Supplementary article data

Toll-like receptors in human chondrocytes and osteoarthritic cartilage

Tarvo Sillat[1,2,5], Gonçalo Barreto[1], Paul Clarijs[1], Antti Soininen[3], Mari Ainola[1], Jukka Pajarinen[1,2], Matti Korhonen[2], Yrjö T Konttinen[1,3,4], Regina Sakalyte[1], Mika Hukkanen[2], Pekka Ylinen[3], and Dan C E Nordström[1]

[1]Institute of Clinical Medicine, Department of Medicine, Helsinki University Central Hospital; [2]Institute of Biomedicine, Department of Anatomy, University of Helsinki; [3]ORTON Orthopaedic Hospital of the ORTON Foundation, Helsinki; [4]COXA Hospital for Joint Replacement, Tampere, Finland; [5]Radiology Centre, North Estonia Medical Centre, Tallinn, Estonia.
Correspondence: dan.nordstrom@hus.fi
Submitted 13-01-11. Accepted 13-08-29

Primary chondrocyte isolation and cultures

Articular cartilage was cut to ~ 1 mm^3 samples, washed 3x in PBS and digested with collagenase (Clostridium histolyticum type XI, 1.0 mg/mL; >1200 IU/mg; Sigma, Steinheim, Germany) in Dulbecco's modified Eagle Medium DMEM/F12 supplemented with 1mM L-glutamine, 50 U/mL penicillin G, 50 µg/mL streptomycin B and 0.3 µg/mL amphotericin B (Gibco, Life Technologies, Paisley, UK) for 16-18 h at 37°C with slow agitation. The digested explant was filtered through a 50 µm pore size nylon mesh filter, cells were washed 3x, counted, resuspended in complete DMEM/F12 with 10% foetal calf serum (FCS, BioWhittaker, Liege, Belgium), and seeded to 10 cm culture dishes maintained at 37°C in a humidified 5 % CO_2-in-air. After an initial expansion for 1-2 weeks, chondrocytes were trypsinised and stored in liquid nitrogen. Passage 1-2 cells were used.

hMSC isolation and chondrogenic cultures

Mononuclear cells were isolated from 10 mL bone marrow aspirates using a density gradient (Ficoll-Paque Plus, GE Healthcare, Uppsala, Sweden) and plated at $4x10^6/cm^2$ in complete culture medium consisting of low glucose DMEM (Gibco, Life Technologies, Paisley, UK) and 10% FCS (StemCell Technologies, Vancouver, Canada) with 100 U/mL penicillin and 100 µg/mL streptomycin (Euroclone, Siziano, Italy). Non-adherent cells were removed by washing after 72 h. Medium was changed twice weekly, and the passage 0 cells were harvested using TrypZean (Sigma) from subconfluent cultures, usually 14 days after plating. The cells were replated at $1000/cm^2$ in complete culture medium and passaged when subconfluent. Multipotency of the established cell-lines was tested with standard osteogenic and adipogenic protocols.

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA from cells was isolated using TRIzol reagent (Invitrogen, Paisley, UK) and mRNA using magnetic Oligo$(dT)_{25}$ polystyrene beads (Dynal, Oslo, Norway). Messenger RNA concentrations were measured spectrophotometrically and cDNA was synthesized from 50 ng mRNA using oligo(dT)12-18 primers and SuperScript enzyme, followed by RNase H treatment (SuperScriptPreamplification System; Invitrogen). Quantitative RT-PCR was run in a LightCycler PCR machine using LightCyclerFastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany), twice for all samples. Primers were designed with Primer3 (SourceForge), the sequences were searched with the NCBI Entrez search system, and sequence similarity search was done using the NCBI Blastn program. Primer sequences used were 5'-GCCGCGGCTGGTACCTTGTAAT-3' and 5'-AGCCAGGTGCTCAAAGGCTAC-3' for SOX9 (amplicon length 301 bp), 5'-TGACCTTTGACACCAGGAAG-3' and GATGGCTGGAGGATTTGATG-3' for COL2A1 (321 bp), 5'-GACAAGCCTGTAGCCCATGT-3' and 5'-TTGATGGCAGAGAGGAGGTT-3' for TNF-α (266 bp), 5'-TTAGGAACGTGGATGAGACC-3' and 5'-ATGCTGCTGTTCAGCTCTTC-3' for TLR1 (amplicon length 316 bp), 5'-TGACCTGTCCAACAACAGGA-3' and 5'-TTCCCACTCTCAGGATTTGC-3' for TLR2 (302 bp), 5'-TCAACCAAGAACCTGGACCT-3' and 5'-CAGCCACCAGCTTCTGTAAA-3' for TLR4 (238 bp), 5'-ACCTGGCCTTCTTTAAGTGG -3' and 5'-TAGTATTTGCAGGGCACTCG-3' for TLR9 (275 bp) and 5'-TCACCCACACTGTGCCCATCTACGA-3' and 5'-CAGCGGAACCGCTCATTGCCAATGG-3' for β-actin (295 bp). For the

DOI 10.3109/17453674.2013.854666

qRT-PCR standard curve, the gene of interest was amplified in PCR, extracted from an agarose gel, and cloned into the pCRII-TOPO vector (Invitrogen). After identification of the plasmid by restriction enzyme analysis and sequencing, the concentrations were determined spectrophotometrically, and serial dilutions were prepared for qRT-PCR analysis. The mRNA copy numbers were determined from each sample in duplicates and normalized against 10^6 β-actin genes.

