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Pharmacological treatment with diacerein combined with mechanical stimulation affects the expression of growth factors in human chondrocytes

Bibiane Steinecker-Frohnwieser[a,*], Heike Kaltenegger[b], Lukas Weigl[c], Anda Mann[c], Werner Kullich[a], Andreas Leithner[b], Birgit Lohberger[b]

[a] Ludwig Boltzmann Department for Rehabilitation of Internal Diseases, Ludwig Boltzmann Cluster for Arthritis and Rehabilitation, Thorerstrasse 26, 5760 Saalfelden, Austria
[b] Department of Orthopaedic Surgery, Medical University of Graz, Graz, Austria
[c] Department of Special Anaesthesia and Pain Therapy, Medical University Vienna, Austria

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ABSTRACT

Background: Osteoarthritis (OA) as the main chronic joint disease arises from a disturbed balance between anabolic and catabolic processes leading to destructions of articular cartilage of the joints. While mechanical stress can be disastrous for the metabolism of chondrocytes, mechanical stimulation at the physiological level is known to improve cell function. The disease modifying OA drug (DMOAD) diacerein functions as a slowly-acting drug in OA by exhibiting anti-inflammatory, anti-catabolic, and pro-anabolic properties on cartilage. Combining these two treatment options revealed positive effects on OA-chondrocytes.

Methods: Cells were grown on flexible silicone membranes and mechanically stimulated by cyclic tensile loading. After seven days in the presence or absence of diacerein, inflammation markers and growth factors were analyzed using quantitative real-time PCR and enzyme linked immune assays. The influence of conditioned medium was tested on cell proliferation and cell migration.

Results: Tensile strain and diacerein treatment reduced interleukin-6 (IL-6) expression, whereas cyclooxygenase-2 (COX2) expression was increased only by mechanical stimulation. The basic fibroblast growth factor (bFGF) was down regulated by the combined treatment modalities, whereas prostaglandin E2 (PGE2) synthesis was reduced only under OA conditions. The expression of platelet-derived growth factor (PDGF) and vascular endothelial growth factor A (VEGF-A) was down-regulated by both.

Conclusions: From our study we conclude that moderate mechanical stimulation appears beneficial for the fate of the cell and improves the pharmacological effect of diacerein based on cross-talks between different initiated pathways.

General significance: Combining two different treatment options broadens the perspective to treat OA and improves chondrocytes metabolism.

1. Introduction

Chondrocytes, as the major cellular compound of the articular cartilage, are constantly confronted with and exposed to a combination of different forces including compression, tension, and shear. Consequently, the resulting mechanical signals act on articular cartilage and therefore represent critical regulators of tissue adaptation, structure, and function [1]. Clinical studies are in line with these findings and postulate that altered mechanical load represents a major risk factor for osteoarthritis (OA) [2,3]. OA, as the main clinical condition affecting joint structure and function, concerns almost forty percent of adults over the age of sixty and causes significant restrictions in the quality of life [4,5]. While excessive or abnormal joint loading patterns can initiate cartilage pathology [6], physical exercise alleviates OA symptoms due to an increase in upper leg strength, a decrease of extension impairments and improvement in proprioception [7–9]. It has been demonstrated that the physiological mechanical loading of joints in OA patients acts as an effective non-drug treatment modality by increasing cartilage thickness [10,11].

Interestingly, even though the prevalence of OA is very high [12], treatment options comprise a limited combination of pharmacological and non-pharmacological therapies aimed at pain reduction and improvement in functionality [13]. Current pharmacologic treatment paradigms for OA like analgesics and nonsteroidal anti-inflammatory

* Corresponding author.
E-mail address: lbi.groebming@aon.at (B. Steinecker-Frohnwieser).

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drugs (NSAIDs) only provide symptomatic relief; neither a remission nor a stop in the progression of the disease can be achieved.

The disease modifying OA drug diacerein functions as a slowly-acting drug in OA by exhibiting anti-inflammatory, anti-catabolic, and pro-anabolic properties on cartilage and synovial membrane [14,15]. The ESCEO regards diacerein as beneficial for the treatment of OA [16,17].

Based on recent findings which describe OA as a heterogeneous disease, combining therapy strategies could more efficiently counteract the progression of this disease [18].

The application of the disease modifying OA drug (DMOADs) diacerein on mechanical stimulated chondrocytes as a non-drug treatment modality could provide a useful combined treatment modality. A change in metabolic activity of chondrocytes might be the consequence. *In vitro*, cell stretching instruments encompass the possibility, in a controlled and defined manner, to cyclically strain cells grown in monolayer on flexible-bottomed devices.

Although, IL-1β signaling and inflammatory processes are in the focus of research, the regulation of growth factors in treating local cartilage defects and/or OA appears promising since the dysregulation of growth factor signaling plays an important role in the pathogenesis of OA [19]. Thus, the interplay of growth factors with different possible treatments could open new options for the diagnosis and therapy of the disease [20].

The aim of this study was to analyze the expression of growth factors and inflammation markers of non-OA and OA chondrocytes in response to tensile strain and diacerein treatment.

