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Norepinephrine modulates osteoarthritic chondrocyte metabolism and inflammatory responses

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SUMMARY

Objective: Norepinephrine (NE) was measured in synovial fluid of trauma patients and sympathetic nerve fibers were detected in healthy and osteoarthritic (OA) joint tissues indicating that cartilage pathophysiology might be influenced by sympathetic neurotransmitters. The aim of this study was to elucidate the mostly unknown role of NE in OA chondrocyte metabolism and inflammatory responses.
Methods: Articular cartilage was received after total knee replacement surgery from OA patients. Expression of adrenergic receptors (AR) and tyrosine hydroxylase (TH) was tested with end point polymerase chain reaction (PCR) and immunohistochemistry. 3-dimensional (3D) cell cultures were employed to analyze effects of NE on chondrocyte cell metabolism and the expression of interleukins (ILs), matrix metalloproteases (MMPs), tissue inhibitor of metalloproteases (TIMPs), glycosaminoglycan (GAG) and collagen II under non- and inflammatory conditions. Chondrocyte monolayer cultures were used to specify AR subtypes, to analyze cell cycle distribution and to determine catecholamines in cell culture supernatants.
Results: AR subtypes and TH were detected in chondrocytes, whereas NE was not released in measurable amounts. 10^{-6} M NE reversed IL-1β induced changes in IL-8, MMP-13, GAG and collagen II expression/production indicating for β-AR signaling. Additionally, NE caused cell cycle slow down and decreased proliferation via β-AR signaling. 10^{-8} M NE increased the number of proliferating cells and induced apoptosis via α1-AR signaling.
Conclusions: NE affects chondrocytes from OA cartilage regarding inflammatory response and its cell metabolism in a dose dependent manner. The sympathetic nervous system (SNS) may have a dual function in OA pathology with preserving a stable chondrocyte phenotype via β-AR signaling and OA pathogenesis accelerating effects via α-AR signaling.

Introduction

OA is the most common form of arthritis and a leading cause of chronic pain. The social burden of OA is immense as it affects 9.6% of men and 18% of woman over 60 years of age worldwide[1] and currently compromising the lives of 40 million Europeans[2]. The etiology of OA is multifactorial and still incompletely understood. Hallmark features are structural changes including articular cartilage destruction as well as alterations in synovium and subchondral bone. Increased proteolytic activity leads to degradation of major cartilage extracellular matrix components as collagens and the large proteoglycans. In this process, MMPs and their inhibitors play a key role. Regulation of several highly expressed MMPs (MMP-2, -3 and -13) in end stage OA cartilage supports this concept[3–5]. Regarding metabolism of chondrocytes, early OA is characterized by proliferation accompanied with increased matrix production, whereas end stage OA chondrocytes express hypertrophy markers such as MMP-13 and collagen X and presumably undergo apoptosis[6–8]. Interestingly, intermittent inflammatory flares also occur in the synovial membrane in the course of OA suggesting

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inflammation is associated with this disease. Although not a primary phenomenon, synovial inflammation contributes to OA progression. Among pro-inflammatory mediators involved in OA, IL-1β seems to play a key role[9]. It increases the synthesis of various MMPs and some short time pro-inflammatory mediators such as IL-6 and IL-8[8,10].

NE belongs to the catecholamine family of tyrosine-derived sympathetic neurotransmitters. This subtype of sympathetic nerve fibres, characterized by the expression of TH, has been identified in synovium, bone marrow, periosteum, and in bone-adherent ligaments[11–13], indicating that growth and metabolic activity of synovial joint tissues are regulated by sympathetic neurotransmitters. Catecholaminergic effects are mediated by the adrenergic receptor (AR) family. At high concentrations ($>10^{-7}$ M) NE acts via α- and β-AR, whereas low concentrations ($\leq 10^{-7}$ M) are mediated mainly via α-AR[14]. Functional α1-, α2-and β2-AR are present on murine costal chondrocytes and chondrogenic ATDC5 cells[15,16]. Previously, we observed a decrease in apoptosis in murine costal chondrocytes after treatment with NE via β-AR signaling and an increase in focal adhesion contacts[15]. Additionally, it is described that NE may also induce apoptosis in several other cell types[17,18] and to regulate the proliferation of osteoblasts, osteoblast-like cells, and mesenchymal stem cell lines through β- and α_1-AR mediated signaling[19–21]. Furthermore, catecholamines play a decisive role in inflammatory processes characteristic for rheumatoid arthritis[22]. With respect to above disease entity, β-AR activation predominantly mediates anti-inflammatory effects, whereas activation of α-AR, if located on immune cells, mediates a priori pro-inflammatory effects (reviewed in[23]).

Articular cartilage is not deeply innervated by nerve fibers, although there are hints in literature that catecholamines influence articular chondrocytes especially in OA. High levels of NE were measured in synovial fluid of patients with joint trauma[24] along with a high incidence to develop post-traumatic OA[25]. Suri *et al.* have localized both sensory and sympathetic nerve fibers in similar distributions to the articular cartilage in human tibiofemoral OA. In the pathogenesis of OA, nerves grow into joint structures through vascular channels mainly from subchondral bone breaching through the tidemark[26].