Immunostaining of TLRs in chondrogenic cultures

Pelleted MSCs induced into chondrogenesis were at day 0, 7, 14 and 21 fixed in 10 % formalin and embedded in agarose, followed by dehydration in ethanol series, clearing in xylene and embedding in paraffin. Samples were cut using a microtome (Leica RM 255, Leica, Microsystems, Wetzlar, Germany) to 4 µm thick sections on microscope slides.

Antigens were retrieved from depparifinized and ethanol dehydrated sections by coating the sections with 10 mM sodium citrate buffer, pH 6, followed by AR 98ºC-S30M program for 24 min in MicroMed T/T Mega Laboratory Microwave Systems (Milestone, Sorisole, Italy).

After quenching endogenous peroxidase in 0.3% H_2O_2 in deionized water for 25 min, sections were blocked with normal goat serum (Vectorlabs, Burlingame, CA) diluted in 0.1 % bovine serum albumin (BSA) in 10 mM phosphate buffered 150 mM NaCl, pH 7.4 (PBS) for 1 h. Sections were incubated 1) overnight at 4°C in 0.8 µg/mL rabbit anti-human TLR1 IgG (H-90), 2.6 µg/mL rabbit anti-human TLR2 IgG (H-175), 0.5 µg/mL rabbit anti-human TLR9 IgG (H-100) (all purchased to Santa Cruz Biotechnology, Santa Cruz, CA), 6.65 µg/mL mouse anti-human type II collagen IgG1 (Chemicon, Temecula, CA, USA) or 1:1000 mouse anti-human COL2A-3/4M IgG1 (Ibex, Montreal, Canada), diluted in 0.1 % BSA-PBS; 2) biotinylated goat anti-rabbit or horse anti-mouse IgG (Vectorlabs, Burlingame, CA, USA) and 3) avidin biotin peroxidase complexes. Colour was developed using H_2O_2 and 3,3'-diaminobenzidine (DAKO A/S, Glostrup, Denmark) for 10 min, followed by counterstaining in Mayer hematoxylin (Merck, Darmstadt, Germany) for 1 min. After washes in deionized water the slides were dehydrated in ethanol series, cleared in xylene and mounted. For negative staining controls non-immune IgG was used instead of and at the same concentration as the primary specific antibodies. All incubations were performed at 22°C, with PBS washes in between the steps if not otherwise stated.

Safranin O staining and OARSI grading of OA tissue samples

Osteochondral cylinders were obtained from total knee arthroplasty operations of 14 patients (10 female and 4 male) after a written informed consent. The tibial plateau was fixed to an in-house purpose designed sample holder and samples were taken from different areas of the joint surface using a hollow 9 mm bore attached to a power drill.

Osteochondral cylinder samples were fixed in neutral buffered 10% formalin for 2 weeks and decalcified in 10% EDTA, pH 7.4, for 5 weeks at 4°C (until bone tissue was pliable) before dehydration in ethanol series, clearing in xylene and embedding in paraffin. Samples were cut using a Leica RM 255 microtome (Leica, Wetzlar, Germany) to 3 µm thick sections on microscope slides.

