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Celecoxib-mediated reduction of prostanoid release in Hoffa's fat pad from donors with cartilage pathology results in an attenuated inflammatory phenotype

U.T. Timur †, *, M.M.J. Caron †, Y.M. Bastiaansen-Jenniskens ‡, T.J.M. Welting †, L.W. van Rhijn †, G.J.V.M. van Osch ‡, P.J. Emans †

† Laboratory for Experimental Orthopedics, Department of Orthopaedic Surgery, Maastricht University Medical Centre +, Maastricht, The Netherlands
‡ Department of Orthopaedics, Erasmus MC, University Medical Center, Rotterdam, The Netherlands

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Objective: The Hoffa's fat pad (HFP) is an intra-articular adipose tissue which is situated under and behind the patella. It contains immune cells next to adipocytes and secretes inflammatory factors during osteoarthritis (OA). In this study, we compared the release profile of prostanoids, which are involved in inflammation, of HFP from OA patients vs patients with a focal cartilage defect (CD) without evidence for OA on MRI and investigated the prostanoid modulatory anti-inflammatory action of celecoxib on HFP.
Design: Prostanoid release was analyzed in conditioned medium of HFP explant cultures from 17 osteoarthritic patients and 12 CD patients, in the presence or absence of celecoxib. Furthermore, gene expression of COX enzymes and expression of genes indicative of a pro-inflammatory or anti-inflammatory phenotype of HFP was analyzed.
Results: Prostanoid release by HFP from knee OA patients clustered in two subgroups with high and low prostanoid producers. HFP from high prostanoid producers released higher amounts of PGE_2, $PGF_{2\alpha}$ and PGD_2 compared to HFP from CD patients. PGE_2 release by OA HFP was positively associated with expression of genes known to be expressed by M1 macrophages, indicating a role for macrophages. Celecoxib modulated prostanoid release by HFP, and also modulated the inflammation ratio towards a more favorable anti-inflammatory M2 phenotype, most effectively in patients with higher prostanoid release profiles.
Conclusion: In knee OA patients with inflamed HFP's, celecoxib may exert positive effects in the knee joint via decreasing the release of prostanoids produced by the HFP and by favorably modulating the anti-inflammatory marker expression in HFP.
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Introduction

Knee osteoarthritis (OA) is a multifactorial disease characterized by cartilage deterioration and subchondral sclerosis[1]. An inflammatory component in this disease is now acknowledged[2]. Next to cartilage and synovium, studies have shown the ability of the Hoffa's fat pad (HFP) to secrete inflammatory mediators able to induce inflammatory and destructive responses in knee OA[3,4]. The HFP is an adipose tissue located intra-articularly behind and under the patella[4]. Important molecules involved in inflammatory processes are prostanoids. While prostanoids have been shown to be secreted by the HFP[5], modulation of prostanoid secretion by HFP and potential consequences of such modulation on inflammatory processes in HFP has not been investigated.

The major rate-limiting step in the biosynthesis of prostanoids is the cyclooxygenase (COX) dependent production of PGH_2[6]. The prostanoid family consists of five different subtypes: PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ and Thromboxane A_2 (TXA_2) and these subtypes are synthesized from PGH_2 by dedicated enzymes[7]. COX-1 is regarded as the ‘housekeeping’ COX, responsible for the production of basal prostanoid levels, which are thought to maintain local homeostasis[8]. On the other hand, COX-2 is considered to be inflammatory, since its expression is upregulated in inflamed joint tissues[8].

* Address correspondence and reprint requests to: U.T. Timur, Maastricht University Medical Center +, Department of Orthopaedic Surgery, P. Debyelaan 25, 6202 AZ, Maastricht, P.O. Box 5800, The Netherlands. Tel: 31-43-3884152.
E-mail address: u.timur@maastrichtuniversity.nl (U.T. Timur).

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Indeed, COX-2 expression in the HFP has been shown to be increased upon inflammatory stimulation[3], and while it is now well established that COX-2 expression is upregulated in inflamed joint tissues[8], the consequences of altered COX-2 expression on the production of its main downstream prostanoid products are poorly documented in OA. Different prostanoid subtypes such as PGE_2 and $PGF_{2\alpha}$ have been shown to be involved in catabolic and pro-fibrotic responses in knee OA[9–11], and thus a broader insight into the involvement of specific prostanoids in OA-related inflammatory processes will be essential to understand their contribution in OA. Since the HFP contains nerve fibers[4] and considering the pronociceptive effects of different prostanoid subtypes[12–14], prostanoids produced by the HFP may provide valuable targets for anti-inflammatory and pain therapy in the OA knee joint. Several risk factors have been described which predispose for knee OA, one of which are cartilage defects (CDs)[15]. Defining the difference between a CD and 'pre-osteoarthritis' still represents a major challenge. Although Gierman and coworker have shown that there was a slight difference in the inflammatory profile of HFP between OA and non-OA donors[5], the HFP from patients with a CD vs HFP from OA patients has never been studied to our knowledge and may provide further insight in the inflammatory pathogenesis of both overlapping diseases OA and CDs. Therefore the main goal of this study was to evaluate the secretion of prostanoid subtypes by the HFP from knee OA patients and compare with prostanoid secretion by the HFP of CD patients. In addition, correlations were investigated between prostanoid secretion from HFP and expression of inflammatory markers in the HFP, to evaluate the general inflammatory status of the tissue. Finally, since COX-2 has been considered as an important inflammatory factor in HFP[3], we sought to determine the anti-inflammatory actions of celecoxib, a specific COX-2 inhibitor, on HFP.