In this study, we have addressed the response of human articular OA chondrocytes to NE with respect to inflammatory markers and chondrocyte metabolism.

Materials and methods

Study design

In this study we analyzed the localization of TH and AR subtypes in human OA cartilage, the response of human OA chondrocytes to NE under inflammatory conditions and their metabolic answer to NE stimulation.

TH and AR subtype gene expression were measured with end point PCR after RNA isolation from monolayer- and 3D (fibrin gel) cultured chondrocytes under non- and inflammatory conditions (w/o 0.5 ng/ml IL-1β) TH, α1D-AR and β2-AR expression were approved by immunohistochemistry staining on cryo-embedded cartilage slides ($N \geq 3$).

Responses of human OA chondrocytes to NE under inflammatory conditions were tested with real time PCR, enzyme-linked immunosorbent assays (ELISA) and dimethyl methylene blue (DMMB) assays under non- and inflammatory conditions (w/o 0.5 ng/ml IL-1β). Chondrocytes of the same donor were cultured under following conditions: Treatment of fibrin gels with (1) vehicle (PBS with 0.5 mM acetic acid), (2) IL-1β and (3) IL-1β + NE (10^{-6} M and 10^{-8} M) respectively (4) vehicle (PBS with 0.5 mM acetic acid) and (5) NE (10^{-6} M and 10^{-8} M). ($N \geq 6$) (Suppl. Fig. 1A).

Metabolic (proliferation and apoptosis) response of OA chondrocytes to NE were analyzed in 3D culture under non-inflammatory conditions after staining of proliferating cell nuclear antigen (PCNA, proliferation) and TdT-mediated dUTP-biotin nick end labeling (TUNEL, apoptosis) on paraffin-embedded micromass pellet sections. To specify AR subtypes involved in metabolic responses, chondrocytes were cultured in monolayer and AR antagonists were included in BrdU (proliferation) and caspase 3/7 (apoptosis) assays. To further analyze proliferation effects, cell cycle was analyzed via flow cytometer. ($N \geq 6$) (Suppl. Fig. 1B).

Isolation and culture of chondrocytes

Articular cartilage was obtained from OA patients after total endoprothesis surgery. This had been approved by the local Ethics Committee (Az:14-101-0189; Ethikkommission an der Universität Regensburg, email: ethikkommission@klinik.ukr.de) and specimens were taken with patients' written consent. For this study knee joints were obtained from 61 different donors (29 male and 32 female) within an age range of 48–84 (mean age of 66,4). Macros-copically normal looking cartilage slices were digested over night with collagenase II (PAA) in Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen) containing 1% penicillin/streptomycin (P/S) at 37°C. Isolated chondrocytes were passed through a 70 μm nylon mesh (Falcon) to remove residual cartilage matrix fragments, centrifuged at 200 × g for 5 min, and resuspended in DMEM/F12 supplemented with 10% fetal calf serum and 1% P/S. Chondrocytes were seeded at a density of 20,000 cells/cm^2 and initially cultured in monolayer for maximal 10 days in a humidified 37°C/5% CO_2 incubator.

RNA isolation, end point PCR and real-time PCR

RNA from chondrocyte monolayer culture was isolated with Aglient RNA Absolutely Miniprep Kit. 3D cultivated chondrocytes were first homogenized with a PowerGen 1000 Homogenizer (Fisher scientific) for 45 sec in 1 ml peqgold trifast (Peqlab) and afterwards RNA was isolated according to manufactures instructions. That was followed by a second RNA isolation step with RNA Absolutely Nanoprep Kit (Aglient). To translate RNA in cDNA, AffinityScript QPCR cDNA Synthesis Kit (Aglient) was applied.

Gene expression of TH and subtypes of α- and β-ARs was determined using one-Step RT-PCR (Invitrogen), where each reaction consisted of 100 ng of cDNA. Gene expression of GAPDH was used as loading control. Primers were designed from universal probe library system assay design center (Roche) and listed in Suppl. Table 1. Commercial available RNA of human brain and arteria tissue served as positive controls and the identity of amplified PCR products was confirmed by sequencing. Pure RNA served as negative control because most AR subtypes contain only one exon, excluding intron-spanning primer design.

Relative quantitative PCR for IL-6, IL-8, MMP-2, MMP-3, MMP-13, TIMP-1, TIMP-2 and TIMP-3 was performed in Mx3005P qPCR system with Brilliant II SYBR Green QPCR Mstr Mx (Aglient), 30 ng cDNA and in triplicates. Primers are listed in Suppl. Table 2. Relative quantification was determined with MxPro QPCR software using GAPDH as endogenous loading control and IL-1β stimulated respectively untreated cells as calibrator. Log fold changes to calibrator (base 2) values were plotted using Graph Pad Prism 5 software.

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