2. Material and methods

2.1. Cell culture

The immortalized human chondrocyte cell lines T/C-28a2 and C-28/I2, originated from rib cartilage of one donor, were used to ensure the best scientific comparability and have become a common tool in cartilage research [21]. C-28/I2 cells exhibit a higher expression of matrix-degrading proteases and the pro-inflammatory cytokine IL-8, wherefore C-28/I2 stimulated with 10 ng/ml IL-1β embodied the model for OA throughout our experiments [22,23]. T/C-28a2 cells have been used as non-OA comparison group.

Cells were seeded at 9.3×10^3 cells per cm^2 and cultured using Dulbecco's modified eagle's medium (DMEM high glucose; GIBCO, Invitrogen, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (all GIBCO, Invitrogen). Cells were kept at 37 °C in a humidified atmosphere of 5% CO_2 and passaged by detaching with Accutase (Sigma-Aldrich, Vienna, Austria).

2.2. Mechanical stimulation of chondrocytes

The Flexcell FX5K-Tension System (Flexcell International Corp, Hillsborough, US) was used to apply mechanical cyclic tensile stretch on chondrocytes. The FX5K is a computer-based system that uses vacuum to strain cells adhered to flexible silicon membranes (BioFlex plates). The deformation of the flexible bottom of the plates causes the attached cells to deform (Fig. 1A). Chondrocytes were seeded (5×10^4 cells/well) onto six well pronectin-coated BioFlex plates. After incubating the cells overnight, cells were subjected to a strain profile consisting of 8 h resting and four repetitions of alternate 2 h slow-moving activity (0.2 Hz, 2% elongation) and 2 h high-intensive activity (1 Hz, 15% elongation). The mechanical stimulation was applied for 7 days. Control cultures were grown under the same conditions but without strain.

The reason why using one loading condition is based on our previous studies were we titrated and optimized the treatment with diacerein. From these investigations, tested via the LDH assay, we concluded that the used concentration is not toxic and that these conditions are optimal to use in cell cultures.

2.3. Lactate dehydrogenase assay

After three and seven days of mechanical stimulation lactate dehydrogenase (LDH) activity was measured using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega, Mannheim, Germany). The amount of measured fluorescence is proportional to the number of lysed cells. After three, and seven days of cultivation, cell culture supernatants were collected and analyzed to examine the state of cellular damage. Fluorescence was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm (Fluostar; BMC Labtech, Ortenberg, Germany). Cells treated with a 0.9% (weight/volume) solution of Triton X-100 in water worked as maximum LDH release control. Culture medium served as zero adjustment.

2.4. Enzyme immuno assay ELISA

Ready-to-use Sandwich ELISAs for human platelet-derived growth factor (PDGF; Abcam, Cambridge, UK), human prostaglandin E2 (PGE2; Abcam), and human vascular endothelial growth factor (VEGF, Abcam) were used to quantify the growth factors. After seven days of treatment supernatants were used undiluted or diluted as required from 1:1 to 1:10 fold and proceeded according to the manufacturer's instruction. All measurements were performed in duplicates at 450 nm with a Spectrostar microplate reader (BMC Labtech, Ortenberg, Germany).

2.5. Real-time RT-PCR

Total RNA was isolated from treated and untreated cells with the RNeasy Mini Kit and DNaseI treatment according to the manufacturer's manual (Qiagen, Hilden, Germany). One µg of RNA was reverse transcribed with the iScriptcDNA Synthesis Kit, (BioRad, Hercules, US) using a blend of oligo(dT) and hexamer random primers for 30 min at 37 °C. Each qPCR run consisted of a standard 3-step PCR temperature protocol followed by a melting curve protocol. Primers used for real-time PCR were designed from sequences available in the database (http://pga.mgh.harvard.edu/primerbank) and listed in Table 1. Amplification was achieved with the RealMasterMix SYBR ROX (5′ Prime, Hamburg, Germany) on a realplex mastercycler (Eppendorf, Hamburg, Deutschland); reactions were performed in duplicates.

Relative quantification of expression levels were obtained by the ΔΔCt method based on the geometric mean of the internal controls GAPDH, aldolase, and ETIF3. The expression level (C_t) of the target gene was normalized to the reference genes (ΔC_t) and the ΔC_t of the test sample was normalized to the ΔC_t of the control ($\Delta\Delta C_t$). Finally, the expression ratio was calculated with the $2^{-\Delta\Delta Ct}$ method. This does not allow absolute quantifications but is based on the expression levels of a target gene versus a housekeeping gene (reference or control gene), a method that is adequate for most purposes to investigate physiological changes in gene expression levels [24]. Since the expression of housekeeping genes in different cell lines may differ, it is not possible to make absolute comparisons.

