Research in Veterinary Science 118 (2018) 466–476

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Time-dependent changes in gene expression induced *in vitro* by interleukin-1β in equine articular cartilage

Maria Löfgren[a,*], Emilia Svala[a,b], Anders Lindahl[b], Eva Skiöldebrand[a,b], Stina Ekman[a]

[a] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Box 7028, SE-750 07 Uppsala, Sweden
[b] Department of Clinical Chemistry and Transfusion Medicine, Institute of Biomedicine, Sahlgrenska University Hospital, Gothenburg University, SE-413 45 Gothenburg, Sweden

ARTICLE INFO

Keywords:
Osteoarthritis
Inflammation
Extracellular matrix
Chondrocyte

ABSTRACT

Osteoarthritis is an inflammatory and degenerative joint disease commonly affecting horses. To identify genes of relevance for cartilage pathology in osteoarthritis we studied the time-course effects of interleukin (IL)-1β on equine articular cartilage.

Articular cartilage explants from the distal third metacarpal bone were collected postmortem from three horses without evidence of joint disease. The explants were stimulated with IL-1β for 27 days and global gene expression was measured by microarray. Gene expression was compared to that of unstimulated explants at days 3, 9, 15, 21 and 27. Release of inflammatory proteins was measured using Proximity Extension Assay.

Stimulation with IL-1β led to time-dependent changes in gene expression related to inflammation, the extracellular matrix (ECM), and phenotypic alterations. Gene expression and protein release of cytokines, chemokines, and matrix-degrading enzymes increased in the stimulated explants. Collagen type II was downregulated from day 15, whereas other ECM molecules were downregulated earlier. In contrast molecules involved in ECM signaling (perlecan, chondroitin sulfate proteoglycan 4, and syndecan 4) were upregulated. At the late time points, genes related to a chondrogenic phenotype were downregulated, and genes related to a hypertrophic phenotype were upregulated, suggesting a transition towards hypertrophy later in the culturing period.

The data suggest that this *in vitro* model mimics time course events of *in vivo* inflammation in OA and it may be valuable as an *in vitro* tool to test treatments and to study disease mechanisms.

1. Introduction

Osteoarthritis (OA) is an inflammatory and degenerative joint disease commonly affecting humans and horses and there is no drug based disease modifying therapy for it (Wieland et al., 2005). For both humans and horses, there is a lack of early diagnosis, problems in monitoring disease progression, and difficulties in evaluating treatment regimens.

The chondrocytes of the articular cartilage respond to mechanical load and inflammatory mediators during the disease process (Guilak et al., 2004; Sandell and Aigner, 2001). Cytokines, such as interleukin (IL)-1β are involved in the initiation and progression of the disease, and chondrocytes are able to produce, as well as respond to, cytokines (Goldring and Goldring, 2007; Goldring and Otero, 2011). Chondrocyte responses include phenotypical changes, as well as changes in synthesis and degradation of the surrounding extracellular matrix (ECM) (Kidd et al., 2001). Phenotypic changes of chondrocytes in OA include proliferation of progenitor cells in the early stages, followed by terminal differentiation towards hypertrophy and apoptosis in the later stages (Tchetina, 2011; van der Kraan and van den Berg, 2012). The balance between anabolic and catabolic factors alters as the OA progresses, with subsequent degradation of the cartilage when the anabolic activities cannot withstand the catabolic activities. Matrix-degrading enzymes, such as matrix metallopeptidases (MMPs), and a disintegrin and metallopeptidase with thrombospondin motifs (ADAMTS), are catabolic factors, causing a gradual degradation of the ECM. Proteoglycans, such as aggrecan, are degraded, followed by disruption of the collagen network (Goldring and Goldring, 2010). The primary collagen degrading enzyme in OA is MMP-13 (Billinghurst et al., 1997; Goldring et al., 2011; Knäuper et al., 1996; Reboul et al., 1996; Troeberg and Nagase, 2012). The release of matrix fragments can induce further production of matrix-degrading enzymes, as well as inflammatory mediators, resulting in continued degradation of the cartilage. The catabolic activities can be counteracted by anti-inflammatory cytokines and growth

* Corresponding author.
E-mail address: maria.lofgren@slu.se (M. Löfgren).

https://doi.org/10.1016/j.rvsc.2018.04.013
Received 31 October 2017; Received in revised form 27 April 2018; Accepted 30 April 2018

factors, whereas pro-inflammatory cytokines further increase catabolic activity (Goldring, 2012).

The dynamic changes in immune response and cartilage matrix degradation are known to occur in a time-dependent pattern (Svala et al., 2015). Our previous work has studied the release of molecules in response to IL-1β in a time-dependent manner for 25 days in equine cartilage explants.

The aim of the current study was to follow the changes in gene expression after IL-1β stimulation of equine cartilage explants at days 3, 9, 15, 21 and 27 by microarray analysis.

Our hypothesis was that differences in expression of genes with known involvement in inflammation, matrix modulation, and phenotypic alterations of chondrocytes are detectable at specific time points for explants stimulated with IL-1β.

