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

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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

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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

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