114-3-3E, a new alarmin candidate, elicits a catabolic and proinflammatory effect involving innate immunity through TLR signaling in osteoarthritis

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Abstract OARSI 2019

14-3-3ε, A NEW ALARMIN CANDIDATE, ELICITS A CATABOLIC AND PROINFLAMMATORY EFFECT INVOLVING INNATE IMMUNITY THROUGH TLR SIGNALING IN OSTEOARTHRITIS.

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Purpose: Osteoarthritis (OA) is now considered as a whole-joint disease characterized by cartilage degradation, synovial inflammation and bone remodeling. Recent studies have shown the implication of the innate immune system and low-grade inflammation in OA progression. Our team identified 14-3-3ε as a novel soluble mediator critical in the communication between subchondral bone and cartilage in OA. This protein acts as a potent MMP-3 and MMP-13-stimulatory factor in chondrocytes leading to catabolic phenotype. 14-3-3ε seems to share common characteristics with alarmins, endogenous molecules with intracellular functions that are released in the extracellular media after infection or tissue damage. Alarmins (such as S100 and HMGB1 involved in cartilage degradation) bind to receptors expressed on the surface of cells engaged in host defense and tissue repair. These receptors include the Toll-like receptors, TLR2 and TLR4. Moreover, innate alarmins can stimulate the activation of resident immune cells of the synovium, particularly macrophages leading to synovitis. As well as alarmins, 14-3-3ε could determine macrophage polarization. Our aim is to investigate the interactions between 14-3-3ε and its potential receptors, TLR2 and TLR4.

Methods: Primary cultures of chondrocytes were performed from murine cartilage of the C57BL/6J mice (5 days old), and from human cartilage samples, obtained from OA patients undergoing total joint replacement surgery. The receptors TLR2 and TLR4 were blocked by monoclonal antibodies and pharmacologic inhibitors in chondrocytes (15mn) and then stimulated by recombinant 14-3-3ε during 24h. The stimulation by 14-3-3ε was also done on primary cultures of chondrocytes from TLR4 KO or TLR2 KO mice pups. Stimulation by 14-3-3ε has been performed with addition of Polymyxin B to avoid endotoxin effects. Immunocytochemistry was realized on murine chondrocytes with receptors antibodies. Chondrocyte gene expression and release of MMP-3, MMP-13 and IL-6 were evaluated by RT-PCR and ELISA. The direct interaction of 14-3-3ε with TLR2 was measured using Surface plasmonic resonance (SPR) on a Biacore 3000 instrument. Their molecular interaction was modeled *in silico* using GrammX and Rosetta programs to model the interaction and optimize the predicted conformations, respectively. The best complexes of 14-3-3ε with TLR2 were visually analyzed using PyMOL. M1 polarization of macrophages was performed by activating THP1 cells (ATCC) with PMA during 24h and and followed by 24h stimulation with LPS or 14-3-3ε. Different markers of the M1 type were analysed by ELISA and RT-qPCR to identify the polarisation of macrophages (**M1**: TNF, IL-6, IL-1β, MCP1, Nos2).

Results: TLR2 and TLR4 receptors were found on murine articular chondrocytes by immunocytochemistry using monoclonal antibodies. Invalidation of TLR2 and TLR4 receptors by blocking antibody in articular chondrocytes reduced significantly mRNA expression and protein release of MMP-3, MMP-13 and IL-6 induced by 14-3-3ε. For MMP3

mRNA expression, the inhibition rate reached 78% with blocking antibodies and 98% with OxPAPC, a TLR2/TLR4 inhibitor. The inhibition rate reached 65% (blocking antibodies), 98% (OxPAPC) for MMP13 and 74% (blocking antibodies) and 93% (OxPAPC) for IL-6. ELISA experiments showed an inhibition rate of 84% (blocking antibodies) and 97% (OxPAPC) for MMP3. Similar rates were observed for IL-6 secretion (83% and 97%). Moreover, the effect of 14-3-3ε on MMP3, MMP13 and IL6 expression was reduced in TLR4-KO and abolished in TLR2-KO chondrocytes. In the Biacore analysis, human recombinant TLR2 and TLR4 were covalently immobilized on CM5 chip. Results demonstrated a direct interaction between recombinant TLR2 and 14-3-3ε (83 RU) and between TLR4 and 14-3-3ε (41 RU). Computational structural analysis identified *in silico* a putative region of interaction, centered on a phosphorylation site in TLR2. This region of binding is consistent as 14-3-3ε is known to accommodate specifically phosphorylated residues. The affinity measurements between 14-3-3ε and peptides of TLR2 designed accordingly to this phospho-site are currently in progress. Finally, polarization of macrophages stimulated by recombinant 14-3-3ε seems to polarize them toward a M1 inflammatory phenotype.

Conclusion: We have discovered 14-3-3ε as a new catabolic factor, produced by the subchondral bone and able to induce a catabolic phenotype on chondrocytes. These effects are mediated, at least in part, by TLR2 or TLR4 signalling, leading to the activation of innate immunity. Taken together, our results designate 14-3-3ε as a novel alarmin in OA, triggering catabolic and inflammatory effects through TLRs signaling. This new alarmin candidate could represent a new target either for therapeutic and/or prognostic purposes.