

NOD2 signaling pathway was involved in fibronectin fragment-induced pro-catabolic factor expressions in human articular chondrocytes

Hyun Sook Hwang^{a,b,#}, Mi Hyun Lee^{a,b,#}, Min Ha Choi^{a,b}, Hyun Ah Kim^{a,b,*}

^a Division of Rheumatology, Department of Internal Medicine, Hallym University Sacred Heart Hospital, Kyunggi, 14068, Korea; ^b Institute for Skeletal Aging, Hallym University, Chunchon, 24251, Korea

Running title: pro-catabolic responses via NOD2 signaling pathway

Keywords

Fibronectin fragments, osteoarthritis, NOD2, IL-6, IL-8, matrix metalloproteinase

#These authors equally contributed to this work.

***Corresponding author:**

Name: Hyun Ah Kim

Address: Division of rheumatology, Department of internal medicine, Hallym University Sacred Heart Hospital, 896, Pyungchon, Anyang, Kyunggi, 14068, Korea

Phone: 82-31-380-1826

Fax: 82-31-381-8812

E-mail: kimha@hallym.ac.kr

Materials

Recombinant human 29-kDa FN-f and an antibody against β -actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were from Cell signaling Technology (Danvers, MA, USA): Myd88, NOD2, RIP2, SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), p38 MAPK (p38), phospho-p38 (Thr180/Tyr182), p44/42 MAPK (Erk1/2), phospho-ERK1/2 (Thr202/Tyr204), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha ($\text{I}\kappa\text{B}\alpha$) and phosphor- $\text{I}\kappa\text{B}\alpha$ (Ser32). An antibody against TLR-2 was obtained from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Small interfering RNAs (siRNAs) against TLR-2 and NOD2 were purchased from Bioneer (Daejeon, South Korea). Primers for NOD1, NOD2, IL-6, IL-8, TLR-2, MMP-1, -3, and -13, and GAPDH were obtained from Cosmo Genetech Co. (Seoul, South Korea). Recombinant human TNF- α and IL-1 β were obtained from R&D Systems (Minneapolis, MN, USA).

Cartilage collection, primary chondrocyte culture, and cartilage explant culture

Cartilage samples were obtained from the knee joints of OA patients at the time of total knee replacement surgery. Patient diagnoses were determined using the criteria developed by the American College of Rheumatology. The collection and use of human tissue samples was reviewed and approved by the Institutional Review Board of Hallym University Sacred Heart Hospital, Anyang, South Korea (approval number 2013-I022). All patients provided written informed consent for the use of their discarded cartilage samples.

Chondrocytes were isolated by carefully dissecting articular cartilage from a relatively lesion-free area, followed by sequential digestion with a protease from *Streptomyces griseus*, collagenase from *Clostridium histolyticum*, and hyaluronidase from bovine testes (Sigma-Aldrich). Chondrocytes were maintained in monolayer culture in Dulbecco's modified Eagle's

medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. First-passage primary chondrocytes were used for all experiments within 1 week after seeding.

IHC analysis

Freshly dissected normal and OA human cartilage tissues were fixed with 4% paraformaldehyde, decalcified, and embedded in paraffin. Five- μ m-thick sections were blocked with 3% bovine serum albumin (BSA) at room temperature for 1 h, followed by incubation with an antibody against NOD2 (1:100 dilution) for 16 h at 4°C. Sections were incubated with biotinylated secondary antibody for 30 min, treated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 30 min, and visualized with 3,3'-diaminobenzidine. The tissue sections were mounted and observed under a microscopy (Nikon, Tokyo, Japan).

