

Osteoarthritis and Cartilage



Oral Presentations OARSI 2014

1 SOD2 DEFICIENCY IN CHONDROCYTES ACCELERATES AGE-RELATED OSTEOARTHRITIS IN MICE

M. Koike^{†‡}, H. Nojiri[‡], Y. Ozawa[‡], K. Watanabe[‡], I. Masuda[‡], Y. Muramatsu[§], H. Kaneko[‡], D. Morikawa[‡], K. Kobayashi^{†‡}, Y. Saita[‡], T. Sasho[§], T. Shirasawa^{||}, K. Yokote[¶], K. Kaneko[‡], T. Shimizu[†]. [†]Dept. of Advanced Aging Med., Chiba Univ. Graduate Sch. of Med., Chiba, Japan; [‡]Dept. of Orthopedics, Juntendo Univ. Graduate Sch. of Med., Tokyo, Japan; [§]Dept. of Orthopedics, Chiba Univ. Graduate Sch. of Med., Chiba, Japan; ^{||}Dept. of Aging Control Med., Juntendo Univ. Graduate Sch. of Med., Tokyo, Japan; [¶]Dept. of Clinical Cell Biology and Med., Chiba Univ. Graduate Sch. of Med., Chiba, Japan

Purpose: Superoxide dismutase 2 (SOD2) is localized in mitochondrial matrix to regulate mitochondrial superoxide. SOD2 expression is significantly down-regulated in the articular cartilage of osteoarthritis (OA) patients. However, pathophysiological role of SOD2 in chondrocytes has not been fully elucidated yet. To investigate the protective role of *Sod2* in chondrocytes *in vivo*, we generated chondrocyte-specific *Sod2*-deficient (*Sod2*-cKO) mice. In the present study, we investigated cartilage pathologies under mechanical stress and aging conditions and cellular phenotypes as well as OA-related gene expression. The aim of this study is to conclude whether SOD2 loss in chondrocytes enhances OA progression.

Methods: Chondrocyte-specific *Sod2* cKO mice were newly generated using a Cre-loxP system (*Col2a1*-Cre;*Sod2*^{fl/fl}). Destabilization of medial meniscus (DMM) was created by surgical transection of the medial meniscotibial ligament under a microscope in knee joints of cKO and *Sod2*^{fl/fl} mice at eight weeks of age. Furthermore, we analyzed knee joints of cKO and *Sod2*^{fl/fl} at 12 months of age as an age-associated OA model. OA pathologies of knee joints were histologically evaluated using the OARSI histopathology grade at eight weeks after DMM surgery and at 12 months of age without surgery. To quantify oxidative damages in chondrocytes *in vivo*, superoxide production in articular chondrocytes isolated from adult cartilage was measured with dihydroethidium using a flow cytometry. Furthermore, to clarify the biological consequence of *Sod2* deficiency in chondrocytes *in vitro*, cellular phenotypes were evaluated in primary articular chondrocytes isolated from knee joint of neonate cKO and *Sod2*^{fl/fl} littermates. Using a flow cytometry, mitochondrial membrane potential was measured with JC-1 staining. In addition, gene expression of anabolic and catabolic genes was also analyzed by qRT-PCR and proteoglycan levels were quantified with alcian blue staining.

Results: We detected increased mitochondrial superoxide generation in articular chondrocytes of adult *Sod2* cKO mice compared with the *Sod2*^{fl/fl} littermates. Although *Sod2* cKO mice showed no obvious skeletal abnormalities, *Sod2* loss exacerbated cartilage degeneration in knee joints at eight weeks after DMM surgery. Notably, *Sod2* loss spontaneously accelerated OA pathologies in the mutant mice at 12 months of age. *In vitro* experiments confirmed that SOD2 insufficiency impaired mitochondrial membrane potential associated with increased mitochondrial superoxide generation. Gene expression analyses also revealed that anabolic genes, including *Col2a1* and *Acan*, were significantly down-regulated, while catabolic genes, including *Mmp13* and *Adamts5*, were significantly up-regulated. Finally, alcian blue staining

revealed a significant decrease of proteoglycan in *Sod2*-deficient chondrocytes.

Conclusions: *Sod2* deficiency caused mitochondrial superoxide overproduction and dysfunction resulted in cartilage degeneration via impaired proteoglycan homeostasis under mechanical stress and aging conditions. Our findings revealed that SOD2 plays a protective role in OA development and progression in aging.

2 ACUTE AND SUSTAINED MOLECULAR CHANGES IN SYNOVIAL FLUID FOLLOWING ACUTE KNEE INJURY MIRROR THE MURINE JOINT INJURY RESPONSE.

