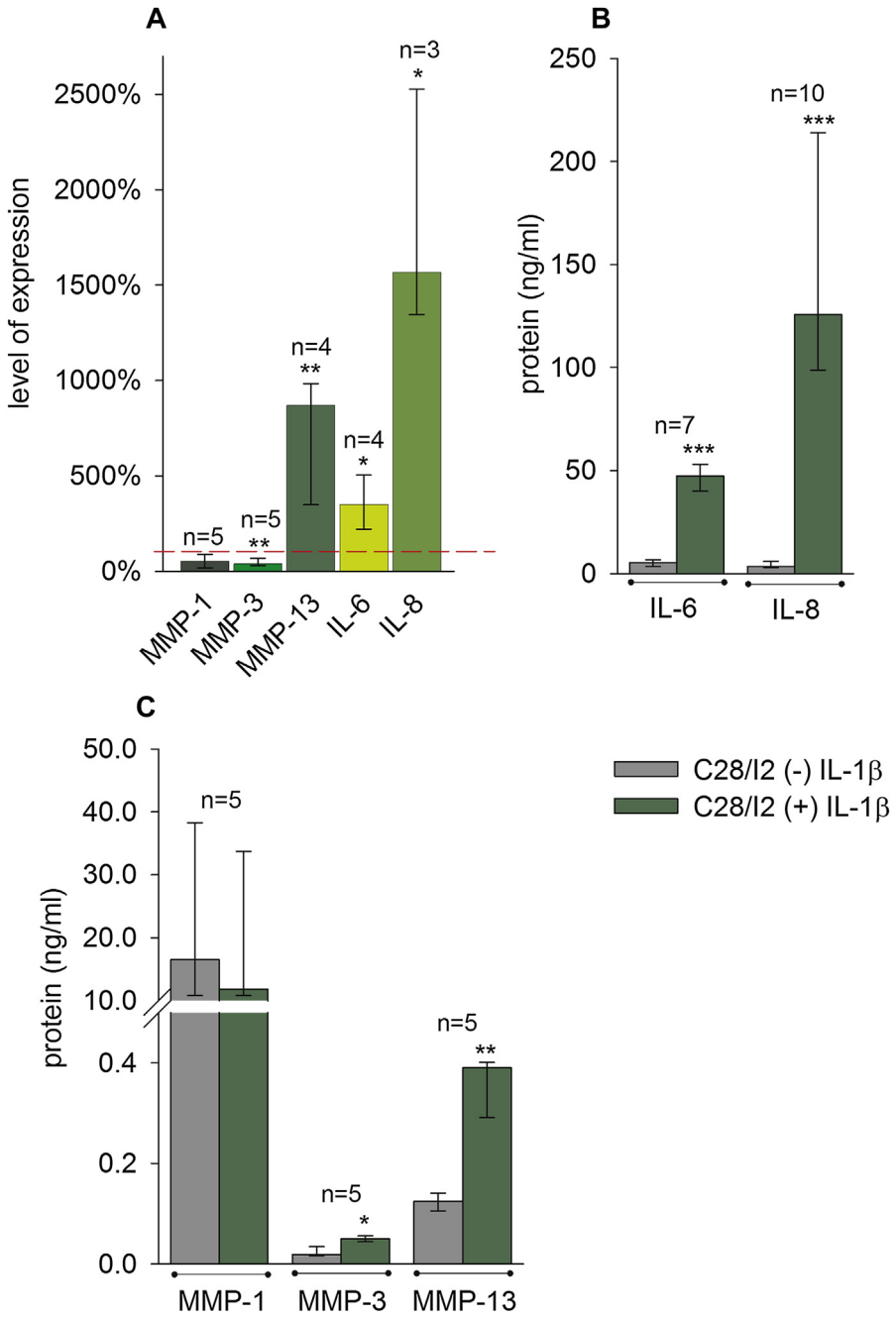

Fig.1. Changes of gene expression in C28/I2 cells under inflammatory conditions. (A) The expression of the matrix metalloproteinases MMP-1, -3, -13 and the interleukins IL-6 and IL-8 were analyzed at RNA and protein levels from C28/I2 chondrocytes treated with IL-1β over a period of 3 days. All three bar charts represent the median values with the 75th and 25th percentile. Gene expression analysis was performed by qPCR. The bar chart under (A) represents the level of gene expression under IL-1β (10 ng/ml) when compared to controls. (B) The concentrations of IL-6 and IL-8 were determined by ELISA and (C) for MMP-1, -3 and -13 by the Fluorokine MAP Human MMP Kit and Luminex technique. Significance levels are given by *P* values of the Mann–Whitney Rank Sum Test (*: $P < 0.05$; **: $P < 0.01$; ***$P < 0.001$); *n* is the number of experiments; all measurements were performed in duplicates. The 100% value for no change is marked by the dashed line.