2.6. xCELLigence cell proliferation/migration assay

The xCELLigence RTCA (ACEA Bioscience, San Diego, US) was used to monitor cell proliferation and cell migration in real-time. For monitoring cell proliferation cells were seeded on electronic microtiter plates (E-Plate), whereas migration was tested on cells seeded on electronic cell invasion and migration plates (CIM-Plate). Cells were treated with conditioned medium (seven days of mechanical stimulation) mixed 1:1 with fresh culture medium and the cell index was measured for 60 h. Cell density measurements were performed in triplicates with signal detection every 20 min. The normalized cell index

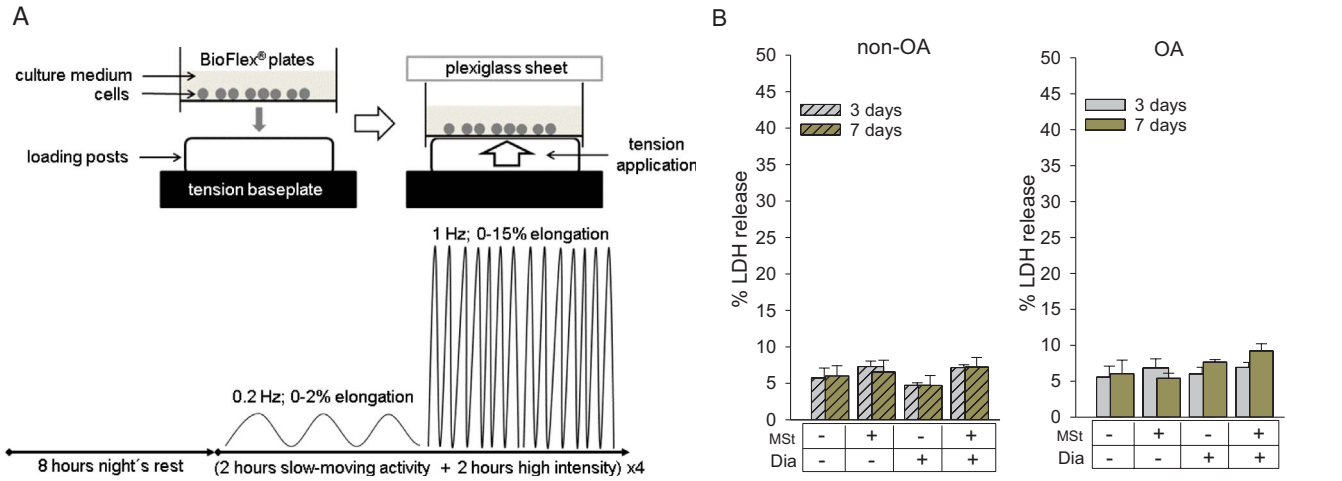

Fig. 1. Profile for the mechanical stimulation and test for treatment's cytotoxicity. A) Chondrocytes were mechanically stimulated via a vacuum applied to the flexible-bottom of the BioFlex culture plates. The mechanical treatment over a period of seven days consisted of a sequence of slow-moving activity (0.2 Hz, 0–2% elongation) for two hours followed by a two hours period of high intense treatment (1 Hz, 0–15% elongation). B) The lactate dehydrogenase (LDH) activity as a measure for damaged cells was assessed by fluorescence measurement at 560 nm (MSt: mechanical stimulation; Dia: 50 μM diacerein).

Table 1
Primer Sequences Used for Real-Time PCR.

Target gene	Primers	Oligonucleotide sequence	Product size (bp)
COX2	forward	5′GGCTTCCATTGACCAGAGCAG3′	194
	reverse	5′GCCGAGGCTTTTCTACCAGA3′	
IL−6	forward	5′TGACAAACAAATTCGGTACATCCT3′	102
	reverse	5′TCTGCCAGTGCCTCTTTGCT3′	
bFGF2	forward	5′ AGAAGAGCGACCCTCACATCA3′	237
	reverse	5′ACTGCCCAGTTCGTTTCAGTG3′	
PDGFa	forward	5′CCAGCGACTCCTGGAGATAGA3′	169
	reverse	5′CGTCCTGGTCTTGCAGACAG3′	
VEGFA	forward	5′CGCAGCTACTGCCATCCAAT3′	192
	reverse	5′GTGAGGTTTGATCCGCATAATCT3′	
GAPDH	forward	5′TGATGACATCAAGAAGGTGGTGAAG3′	102
	reverse	5′TCCTTGGAGGCCATGTGGGCCAT3′	
aldolase	forward	5′ATGAGTCCACTGGGAGCATTG3′	209
	reverse	5′ACCGCCCTTGGATTTGATAAC3′	
ETIF3	forward	5′CTACCAGCCGTTCAGCAAAG3′	110
	reverse	5′CACCACCAAACTGAGAGGAGT3′	

COX2: cyclooxygenase-2; IL-6: interleukin-6; bFGF2: basic fibroblast growth factor 2; PDGFa: platelet-derived growth factor A; VEGFA: vascular endothelial growth factor A; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ETIF3: eukaryotic translation initiation factor 3.

(CI) is a measure for the density of cells. Acquisition and analysis were performed with the RTCA software (Version 1.2, Roche Diagnostics). Cell migration was performed in CIM plates containing electronically integrated Boyden chambers that enables the gathering of quantitative kinetic data for migration in real-time and without the use of labels. As cells move from the upper chamber towards the lower chamber they pass through a membrane containing 8 μm pores and then adhere to gold impedance microelectrodes. Migration through the membrane was induced by 10% FBS as a chemoattractant placed in the lower chamber. The resultant change of the impedance of the microelectrodes correlates with the number of cells migrated through the membrane.

