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

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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

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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,