The impact of diacerein on the viability and proliferation of C28/I2 cells

The influence of different concentrations of diacerein on cell viability and proliferation over a period of 24 h was tested with the MTS tetrazolium assay. Cells grown in the presence of 100 μM diacerein for 24 h demonstrated a significant reduction in cell viability by 28.6 ± 1.07% [Fig. 2(A)].

Analysis of cell proliferation monitored in real time revealed no changes for 1 μM or 10 μM but for 100 μM diacerein compared to control conditions as demonstrated by the CI [Fig. 2(B)]. The influence of IL-1β in combination with different concentrations of diacerein was also tested [Fig. 2(C)]. The addition of IL-1β changed the CI only at concentrations of 30 μM and 100 μM diacerein [Fig. 2(C)]. Lower concentrations of diacerein were without effects. Accordingly, 100 μM diacerein caused an increase of the calculated

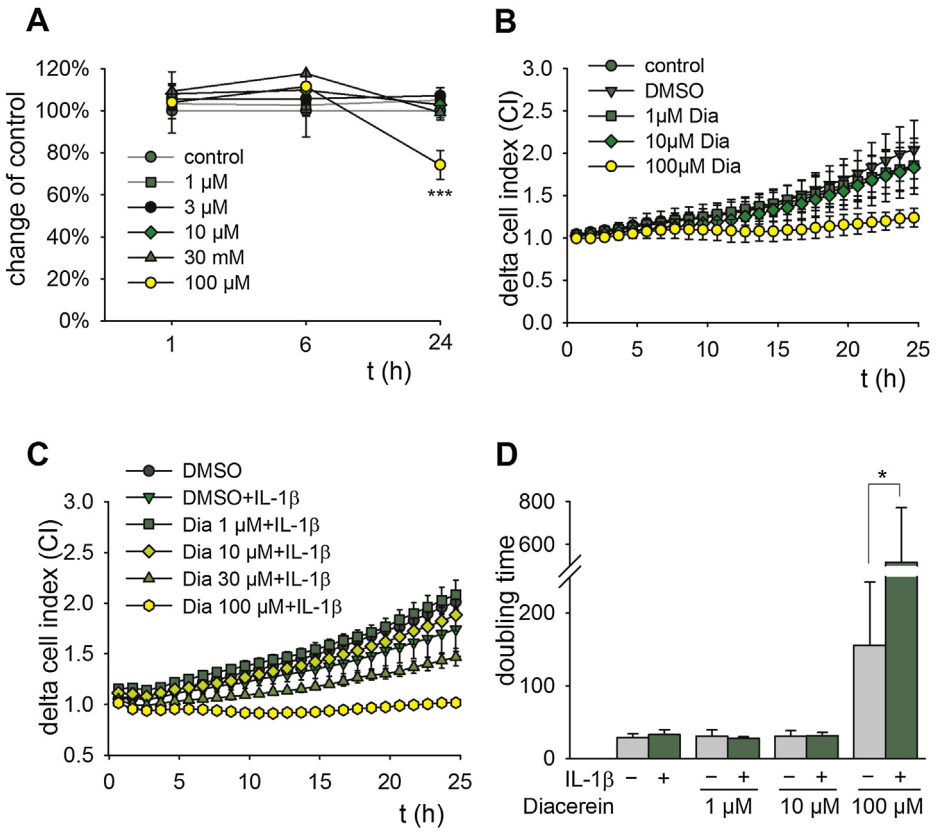

Fig. 2. Viability and cell proliferation of C28/I2 cells treated with different concentrations of diacerein. (A) Viability of cells treated with different concentrations of diacerein was assessed with the MTS assay. Shown are the mean values and standard deviations of four experiments whereas per experiment each value was determined in quadruplicates. (B)The influence of different concentrations of diacerein on cell growth was measured in real time with an impedance based cell proliferation assay (xCELLigence) over a period of 24 h. Mean values ± standard deviations of the normalized CI of four experiments with 3–5 fold determinations are given. (C) Time course of the CI with IL-1β treatment in the presence of different concentrations of diacerein. (D) From the dynamic monitoring of cell proliferation the doubling time was calculated (mean ± standard deviation, $n = 7–9$). Level of significance (*: $P < 0.05$) was determined by the Student's t test.

doubling time from 28 ± 2.3 h to 154 ± 35 h in the absence of IL-1β and from 32.7 ± 2.6 h to 517.3 ± 146 h in the presence of IL-1β [Fig. 2(D)].