2.7. Statistical analysis

Data from RT-qPCR are presented as the median values (25th percentile/75th percentile) of at least four single experiments, whereby RT-qPCR as well as ELISA experiments were performed in duplicates. Statistical significance was determined by the two-sample Student *t*-test (parametric data), if normality test failed, the Mann-Whitney Rank Sum Test (non-parametric) was used and particularly cited in the figure legends. P-values < 0.05 are considered to be significant (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$), all p-values are two-sided. Data analysis and display were performed with the SigmaPlot software (Systat Software Inc., Erkrath, Germany).

3. Results

3.1. Treatment modalities and their influence on cytotoxicity

Moderate mechanical stimulation for seven days was applied to non-OA and OA chondrocytes by use of the FX5K-tension system. Diacerein at a concentration of 50 μM was applied to the mechanical stimulated and unstimulated cells. Neither the mechanical stimulation nor the diacerein treatment - applied separately or in combination - exhibited any cytotoxic effect (Fig. 1B).

3.2. Modulation at the mRNA levels of mediators of inflammation and FGF-2

To evaluate the inflammatory background dependent on the treatment modalities, the relative mRNA expression levels for the two inflammatory markers, IL-6 and COX2, were analyzed. While untreated control cells served as reference (ratio = 1), the expression of the cytokine IL-6 was significantly down-regulated in the presence of diacerein both, in non-OA cells (Dia: 0.3, 25th percentile: 0.2/ 75th percentile: 0.4, $p < 0.001$) and OA chondrocytes (Dia: 0.6, 0.5/0.7, $p < 0.001$) (Fig. 2A). The observed effect on cells under mechanical stimulation alone (without diacerein) was weaker (MSt: non-OA, 0.6, 0.2/0.9, $p = 0.003$; OA, 0.8, 0.3/1.0, $p = 0.061$). Further down-regulation of IL-6 expression by diacerein was achieved by the combination of both treatment options only in OA cells (MSt/Dia: 0.2, 0.2/0.7, $p = 0.025$), whereby cells under non-inflammatory conditions showed no further decline in IL-6 expression (MSt/Dia: 0.4, 0.1/0.9, $p = 0.013$).

In contrast, COX2 mRNA expression was increased under mechanical modulation applied to both cellular systems (MSt/non-OA, 3.3, 2.5/4.7, $p < 0.001$; MSt/OA, 3.1, 1.1/6.6, $p = 0.04$). Diacerein alone did neither induce an increase in COX2 expression in non-OA cells (Dia/non-OA: 1.1, 0.8/1.4) nor under OA conditions (Dia/OA: 1.0, 07/6.3) when compared to controls. Combining both treatments, the COX2 elevation was lowered compared to the mechanical treatment alone, although a significant overall increase compared to controls remained (MSt/Dia/non-OA: 2.2, 1.2/5.0, $p = 0.006$; MST/Dia/OA: 2.6, 1.5/7.7, $p = 0.031$, Fig. 2B). FGF-2 has been associated with anabolic or

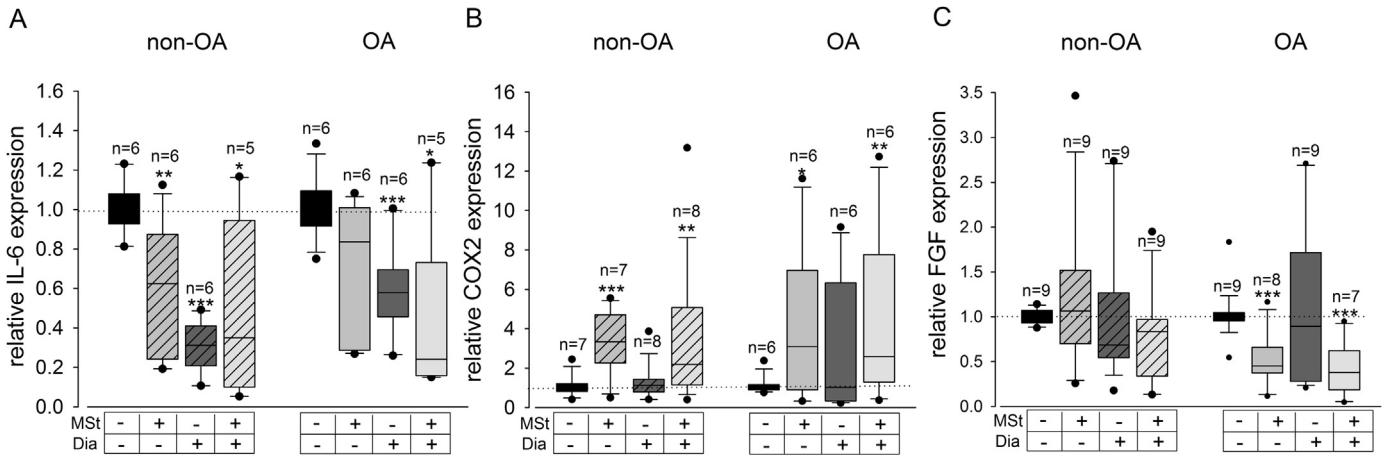

Fig. 2. Changes in the expression of components involved in inflammation. Changes in the expression of A) Interleukin-6 (IL-6), B) cyclooxygenase-2 (COX2), and C) basic fibroblast growth factor (FGF) given by the ratio values to the untreated control groups (MSt: mechanical stimulation, Dia: diacerein, MSt/Dia: mechanical stimulation and diacerein). The bars show the median values, the 25th and 75th percentiles with the whiskers at the 5th/95th percentile. The number of experiments for each group is given (n), each reaction was performed in duplicates. The Mann-Whitney Rank Sum test was used to test for statistical significance with * is < 0.05, ** is p < 0.01 and *** is p < 0.001.

catabolic events in human articular chondrocytes [25]. In OA cells we found that the mechanical stimulation reduced the FGF-2 expression at the RNA level by 50% (0.5, 0.4/0.7, p < 0.001) (Fig. 2C). Although, diacerein alone was ineffective, the added mechanical stimulation down regulated the FGF-2 expression to a ratio of 0.4 (0.2/0.6, p < 0.001).