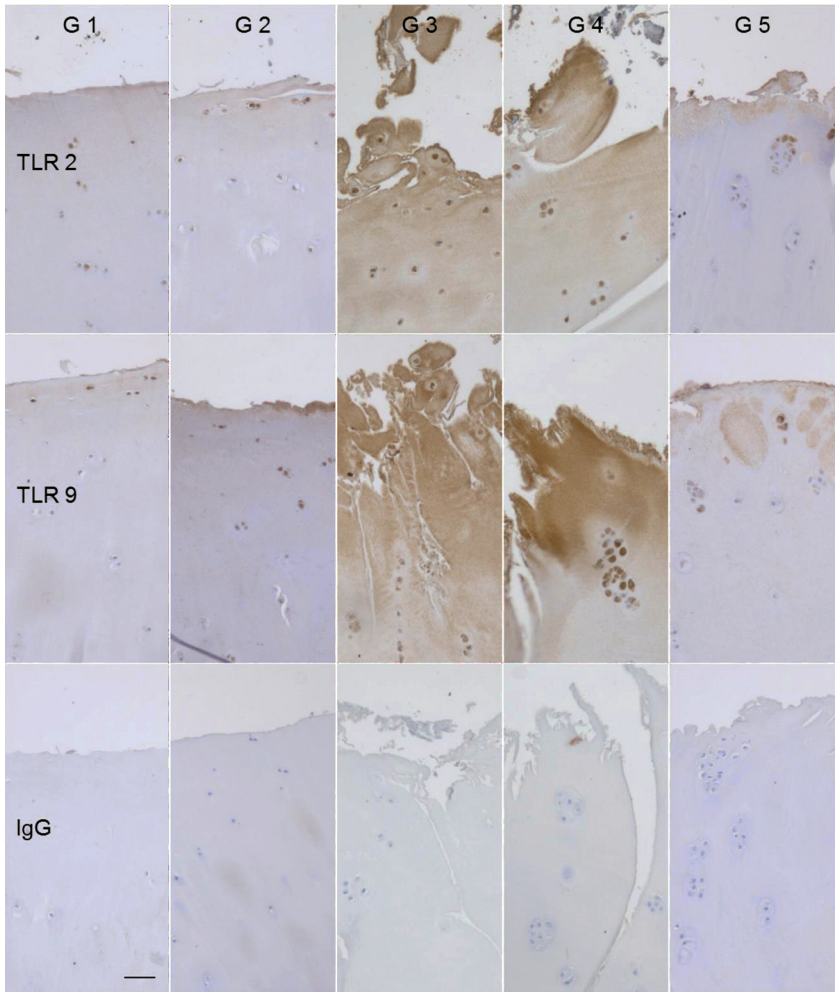
After deparaffinization in xylene and ethanol rehydration OA tissue sections (and also the 3D-cartilage pellets produced in vitro from human bone marrow MSC) were stained using Safranin O and toluidine blue. OA tissue sections were graded based on histopathology and OARSI grades evaluated blindly by three independent researchers from coded samples using light microscope (Pritzker et al. 2006); grade 1: 3 samples; grade 2: 8 samples; grade 3: 10 samples; grade 4: 6 samples; grade 5: 2 samples. Our samples did not contain any totally healthy grade 0 cartilage samples. Grade 6 OA samples only contain subchondral bone and, therefore, grade 6 samples lacking cartilage were not analyzed.

Samples were studied using Nikon LV-DIA-BASE microscope (Nikon, Tokyo, Japan) with a motorized XY-stage (Scan Prior III, Prior, Rockland, MA, USA), connected to a DS-Fi1 digital camera, using Nikon Imaging Software (NIS) - Element Basic Research (BR) analysis (Nikon, Tokyo, Japan). This enabled capture of several images (taken using 100x Magnification) in a defined sequence and mounting them together to one composite microphotograph.

Immunohistochemical staining of different OARSI-grade OA cartilage tissue sections

Representative samples from all different OARSI-grades were used in immunohistochemistry. After deparaffinization and dehydration, antigens were retrieved in 10 mM citrate buffer, pH 6.0, for 1 hour at 70°C, because otherwise the relatively stiff cartilage-bone tissue sections detached from the microscope slides. After quenching of the endogenous peroxidase in 0.3% H_2O_2 in deionized water for 25 min, sections were blocked with 10% normal goat serum (Vectorlabs, Burlingame, CA) diluted in 0.1% BSA-PBS for 1 h. Immunohistochemistry was done using the ABC method described above, using 2 µg/mL rabbit anti-human TLR1 IgG (H-90), 1 µg/mL rabbit anti-human TLR2 IgG (H-175), 1 µg/mL rabbit anti-human TLR4 IgG (H-80),1 µg/mL rabbit anti-human TLR9 IgG (H-100) (all TLR antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA) and 10 µg/mL mouse anti-human TNF-α (Research Diagnostics Inc., Concord, MA) as the primary antibodies. The MSC markers were stained using monoclonal 10 µg/mL mouse anti-human CD105 IgG2a (Abcam, Cambridge, UK) and 1 µg/mL rabbit anti-human CD166 IgG (Abcam, Cambridge, UK), and the hematopoietic stem cell markers were stained using 18 µg/mL monoclonal mouse anti-human CD31 IgG1 (Dako, Glostrup, Denmark) and 2 µg/mL monoclonal mouse anti-human CD34 IgG1 (Cymbus Biotech Ltd, London, UK).

Samples were evaluated using a Leica DM6000 B/M microscope (Leica Microsystems, Wetzlar, Germany) with a motorized Leica XY-stage connected to a Leica DFC 420 digital camera using Leica Application Suite version 3.8.0 software. The percentages of TLR positive cells were evaluated from at least 5 high magnification (200x) fields and separately in the superficial, middle and deep zone of the hyaline articular cartilage.



Supplementary figure. TLR2 and TLR9 immunostaining and negative staining controls (third row) of OARSI-graded osteoarthritis (OA) samples (grades G1–G5). Magnification 100×.