Changes in gene expression by diacerein and mechanical stimulated C28/I2 cells

To elicit an inflammatory state at the cellular level chondrocytes were grown in the presence of IL-1β. Mechanical stimulation slightly increased the expression of IL-6 by 20% and significantly decreased the expression of IL-8 to 84% (77%, 92%) of control [Fig. 3]. Diacerein at a concentration of 100 µM had no additional effect on IL-6 but further reduced the pro-inflammatory modulator IL-8 to 58% (55%, 62%) of its control value. Even though in IL-1β treated cells, the expression of NF-κB was not changed under mechanical stimulation, the addition of diacerein produced a 1.5 fold (to 154% (134%, 238%)) increase. While expression of the transforming growth factor beta (TGF-β) was not influenced, induction in expression of the mitogen-activated protein kinase 8 (JNK1) was demonstrated when the cells were simultaneously stimulated mechanically and with diacerein (to 198% (175%, 213%), Fig. 3).

Influence on MMP-1 activity and GAG production of chondrocytes

To evaluate the influence of diacerein and the mechanical stimulation separately and as a combined treatment modality, the

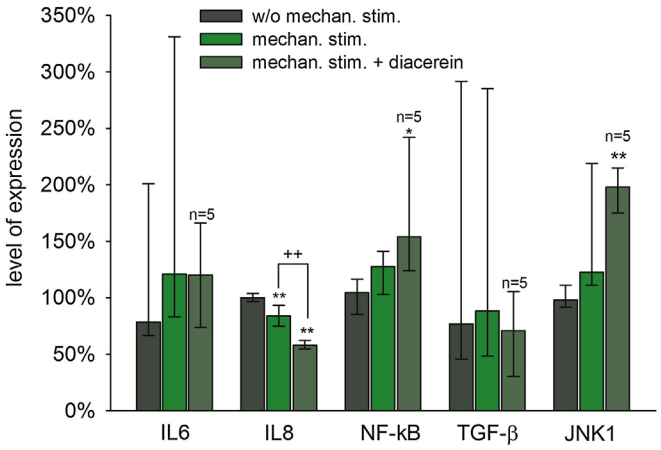

Fig. 3. Influence of diacerein and mechanical stimulation on gene expression under inflammatory conditions. C28/I2 cells were grown on pronectin coated six-well BioFlex plates in the presence of IL-1β. The bar chart represents the qPCR data as for the level of expression in mechanical stimulated and mechanical stimulated plus diacerein (100 µM) treated chondrocytes compared to control. The single bars represent the median and the 75th and 25th percentile. Percent values are based on the ratio values which were calculated by normalization to the results gained from cells treated only with IL-1β. Statistic evaluation is based on differences in ΔCt values. Significance levels evaluated by the Mann–Whitney Rank Sum Test are *: $P < 0.05$; **: $P < 0.01$; ++: $P < 0.01$. Cells grown with IL-1β and without mechanical stimulation or diacerein served as control.

production of GAG and the activity of MMP-1 were measured. Cell supernatants of control chondrocytes stimulated only with IL-1β were compared to supernatants of cells which were grown in IL-1β containing medium and stimulated mechanically or stimulated in the presence of diacerein. The application of diacerein in the absence of mechanical stimulation induced GAG production (29.5 ± 3.9 μg/ml) compared to control values (1.9 ± 0.7 μg/ml). Mechanical stimulation had no influence on diacerein induced production of GAG. Cell supernatants of cells treated with diacerein and mechanically displayed 26.1 ± 3.8 μg/ml GAG (control: 0.76 ± 0.65 μg/ml, Fig. 4(A)). The MMP-1 activity was unchanged by the IL-1β treatment in C28/I2 cells; IL-1β combined with diacerein significantly reduced MMP-1 activity to 57% [Fig. 4(B)]. Under mechanical stimulation even though IL-1β had no effect, the presence of diacerein decreased the MMP-1 activity by 54%. This decrease was less marked in cells additionally stimulated mechanically (1.2 ± 0.05 ng/ml) when compared to cells without mechanical treatment (0.94 ± 0.05 ng/ml).