3.3. Diacerein and mechanical stimulation act on the expression of PGE2 and growth factors at the protein level

In the non-OA cells no reduction in PGE2 synthesis was observed even when both treatments were combined (MSt/Dia: 1957.7 ± 207 pg/ml vs. control 2558.5 ± 174.8 pg/ml, p = 0.055). In OA chondrocytes the mechanical stimulation reduced the PGE2 production (MSt: 227.1 ± 38.9 pg/ml, p = 0.001 vs. control 770.9 ± 152.7 pg/ml), even in the presence of diacerein (MSt/Dia: 340.8 ± 77.6 pg/ml, p = 0.013) while the application of diacerein alone showed no alterations (Dia: 738.9 ± 112.3 pg/ml, p = 0.87) (Fig. 3A).

The platelet-derived growth factor (PDGF) is known to be a potent biological regulator of chondrocytes in cartilage repair [26]. In non-OA and OA cells, as a response to diacerein, PDGF protein expression significantly decreased by more than 80% compared to untreated control cells (Dia/non-OA: 3.63 ± 1.11 pg/ml vs. control/non-OA 20.65 ± 4.26 pg/ml, p = 0.002; Dia/OA: 6.74 ± 0.74 pg/ml vs control/OA 59.91 ± 11.08 pg/ml, p < 0.001, Fig. 3B). Mechanical stimulation alone did not induce changes in the PDGF expression. The VEGF has been demonstrated not only to control angiogenesis but also to modulate the metabolism of chondrocytes [27]. The VEGF-A protein expression was strongly down regulated by diacerein from 9667 ± 1051 pg/ml to 1762 ± 235 pg/ml, p < 0.001 in non-OA cells. Mechanical stimulation applied in addition partially reversed the decline of VEGF-A production by diacerein (MSt/Dia: 4744 ± 957 pg/ml, p = 0.03, Fig. 3C). An increase by mechanical stimulation in the presence of diacerein was also detected in OA cells (Dia: 692 ± 201 pg/ml vs. MSt/Dia: 1695 ± 245 pg/ml, p = 0.004). Important to mention, combined treatments applied to the OA cells induced a 2-fold increase in VEGF-A expression over the control value (control: 840 ± 223 pg/ml) whereas in non-OA cells the expression of VEGF-A under these conditions was inhibited by about 50%.

3.4. Conditioned medium from mechanically stimulated and diacerein treated chondrocytes influences cell growth and cell migration

Conditioned medium (CoM) was collected after seven days of mechanical stimulation. Freshly plated cells were treated with a one-to-one dilution of the respective CoM. The cell proliferation and the cell