F.E. Watt[†], E. Paterson[‡], A. Freidin[†], J. Saklatvala[†], A. Williams[‡], T.L. Vincent[†]. [†]Kennedy Inst. of Rheumatology, Univ. of Oxford, Oxford, United Kingdom; [‡]Chelsea & Westminster Hosp., London, United Kingdom

Purpose: Acute joint injury predisposes to OA, and is an ideal setting in which to study early disease pathogenesis. We have shown that an immediate inflammatory response occurs in connective tissues after injury, and a discrete group of mRNAs is up-regulated within hours of medial meniscal destabilisation (DMM) of the mouse joint. Some of this molecular response appears necessary for subsequent OA. We investigated a) whether these same molecules were up-regulated in the joint, and in the blood in the analogous human setting of acute knee injury, and b) how this molecular response varies, between individuals and over time.

Methods: KICK (the Knee Injury Cohort at the Kennedy) has recruited young (aged 16–50) active individuals with a recent (within 8 weeks) history of acute structural knee injury and associated effusion. Those with existing OA, other inflammatory co-morbidities, or recent surgery or other trauma were excluded. In planned validation work, 7 candidate molecules (selected as up-regulated following murine DMM and known to be secreted) were measured in the first 50 participants where both blood and synovial fluid samples were available at baseline. Control synovial fluid (from healthy joints at amputation) and blood (healthy age- and sex-matched volunteers) were compared. Serum or plasma (depending on assay) and synovial fluid were analysed by validated plate ELISA or Mesoscale Delivery™ platform. Clinical data were stored in a secure online database (SCTR, HSS, USA). Analysis was carried out using Excel and Graphpad prism. Statistical testing was by Mann Whitney U test or ANOVA, with Dunnett's correction; $P < 0.05$ was considered significant. Spearman R correlation coefficient for plasma/serum and synovial fluid pairs was calculated.

Results: 5/7 of the molecules from the murine studies were also significantly elevated in human synovial fluid immediately after joint injury. These were IL-6, MCP-1, MMP-3, TIMP-1 and TSG-6. IL-1 β was below the limit of detection for the assay used. SAA-1 was found in far greater amounts in blood than synovial fluid, reflecting the difference between the murine tissue SAA-3, and the serum acute phase human protein SAA-1. MMP-3 was markedly (between 10 and 100 fold) elevated in all KICK synovial fluid samples; other molecules were differentially raised in many, but not all individuals. A significant negative relationship existed for most, but not all molecules with the time from injury to sampling (MMP-3 showed no such association). In contrast, none of the analytes were significantly higher in KICK blood than

controls. Furthermore, there was no significant correlation between the paired serum and synovial fluid levels at baseline (Spearman $R < 0.2$ for all markers). The positive predictive value of a raised serum level of IL-6 or TSG-6 for an elevated synovial fluid level respectively were 80% and 89%, but with negative predictive values of 49% and 20%. At 3 months from baseline, analytes generally remained low in blood, and had also normalised in synovial fluid, where the latter was available. Exceptions were MMP-3 and TSG-6, where elevated levels persisted in the serum and synovial fluid in a proportion of individuals at this later time.

Conclusions: Our findings validate the utility of the murine post-traumatic OA model in identifying potential human joint injury response biomarkers. In this translational setting, analysis of synovial fluid rather than blood appears essential for the accurate assay of cellular products secreted into the joint in response to injury. Neither serum nor plasma levels are clinically sensitive enough to be surrogates for the intra-articular response. Investigation of the pathogenetic relevance and prognostic value of an individual's initial molecular response to joint injury in prospective cohorts such as KICK is essential to our understanding of post-traumatic OA.

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FIBULIN-3 IN JOINT AGING AND OSTEOARTHRITIS PATHOGENESIS

A. Hasegawa^{††}, T. Yonezawa[†], N. Taniguchi[†], K. Otabe[†], Y. Akasaki[†], T. Matsukawa[†], M. Saito[†], M. Neo[†], L. Marmorstein[§], M. Lotz[†]. [†]The Scripps Res. Inst., La Jolla, CA, USA; ^{††}Osaka Med. Coll., Takatsuki, Osaka, Japan; [§]Univ. of Arizona, Tucson, AZ, USA

Purpose: Osteoarthritis (OA) is the most common joint disease. Among the earliest lesions during OA development is disruption of the superficial zone (SZ) of articular cartilage. Our recent studies showed that the chromatin protein high mobility group protein B2 (HMGB2) is uniquely expressed in the SZ and its expression declines with aging in human and mouse joints. Deletion of the hmgb2 gene leads to increased severity of aging-related OA. HMGB2 supports SZ cell survival through interactions with Wnt signaling and maintains the immature differentiation status of these cells. To further elucidate HMGB2 functions we performed gene expression analyses and identified EFEMP1 encoding fibulin-3 as a HMGB2 regulated gene. Fibulin-3 is one of seven members of the mammalian fibulin family of glycoproteins. Fibulin-3 is expressed in cartilage and bone structures in the embryo and fibulin-3 peptides are potential biomarkers for the diagnosis of OA. However, the expression pattern and function of fibulin-3 in adult joint tissues is not well characterized. The purposes of this study were to examine fibulin-3 expression patterns in joint aging and OA and to investigate the role of fibulin-3 in OA pathogenesis.