Regulation of the ECM by mechanical stimulation and diacerein

Cells were mechanically stimulated with and without diacerein in the presence of IL-1β for 24 h. The mechanical stimulation significantly reduced both collagen type XII (Col 12) and fibronectin (FN1) expression levels by 25–30%. Diacerein further augmented these effects to 42% for Col 12 and to 55% of control for FN1 [Fig. 5]. A decrease in collagen type I (Col 1) and MMP-1 by 50%, integrin-β1 (ITG-β1) by 19 ± 9% as well as, an increase in collagen type II (Col 2) by 54 ± 40% were only detectable when mechanical stimulation was combined with the diacerein treatment. The mechanical stimulation alone induced significant changes only for FN1 and Col 12 [Fig. 5].

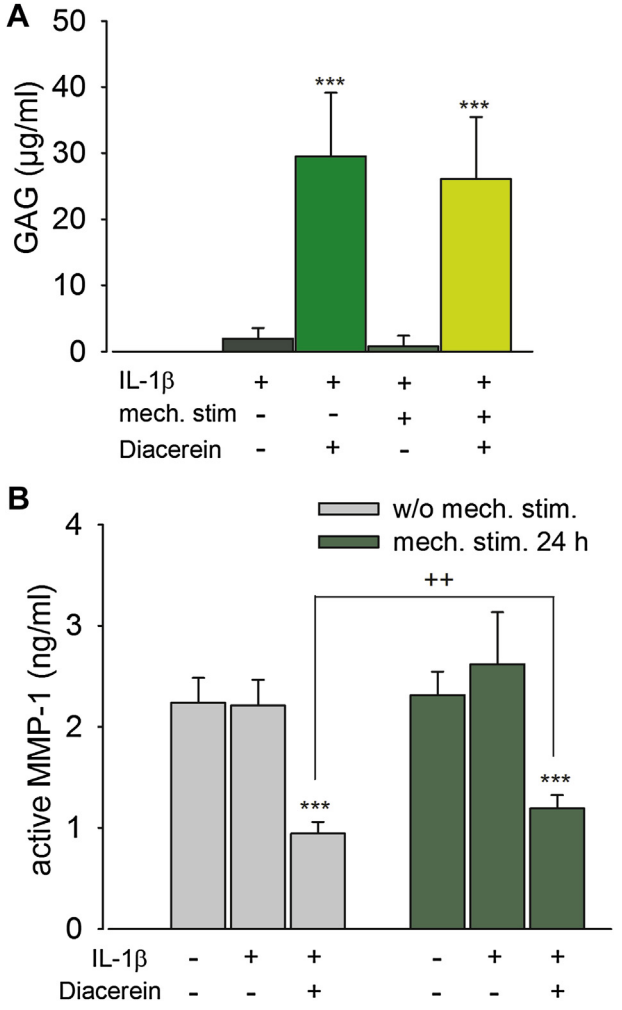

Fig. 4. GAGs content and matrix metalloproteinase-1 (MMP-1) activity changes by the treatment with diacerein. (A) Cell supernatants of C28/I2 cells were analyzed for the GAG content and in addition, (B) the activity of MMP-1 was measured. Data are given as mean ± standard deviation, ***$P < 0.001$, ++: $P < 0.01$.

Changes in expression of interleukin-6 receptor (IL-6R), fibroblast growth factor receptor (FGFR) and vascular endothelial/growth factor-A (VEGF-A) by diacerein and mechanical stimulation

The expression levels of important receptors involved in inflammatory signal transduction were determined under diacerein and/or mechanical stimulation. Both the expression of the IL-6R and the FGFR were up regulated more than 2 fold by diacerein. This up regulation was independent of the mechanical stimulation [Fig. 6]. In contrast, the expression of the EGFR appeared to be down regulated by the mechanical stimulation by 30% [Fig. 6]. Diacerein without mechanical stimulation induced the up regulation of the vascular endothelial growth factor-A (VEGF-A) to 270 ± 20%, a change to 230 ± 18%was detected under diacerein plus mechanical stimulation [Fig. 6].