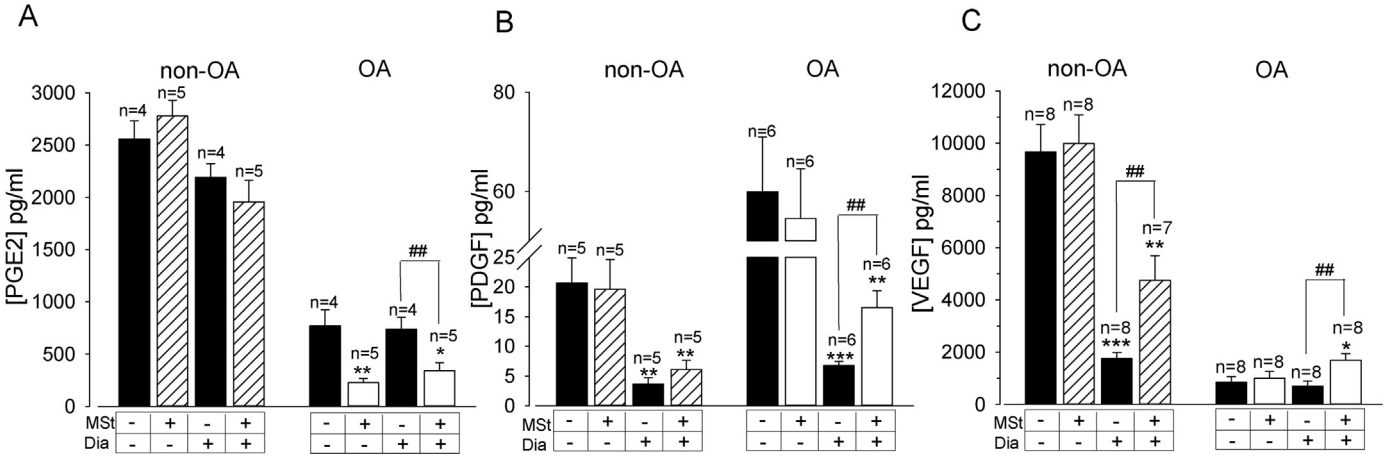

Fig. 3. Growth factors expression is regulated by mechanical stimulation and diacerein treatment. Growth factors expression was determined at the protein level with the ELISA technique in pg/ml from supernatants collected after seven days of treatment. Concentrations for measured PGE2 (A), PDGF (B), and VEGF-A (C) protein expression are given (MSt: mechanical stimulation, Dia: diacerein, MSt/Dia: mechanical stimulation and diacerein) as mean values ± standard error; number of single experiments (n) performed in duplicates are given. The Student's *t*-Test was used to test for statistical significance with * is p < 0.05, **, ## is p < 0.01 and *** is p < 0.001, respectively.

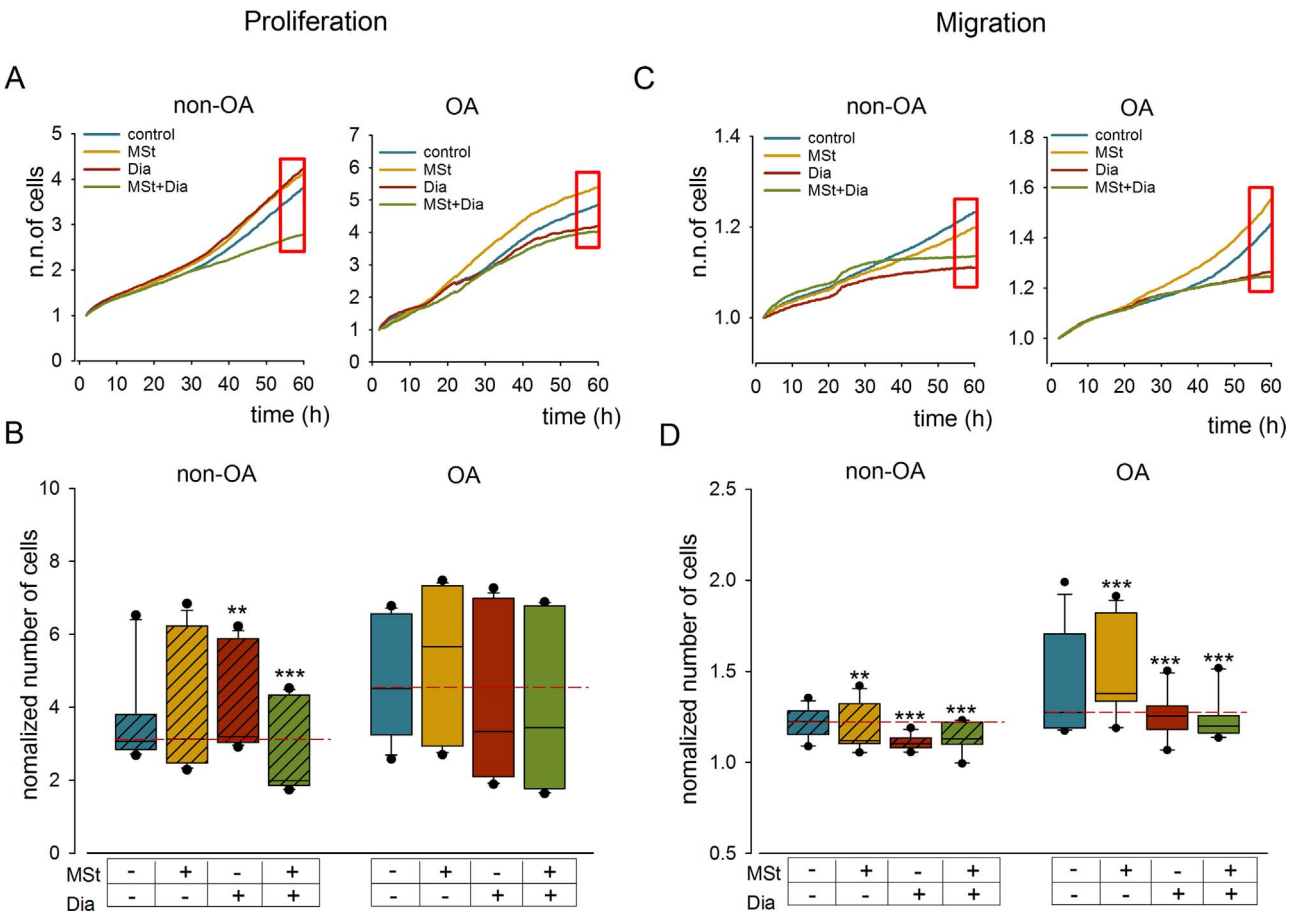

Fig. 4. Cell proliferation and migration behavior under the influence of conditioned medium. Conditioned medium from untreated cells (control), mechanically stimulated cells (MSt), diacerein treated cells (Dia) and from combined treatment (MSt/Dia) was applied. A) Their impact on cell growth and C) migration behavior were measured in real time with the xCELLigence RTCA device over a period of 60 h. Mean values of the normalized cell number from three experiments measured in duplicates are given as a line graph for non-OA and OA chondrocytes. B, D). Box plot data represent the averages calculated from values measured during the last 5 h of each single experiment; areas from which the data originated are marked by the red box. Values from untreated cells functioned as control, the Mann-Whitney Rank Sum test was used to test for statistical significance with ** is $p < 0.01$ and *** is $p < 0.001$.