Transfection with siRNA

Transfection of chondrocytes with siRNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Human chondrocytes were transfected with control (sense, 5'-CCU-ACG-CCA-CCA-AUU-UCG-U-3'; antisense, 5'-ACG-AAA-UUG-GUG-GCG-UAG-G-3'), NOD2 (sense, 5'-UAU-UGU-UAU-CGC-GCA-AAU-ACA-GAG-C-3'; antisense, 5'-GCU-CUG-UAU-UUG-CGC-GAU-AAC-AAU-A-3'), and TLR-2 (sense, 5'-GGC-UUC-UCU-GUC-UUG-UGA-C-3'; antisense, 5'-GUC-ACA-AGA-CAG-AGA-AGC-C-3') at a concentration of 50 nM for 48 h using Lipofectamine 2000. Cells were then exposed to 29-kDa FN-f (300 nM) for 6 or 24 h and used in all subsequent experiments.

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

Total RNA was extracted from chondrocytes or cartilage tissues using TRIzol reagent as previously described (37). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed using a StepOnePlus real-time PCR system with the following primers: IL-6 forward, 5'-TAC-CCC-CAG-GAG-AAG-ATT-CC-3'; IL-6 reverse, 5'-TTT-TCT-GCC-AGT-GCC-TCT-TT-3'; IL-8 forward, 5'-CTG-GCC-GTG-GCT-CTC-TTG-3'; IL-8 reverse, 5'-CTT-GGC-AAA-ACT-GCA-CCT-TCA-3'; TLR-2 forward, 5'-TTG-TGA-CCG-CAA-TGG-TAT-CTG-3'; TLR-2 reverse, 5'-GCC-CTG-AGG-GAA-TGG-AGT-TT-3'; NOD1 forward, 5'-TTC-CGT-GCT-GCC-TTT-GAA-G-3'; NOD1 reverse, 5'-GAC-ATC-TGT-CAG-GGT-CAT-CGT-3'; NOD2 forward, 5'-CTG-TCC-AGA-CCC-TGC-TCT-TC-3'; NOD2 reverse, 5'-CAG-AGA-AGC-CCT-TGA-GGT-TG-3'; MMP-1 forward, 5'-CCT-CGC-TGG-GAG-CAA-ACA-3'; MMP-1 reverse, 5'-TTG-GCA-AAT-CTG-GCG-TGT-AA-3'; MMP-3 forward, 5'-AGG-CAT-CCA-CAC-CCT-AGG-TTT-3'; MMP-3 reverse, 5'-ATC-AGA-AAT-GGC-TGC-ATC-GAT-3'; MMP-13 forward, 5'-CCT-TCA-AAG-TTT-GGT-CCG-ATG-T-3'; MMP-13 reverse, 5'-CAG-CAA-TGC-CAT-CGT-GAA-GT-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-ATG-GAA-ATC-CCA-TCA-CCA-TCT-T-3'; GAPDH reverse, 5'-CGC-CCC-ACT-TGA-TTT-TGG-3'. GAPDH was used as an internal control.

Immunoblot analysis

Proteins from chondrocytes were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer (Biosesang, Kyunggi, South Korea) and protein concentrations were quantified using bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a polyvinylidene difluoride membrane (Bio-Rad

Laboratories, Hercules, CA, USA). The membrane was blocked with 5% (w/v) nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with primary and secondary antibodies at the appropriate dilutions. The membrane was developed using an enhanced chemiluminescence kit (GE Healthcare Life Science, Buckinghamshire, UK).

Immunoprecipitation (IP) assays

Proteins were extracted from chondrocytes with RIPA lysis buffer and the supernatants were obtained by centrifugation at $13,000 \times g$ for 10 min at 4°C. The supernatant containing equal amounts of proteins were pre-incubated with protein A agarose bead slurry for 4 h at 4°C on a rotating shaker and then incubated with specific antibodies against RIP2 or IgG in the presence of protein A agarose beads at 4°C overnight with gentle rotation. Proteins eluted in 2x SDS sample buffer were separated with SDS-PAGE and subjected to immunoblot analysis as described above. A portion of the protein extracts was used as an input control.

Statistical analysis

Data are expressed as the mean \pm standard deviation (SD). Statistical analyses were performed using Mann–Whitney *U* test or two-way analysis of variance (ANOVA) in GraphPad Prism 6 (GraphPad software). A value of $P < 0.05$ was considered statistically significant.