Discussion

The mechanical stimulation applied to IL-1β treated chondrocytes consisted of a dynamic tensile stretch protocol. Whether this simplified mechanical model represents the actual physiological stress situation in articular cartilage is unknown. Chondrocytes in the superficial layers of the cartilage are exposed to tensile strain and a frequency of 0.5 Hz is typical for jogging or fast walking and it is conceived that stretching of the chondrocytes is a mechanical input leading to initiation of downstream signaling events and adaption of cells and cartilage to mechanical stress[35]. Dynamic compression[36] as well as hydrostatic pressure[37] has been shown to increase expression of aggrecan and Col 2. We did not observe an increase in Col 2 due to our mechanical loading protocol but this could also reflect an effect of the IL-1β treatment.

C28/I2 human chondrocytes under IL-1β changed into an inflammatory state, demonstrated by an increase in expression of the pro-inflammatory factors IL-6, IL-8 and MMP-13. IL-6 levels have been demonstrated to be increased significantly in the synovial fluid of patients with rheumatoid arthritis (RA) or OA[38], IL-8 is known for its role in inflammation and plays a critical part in developing pain[39]. MMP-1 and MMP-13 function as interstitial collagenases and are well characterized to degrade type II collagen in cartilage, a crucial step in the progression of OA. Regarding these pro-inflammatory factors diacerein preserved its chondroprotective properties even under mechanical stimulation demonstrated by down regulating the expression of MMP-1, IL-6 and IL-8.

The applied concentration of diacerein (10^{-4} M) reduced the cell viability to 30% while in accordance with the slowdown of cell growth both facts point to diacerein modulating cellular physiology. Rhein, the metabolite of diacerein, has been demonstrated to induce anti-catabolic and anti-proliferative effects on chondrocytes stimulated by IL-1β at similar concentrations (10^{-5} M and 10^{-4} M). Both effects were explained by alterations in cell cycle regulation and not by induction of apoptosis[40]. In our observation, the increase in the doubling time of diacerein treated cells was more pronounced in cells under IL-1β treatment, suggesting that diacerein is more effective under inflammatory conditions. A higher sensitivity of cells in an inflammatory state against the toxic effects of