Table 2
Data describing cell proliferation and cell migration of chondrocytes treated with conditioned medium. Delta cell indexes for cells under the influence of conditioned medium are given. Data were gathered from control cells (w/o stimulation), cells treated with mechanical stimulation (MSt) or diacerein (Dia) and with a combination (MSt/Dia), three experiments (n = 3) in duplicates were performed.

	Cell proliferation (Δ cell index), n = 3 in duplicates													
OA		mean	Std. err.	median	25%	75%	Mann-Whitney R.S. Test	**healthy**	mean	Std. err.	median	25%	75%	Mann-Whitney R.S. Test
	control	4.74	0.15	4.52	3.26	6.55			3.66	0.11	3.07	2.85	3.80	
	MSt	5.30	0.17	5.67	2.94	7.34			3.99	0.16	3.13	2.48	6.23	
	Dia	4.15	0.19	3.34	2.10	6.99			4.07	0.12	3,19	3.04	5.88	$p < 0,01$
	MSt/Dia	4.00	0.19	3.45	1.78	6.78			2.74	0.11	1.99	1.87	4.33	$p < 0001$
	Cell migration (Δ cell index), n = 3 in duplicates													
OA	control	1.43	0.026	1.27	1.19	1.70		healthy	1.22	0.007	1.22	1.15	1.28	
	MSt	1.52	0.027	1.38	1.34	1.82	$p < 0001$		1.19	0.012	1.12	1,10	1.32	$p < 0,01$
	Dia	1.26	0.012	1.26	1.19	1.31	$p < 0001$		1.11	0.004	1.10	1.08	1.13	$p < 0001$
	MSt/Dia	1.24	0.011	1.20	1.16	1.26	$p < 0001$		1.13	0.008	1.13	1.10	1.22	$p < 0001$

migration of non-OA and OA chondrocytes were measured in real time via the noninvasive electrical impedance monitoring over a time period of 60 h. Cell proliferation under OA conditions slightly increased in the presence of CoM, while CoM collected from cells cultured in the presence of diacerein significantly slowed down cell growth (Fig. 4 A-B). CoM from mechanically stimulated cells with the additional diacerein treatment did not compensate this effect. Although a highly significant increase in cell growth was observed for non-OA cells cultured in the presence of the CoM from diacerein or mechanically stimulated cells, CoM of the combined treatment inhibited cell proliferation. Detailed data concerning cell proliferation and the migration behavior are given in Table 2. CoM derived from mechanical stimulated cells significantly augmented the migration of OA cells. CoM collected from treatments with diacerein alone or diacerein plus mechanical stimulation reduced the migration frequency (Fig. 4 C-D). While a similar effect of CoM/diacerein and CoM/diacerein plus mechanical stimulation on non-OA cells was observed, CoM from mechanical stimulated non-OA cells elicited a reduction in cell migration.

4. Discussion

OA represents the major disease associated with degenerative joint

disorder. Treatment options for OA are an actual challenge due to the poor self-healing capacity of cartilage and lack of appropriate diagnostic biomarkers [28]. Inflammatory mediators (cytokines, prostaglandins) can enhance catabolic processes targeted to matrix destruction and gave rise to the "inflammatory" theory for OA [29]. While current treatment modalities aim at pain reduction and modest improvements in functions, treatment for OA should target tissue repair and cartilage regeneration [30]. The present study, deals with the effect of combining mild mechanical stimulation and diacerein treatment at the cellular level to target inflammation and growth factor regulation.

While the inflammatory cytokine IL-6 has already been discussed as a therapeutic target in OA [31], we were able to reduce the IL-6 expression by diacerein and mechanical stimulation under non-OA and OA conditions. A population-based study of older adults showed an association between circulating inflammatory markers such as IL-6 and cartilage loss [32]. PGE2 has been characterized to induce IL-6 expression via the cAMP/protein kinase A (PKA) dependent NF-κB activation in human cultured chondrocytes [33,34]. The down-regulation of IL-6 and PGE2 due to our selected program of moderate mechanical stimulation may result from a linked action between the integrin/focal adhesion kinase (FAK) pathway for mechanical signaling and the PGE2/IL-6 pathway by triggering NF-κB. Several lines of evidence indicate that the cellular metabolism is contingent on the communication between receptors to modulate their signaling pathways, a phenomenon known as "cross talk" [35] (Fig. 5). Diacerein has been shown to reduce IL-6 expression by blocking the IL-1β pathway via MEK/ERK and NF-κB DNA binding [36]. Mechanical stimulation seems to strengthen this down regulation via the integrin/FAK signaling pathway. The increase in COX2 seen by the mechanical stimulation is in contrast to the reduction of PGE2. However, COX2 synthesizes PGH2 and not PGE2, which is a downstream product of the PGE synthase. The reduced PGE2 level could be the result of an inhibition of the PGE synthase caused by the mechanical stimulus [37]. In line with earlier in vitro findings describing diacerein not being able to reduce PGE2 [38], our results demonstrate a reduction only in combination with mechanical stimulation.