2. Materials and methods

2.1. Collection of equine cartilage explants

Three horses, euthanized due to other reasons than joint problems and with no clinical history of joint disease, were included in the study: a Standardbred trotter (4 years old) and two Swedish Warmblood riding horses (6 and 8 years old) Explants of non-calcified articular cartilage were harvested aseptically with a 5-mm biopsy punch postmortem from the third metacarpal bone of macroscopically and microscopically normal joints. A total of 33 explants from both forelimbs were pooled for each horse and stored in sterile phosphate buffered saline (PBS) during transport to the laboratory. The three horses were run as three separate experiments and all analyses were performed for each horse. This study was approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (No·C62/13).

2.2. Stimulation of cartilage explants

The equine cartilage explants (3 explants/well) were incubated in Dulbecco's modified Eagle's medium nutrient mixture F-12 (Life Technologies, Paisley, UK) supplemented with 0.1 mg/ml cell culture-tested bovine serum albumin, 0.1 mg/ml ascorbic acid, 100 units/ml penicillin and 0.1 mg/ml streptomycin (all from Sigma Aldrich, St Louis, MO, USA) in Nunc Non-Treated Multidishes (Thermo Scientific, Roskilde, Denmark) at 37 °C for in 7% CO_2/93% air.

After adaptation of the explants to the culturing conditions for 48 h, the medium was exchanged for fresh medium containing 10 ng/ml recombinant equine IL-1β (R&D Systems; Minneapolis, MN, USA) and added to 5 wells containing 3 explants each. Medium without IL-1β was added to 5 wells as unstimulated controls. The explants were cultured at 37 °C in 7% CO_2/93% air, and the medium was changed every third day.

2.3. Harvest of cartilage explants and media

Cartilage explants from one well were harvested at the start of stimulation (day 0). Cartilage explants from one well containing medium with IL-1β and one well without IL-1β were then harvested at days 3, 9, 15, 21, and 27 post-stimulation. These time points were selected on the basis of results from a previous experiment (Svala et al., 2015). The explants were weighed and two explants from each group were transferred to sterile PBS for isolation of RNA immediately after harvest. One explant from each treatment group for all horses was immersed in 10% neutral buffered formalin for 24 h, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin & eosin and toluidine blue. The medium was collected and stored at −80 °C.

2.4. Isolation of RNA

Chondrocyte RNA was isolated by a method modified from that of Ali and Alman (2012). Briefly, cartilage explants (two explants from each treatment group and time point) were minced with a scalpel before digestion of the matrix in polypropylene tubes with 0.25% trypsin (Life Technologies) and three added tungsten carbide beads (Qiagen, Hilden, Germany) for 60 min at 37 °C with agitation (170 rpm). Centrifugation was performed at 15,000 ×*g* for 60 s, at 20 °C, followed by removal of the supernatant with the trypsin. After a sterile PBS wash of the pellet, centrifugation was repeated. After removal of the PBS, freshly prepared and filtered collagenase type II (Worthington, Lakewood, NJ, USA) (3 mg/ml) was added and the pellet agitated at 170 rpm and 37 °C until the cartilage was 95% digested. The cell suspension was pipetted through a 70-μm filter, transferred to a new polypropylene tube, and centrifuged at 15,000 ×*g* for 5 min at 20 °C. After removal of the supernatant, the cell pellet was resuspended in PBS, and centrifuged at 15,000 ×*g* for 5 min at 20 °C. This washing step was repeated twice more before the cells were lysed with 1 ml TRIzol reagent (Life Technologies). Thereafter, the samples were stored at −80 °C until further processing.

RNA was extracted from thawed samples using TRIzol® and 1-bromo-3-chloropropane (Sigma Aldrich), followed by purification with RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Genomic DNA was removed using RNase-Free DNase Set (Qiagen), and the samples were cleaned using RNeasy MinElute Cleanup Kit (Qiagen). RNA concentration and quality were evaluated using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc., Palo Alto, CA, USA).

2.5. Microarray expression analysis

Stimulated and unstimulated samples for all time points from all three horses were subjected to microarray analysis, each horse representing one replicate. Two ng of total RNA from each sample was used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome, following the user manual for GeneChip WT Pico Reagent Kit (Affymetrix Inc. Santa Clara, CA, USA). GeneChip Equine Gene 1.0 ST Arrays (Affymetrix Inc.) were hybridized for 16 h in a 45 °C incubator, with agitation at 60 rpm. The arrays were washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip Scanner 3000 7G (both instruments were from Affymetrix Inc.).

2.6. Microarray data analysis

The raw data were normalized in an Expression Console (Affymetrix Inc.), using the robust multi-array average method first suggested by Li and Wong (2001) and Irizarry et al. (2003). Subsequent analysis of the gene expression data was carried out in the statistical computing language R, (www.r-project.org) using packages available from the Bioconductor project (www.bioconductor.org). To search for the genes expressed differentially between the IL-1β stimulated and unstimulated explants, a paired empirical Bayes moderated *t*-test was applied using the "limma" package (Smyth, 2004, 2005). To address the problem with multiple testing, the *p*-values were adjusted using the method of Benjamini and Hochberg (1995). Differences between IL-1β stimulated and unstimulated explants were considered significant if the logarithmic fold change (log2 FC) was ≥ 1 or ≤ -1 with $p < 0.05$. The microarray data have been submitted to Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), accession number GSE100083.

DAVID v6.7 was used to identify enriched GO terms and KEGG pathways in the data sets (Huang et al., 2009a, 2009b).

2.7. Proximity extension assay (PEA) technology

Inflammatory proteins released into culture media were measured