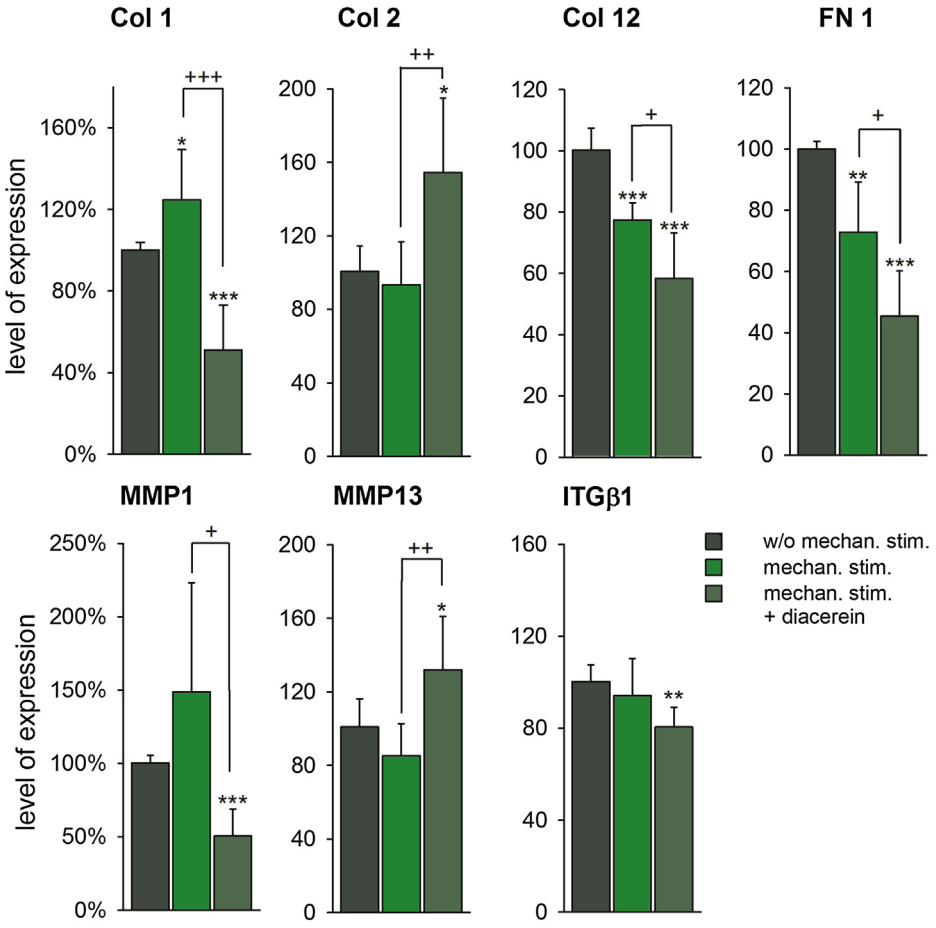

Fig. 5. Changes in expression of relevant genes controlling ECM as tested by qPCR. RNAs of the three different groups: IL-1β alone, IL-1β/mechanical stimulation and IL-1β/mechanical stimulation/diacerein (100 μM) were used to perform qPCR reactions. Controls for calculating ratio values were C28/I2 cells not mechanically or diacerein stimulated but grown in the presence of IL-1β. Levels of expression compared to control are given in percent. Changes of the expression profile of Col 1, Col 2, Col 12, fibronectin (FN-1), metalloproteinases MMP-1, and -13 as well as ITG-β1 are given. Statistical evaluation by the Student's *t* test is based on changes in ΔCt values (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$). Significant changes induced through diacerein application under mechanical stimulation are marked by +: $P < 0.05$; ++: $P < 0.01$; +++: $P < 0.001$.

diacerein might be the case. The increase in IL-6 expression seen under mechanical stimulation may reflect an irritating effect which is completely blunted by diacerein and could be part of the anti-inflammatory effects of diacerein. The mechanical as well as the diacerein induced decrease in IL-8 expression coincides further with findings that physiological mechanical signals antagonize effects of catabolic mediators involving pro-inflammatory cytokines and transcription factors[24]. In articular chondrocytes diacerein has been found to antagonize the IL-1β triggered extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK) and p38 kinase pathways, for their part activating NF-κB and AP-1[15,16]. Interestingly, magnitude-dependent signals of mechanical strain utilize the NF-κB transcription factors as common elements to abrogate or aggravate pro-inflammatory responses[41]. Although its transcriptional up regulation is not equivalent to its activation in a signaling pathway, the observed effects indicate an increased turnover and interference of diacerein with this pathway. However, the down regulation by diacerein of the inflammatory cytokines IL-6 and IL-8, which are NF-κB target genes, does not suggest that activation of the NF-κB pathway is an important element of the diacerein effect.

The TGF-β expression was unchanged even though diacerein has been reported to stimulate cartilage repair by up-regulating the expression of TGF-β1 and β2 even in the absence of IL-1β[17].

The progressive degeneration of ECM components is one of the hallmarks of OA. Independent of the mechanical stimulation, diacerein increased the concentration of GAGs in the cell culture supernatant of chondrocytes which might contribute to the recovery of damaged cartilage.

The ability of diacerein in antagonizing the inflammatory activity of IL-1β is further reflected by the strong substances-induced reduction of the MMP-1 activity even in the presence of IL-1β and under mechanical stimulation. Diacerein decreased the MMP-1 activity whereas the mechanical stimulation slightly attenuated this reduction. The expression of MMP-1 and of MMP-13, two major enzymes responsible for Col 1 breakdown, has already been found to be down regulated by diacerein[14,15]. In addition to a reduced collagen proteolysis, in diacerein treated cells also the mRNA level of Col 2 was increased, accompanied by a reduction in Col 1 expression[12,42]. Under diacerein treatment combined with mechanical stimulation the same converse effect was observed, indicating that mild mechanical stimulation does not negatively influence the anabolic action of diacerein to ECM.

In OA, the fibronectin content is markedly increased in the altered matrix due to an increased synthesis by chondrocytes[43]. Proteolytic fragments of fibronectin could stimulate the catabolism of articular cartilage thus, promoting the degeneration of cartilage in OA[44]. Mechanical stimulation and the diacerein treatment could exert their antirheumatic effect by a reduction in the expression of fibronectin.