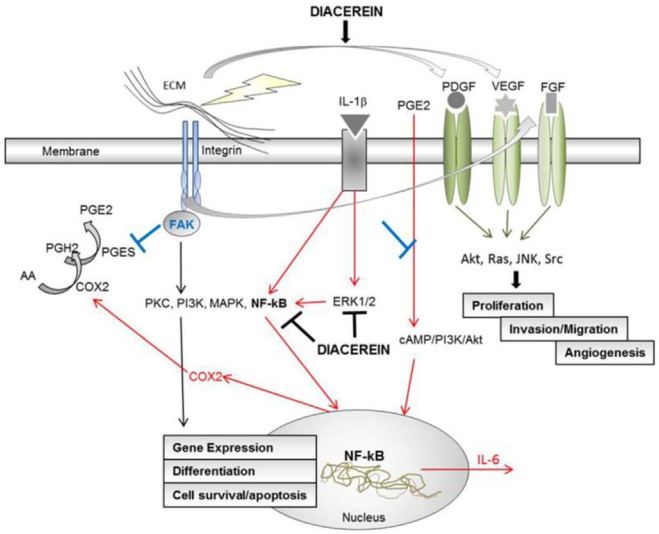

Fig. 5. Cross-talk between the signal transduction pathways involved in mechanical stimulation and diacerein treatment of chondrocytes. Mechanical stimulation (illustrated with a flash) is transduced via the integrin/FAK (focal adhesion kinase) pathway, modulating proteinkinase C (PKC), Phosphoinositid-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB). Interleukin-1β (IL-1β) activates the extracellular signal–regulated kinases (ERK1/2) and NF-κB, which subsequently regulate cyclooxygenase 2 (COX2) and interleukin-6 (IL-6). Arachidonic acid (AA) via COX2 is converted to prostaglandin H2 (PGH2) which is further processed into and prostaglandin E2 (PGE2) by prostaglandin synthase (PGES). PGE2 acts via cyclic adenosine monophosphate (cAMP)/PI3K/ protein kinase B (PKB, Akt). Receptors for platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) function via the Akt, Ras, c-Jun N-terminal kinases (JNK), Scr signaling. Inhibition by mechanical stimulation is given by a blue T, inhibition by diacerein is represented by a black T. The inflammatory pathway is marked by red arrows.

Growth factors are known to stimulate cell division, growth or differentiation. In articular cartilage, numerous growth factors work in concert to regulate development and homeostasis. Studying growth factor regulation under the influence of therapeutic treatments for OA should clarify their role in cartilage regeneration and help us to improve our current cartilage repair techniques [19]. While PDGF functions as a potent mitogen and chemotactic factor for chondrocytes, we observed a diacerein induced decrease in PDGF expression at the protein level. Dependent on the presence of IL-1β, mechanical treatment was able to reverse this effect, an observation possibly ascribed to effects on the translational level since under both conditions the mRNAs are equally upregulated. While VEGF plays an important role in tissue regeneration, recent research suggests VEGF as a key factor in the development of OA [39]. Its production is mainly induced by mechanical overload and increases the metalloproteinases MMP-1, −3 and −13 [40]. The unchanged expression of VEGF at the protein level under the applied mechanical stimulation reflects the moderate nature of our application. Diacerein lowers the VEGF concentration under non-inflammatory conditions and mechanical stimulation partially reverses this effect. These divergent results once more imply the antagonistic impact of two or more signaling cascades.

Some studies have identified FGF-2 as a catabolic inducer in human adult articular cartilage. In articular chondrocytes, FGF-2 induces MMP-13 [41], suppresses the aggrecan gene, and promotes the expression of aggrecanases (ADAMTS-5) and tumor necrosis factor (TNF) receptor [42,43]. In our study mechanical stimulation under OA conditions reduced FGF-2 expression, an effect that might exhibit a beneficial effect for OA patients.

Even though moderate mechanical stimulation did not induce a significant increase in growth factor expression, cell proliferation and migration was enhanced in cells treated with conditioned medium from mechanically stimulated cells under OA condition. Joos et al., 2013 highlighted a significantly reduced basal cell migration and abrogation of a stimulating effect of the growth factors in chondrogenic progenitor cells by IL-1β and TNF-α [44]. Our observed increase in cell growth might therefore be based on changes in the inflammatory framework generated by mechanical stimulation and the reduction in FGF-2, for FGF-2 is known to inhibit cell growth [45]. Conditioned medium from diacerein treated plus/minus mechanical stimulated cells mostly reduced cell proliferation/migration. Under non-OA conditions we detected augmented cell proliferation associated with the reduction of VEGF expression, underlining the role of VEGF in chondrocytes noticeable by increased cell growth [27].

5. Conclusion

Treatment of chondrocytes with either mechanical strain or with diacerein alone or in combination influenced the inflammatory status and growth factor expression of the cells. Changes in the metabolism of OA chondrocytes induced by mechanical stimulation positively affected cell proliferation and cell migration. We conclude that mild mechanical stimulation appears to be beneficial for the fate of the cell and when combined with diacerein partly improves its pharmacological effects. By testing the outcome of the combination of two different treatment options a foundation for a more widened perspective to treat OA might be laid.

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Appendix A. Transparency document

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