Material & methods

Preparation of Hoffa's fat pad conditioned medium

The Medical Ethical Committee (MEC) of Maastricht University approved this study and assigned approval ID: MEC 11-4-040.17. HFPs were obtained as anonymous material from human subjects with endstage OA (Kellgren & Lawrence grade three–four) who underwent total knee arthroplasty (TKA). Since the autologous chondrocyte transplantation (ACT) procedure is performed less frequently than a TKA for endstage OA, 12 HFPs were obtained from human subjects with a single CD who underwent an ACT procedure (ACT; Chondrocelect Tigenix, Leuven Belgium). CDs were either due to trauma or osteochondritis dissecans and patients did not show any signs of OA on X-ray and MRI. These HFP's were used to generate HFP conditioned medium (HCM) at a concentration of 100 mg tissue/mL using DMEM/F12 (Life Technologies, Carlsbad US), 1% ITS, 1% antibiotic/antimycotic (Life Technologies) in a humidified atmosphere at 37°C, 5% CO_2 as reported previously[16]. The inner part of HFP was cut into small explants to avoid the presence of synovial tissue. Explants were selected randomly throughout the entire tissue and used at a concentration of 100 mg tissue/mL medium to create OA HCM or CD HCM. A stock solution of 10 mM celecoxib (LC Laboratories, Woburn, MA, US) was made in dimethylsulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, US) and added 1:1,000 to medium with pieces of HFP to obtain a 10 μM concentration. DMSO was also added 1:1,000 to HFP cultures without celecoxib as a control. In addition, three patients in the OA group were cultured with 2.5, 10 and 100 μM celecoxib to examine a dose response. In accordance with the method described by Clockaerts *et al.*, medium was harvested after 24 h, centrifuged at 1,200 RPM for 8 min and the supernatant was frozen at −80°C[16]. Media were stored for a maximum of 8 weeks before analysis. HFP explants were frozen in liquid nitrogen and stored at −80°C until being processed for RNA isolation.

Prostanoid, Interleukin-6 and Interleukin-1β measurement in HCM

Concentrations of PGE_2, $PGF_{2\alpha}$, PGD_2 and TXB_2 (a stable metabolite of TXA_2) were determined in OA HCM and CD HCM by a competitive Enzyme-Linked Immunosorbent Assay according to the manufacturer's instructions (ELISA, Cayman Chemicals, Ann Arbor, MI, US) as reported previously[17]. Concentrations of interleukin-6 and interleukin-1β in HCM was determined according to the manufacturer's instructions of the human IL-6 and IL-1β Quantikine ELISA Kits (R&D Systems, MI, US).

RNA isolation and quantitative RT-PCR

Frozen HFP was homogenized with a Mikro-Dismembrator and suspended in 1 ml TRIzol/100 mg tissue. RNA was extracted and cDNA was made as reported previously[16]. Real time quantitative PCR (RT-qPCR) was performed using Mesagreen qPCR mastermix plus for SYBR Green (Eurogentec). A CFX96 Real-Time PCR Detection system (Biorad) was used for amplification: initial denaturation 95°C for 10 min, followed by 40 cycles of amplification (denaturing 15 s at 95°C and annealing 1 min at 60°C) as reported previously[17]. Validated primer sequences can be found in Supplementary Table 1. Gene expression was normalized to a best housekeeper ratio based on gene expression levels of *GAPDH*, *PPIA* and *28S*[18] and data were analyzed according to the 2ˆ−ΔΔCT method[19]. Housekeeper gene expression was found to be stable (data not shown).

Statistical analysis

SPSS Inc., Chicago, USA (version 20) was used for statistical analyses. A minimum of five explants was used per experimental condition. All samples for gene expression analysis and cytokine measurements in medium were processed and analyzed individually with a single measurement per donor. Continuous variables were tested for normality using Kolmogorov–Smirnov test.

PGE_2 release data were not normally distributed and a Mann–Whitney *U* test was performed to evaluate differences between PGE_2 release by OA HFP and CD HFP. Based on PGE_2 release data, OA patients were further divided into two subgroups, which was based on an arbitrarily chosen PGE_2 release threshold. An inflammation ratio was calculated, using *IL6*, *IL1B*, *CD86* and *TNFA* as markers associated with pro-inflammatory or M1 macrophages and *CD206*, *IL10*, *CD163* and *CCL18* as markers associated with anti-inflammatory or M2 macrophages[20]. The mean of the relative expression of pro-inflammatory genes per sample, was divided over the mean of the relative expression of anti-inflammatory genes per sample. Gene expression data, the inflammation ratio, prostanoid release and cytokine release data of these two OA subgroups and CD patients were then log transformed and subjected into a general linear model. For celecoxib treatments in the patient groups, the celecoxib treatment itself was defined as a fixed factor in a general linear model. Correlations between prostanoid levels and expression of markers associated with inflammation were calculated using the Spearman's rank correlation test.

Differences in clinical parameters BMI, and age between OA and CD patients were analyzed using an independent Student's *t*-test, differences in duration of symptoms and K&L grade between OA and CD patients were analyzed using a Mann–Whitney *U* test and differences in gender using a Pearson Chi-square test.

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