Mild mechanical stimulation up-regulated Col 1 and MMP-1. These potentially pro-inflammatory effects of the mechanical

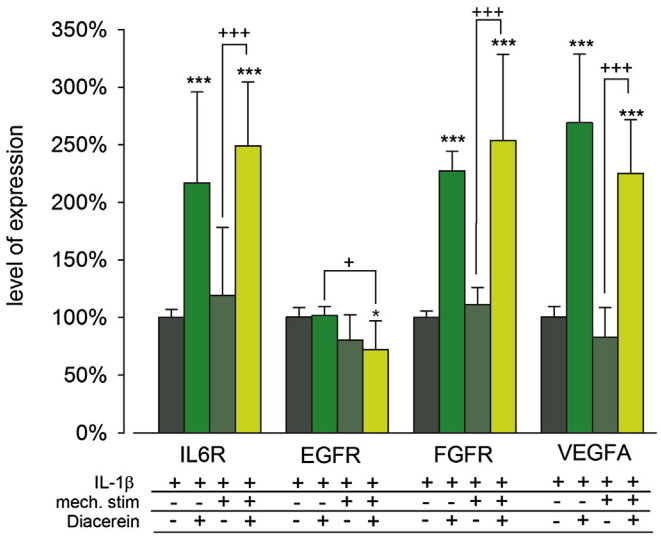

Fig. 6. Increased expression of IL-6R, FGFR and VEGF-A under non-mechanical and mechanical stimulation of C28/I2 cells by diacerein. Cells were stimulated with IL-1β alone, IL-1β/diacerein (100 μM), IL-1β/mechanical stimulation and IL-1β/mechanical stimulation/diacerein (100 μM) for 24 h; the isolated RNA was used for qPCR reactions. Changes of the expression of IL-6R, epidermal growth factor receptor (EGFR), FGFR and vascular endothelial growth factor A (VEGF-A) are shown. Data are given as mean ± standard deviation. Controls for calculating ratio values were C28/I2 cells not mechanically or diacerein stimulated but grown in the presence of IL-1β. Statistical evaluation by one way ANOVA and the Holm–Sidak test with *: $P < 0.05$; ***: $P < 0.001$; and +: $P < 0.05$; +++: $P < 0.001$.

stimulation on the cellular level however, were counteracted by the diacerein treatment. A possibly important effect because diacerein also increased the expression of the IL-6 receptor, thus, sensitizing cells for the effect of IL-6. On the other hand an increase in expression of the potentially pro inflammatory factors MMP-13 and VEGF-A induced by diacerein was hardly modulated by the mechanical stimulation. A synergistic negative regulatory effect was observed on Col 12, fibronectin and to a lesser extent on the fibronectin receptor ITGβ1 so that one would expect a mutual amplification of the effects of mechanical stimulation and diacerein treatment. The up regulation of the FGFR3 expression by diacerein alone and under mechanical stimulation might contribute to the increase of GAGs due to the fact that FGFR3 and its selective ligand FGF18 were identified to stimulate proteoglycan synthesis[45]. The down regulation of EGFR by the combined diacerein and mechanical stimulation may contribute to its anti-inflammatory effect since under inflammatory conditions EGFR is overexpressed in RA synovial tissues[46].

The aim of our study was to test whether the combination of two methods involved in OA treatment differs from applications with only a single method and how diacerein treatment interferes with effects of the mechanical stimulation. In order to first achieve insights into affected molecular mechanisms we performed our study at the cellular level.

Taken together diacerein treatment, even in the presence of mechanical stimulation could have a chondroprotective effect. These data for the first time indicate that mechanical tension and diacerein treatment applied in combination might be beneficial in OA treatment but this has to be shown in clinical trials. Additionally, due regard must be given to the fact that our test system is an immortalized cell line and clinical studies for conformation are of profound necessity.

Authors' contributions

Bibiane Steinecker-Frohnwieser made substantial contributions to the conception and design of the project, the acquisition, analysis and interpretation of the data, drafted the article and approved the version to be submitted. Lukas Weigl participated in study's design and interpretation of the data, revised the article for important intellectual content and contributed to the final approval of the article. Werner Kullich contributed to the conception and design of the project, revised the article critically and approved the version to be submitted. Birgit Lohberger was involved in acquisition of the data, revising the article and the final approval of the version to be submitted. Werner Kullich head of the Boltzmann Institute Saalfelden (lbirehab@aon.at) takes responsibility for the integrity of the work, from inception to finished article.

Role of the funding source

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Competing interest statement

The authors declare that they have no competing interests.

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