Methods: Human knee joints were obtained at autopsy with approval of the Human Subjects Committee. Joints were processed within 72 hours post-mortem. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee. Immunohistochemical analysis was performed on human and mouse knee cartilage to determine changes in fibulin-3 expression during joint aging and in OA. To examine the role of fibulin-3 in cartilage homeostasis, aging-related OA and experimental OA (induced by transection of the medial meniscotibial ligament and the medial collateral ligament) were investigated in wild type and fibulin-3^{-/-} mice. To address whether fibulin-3 is involved in regulating chondrocyte differentiation and cell viability, human articular chondrocytes were transfected with fibulin-3-specific siRNA. Cells were harvested at 48 hours and 72 hours for quantitative PCR and Western blot analyses. Following transfection with siRNA and incubation for 72 hours, cell viability was determined using MTT assay. To further address the role of fibulin-3 during chondrogenesis, bone marrow mesenchymal stem cells (MSC) were transduced with lentivirus (LV) encoding EFEMP1 or control LV expressing LacZ, then MSC pellets were prepared and analyzed for chondrogenesis. Statistically significant differences between two groups were determined by Mann-Whitney's U test (non-parametric) or Student's t-test (parametric). Differences among 3 groups were determined by Kruskal-Wallis (non-parametric one-way analysis of variance) or Tukey-Kramer (parametric) procedures. P values of less than 0.05 were considered statistically significant.

Results: Fibulin-3 was specifically expressed in the SZ of normal cartilage in human and mouse knee joints. Fibulin-3 expression was intracellular and in the extracellular matrix (ECM) and declined with aging. Both aging-related OA and experimental OA were significantly more severe in fibulin-3^{-/-} mice compared with wild type mice. Fibulin-

3^{-/-} mice showed significantly fewer SZ cells compared with WT mice in 9 months. There was a significant reduction in chondrocyte viability following fibulin-3 knockdown under low serum conditions. Fibulin-3 expression was high in MSC and decreased during chondrogenesis. Suppression of fibulin-3 by siRNA in MSC significantly increased SOX9, collagen II and aggrecan in articular chondrocytes, while the over-expression of fibulin-3 inhibited chondrogenesis in MSC.

Conclusions: We found that fibulin-3 is specifically expressed in the SZ of articular cartilage and its expression is reduced in aging. Fibulin-3 regulates survival and differentiation of adult progenitor cells in the SZ of articular cartilage and its aging-related decline is an early event in OA pathogenesis. Preventing or restoring aging-associated loss of fibulin-3 in SZ chondrocytes has potential to delay or prevent onset of OA.

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WNT16 IS INDUCED BY OXIDATIVE STRESS FOLLOWING CARTILAGE INJURY AND CONTRIBUTES TO CARTILAGE HOMEOSTASIS IN OSTEOARTHRITIS

G. Nalesso[†], B.L. Thomas[†], J. Sherwood[†], G. Schett[†], L. Dale[§], O. Addimanda^{||¶}, N. Eltawil[#], C. Pitzalis[†], F. Dell'Accio[†]. [†]William Harvey Res. Inst., London, United Kingdom; [‡]Univ. of Erlangen-Nuremberg, Erlangen, Germany; [§]Univ. Coll. London, London, United Kingdom; ^{||}Rizzoli Orthopaedic Inst., Bologna, Italy; [¶]Arcispedale Santa Maria Nuova, Reggio Emilia, Italy; [#]Univ. of Edinburgh, Edinburgh, United Kingdom

Purpose: The purpose of this study is to investigate the role of Wnt16 following cartilage injury and osteoarthritis development. Our previous studies showed that Wnt16 is up-regulated upon acute trauma – a condition known to predispose to osteoarthritis (OA) – in an ex vivo setting. Activation of Wnt signalling is associated with cartilage breakdown and OA development, although the mechanisms and the ligands responsible for it are still unclear. The aims of this study, therefore, are i) to investigate whether Wnt16 contributes to the outcome of experimental OA *in vivo*; ii) The molecular mechanism leading to WNT16 up-regulation following injury and iii) identify which signalling pathway is activated by Wnt16 *in vivo*.

Methods: Articular chondrocytes were isolated from bovine metatarsal joints. The cells were then stimulated with recombinant Wnt16 at different doses and gene expression analysis performed. OA was induced by destabilization of the medial meniscus (DMM) in 8 week-old 129/Sv mice Wnt16^{-/-} or WT littermate. The animals were culled at 2, 7 days and 8 weeks post surgery. The joints were either processed for gene expression analysis, fixed for bone analysis (μ Ct) or de-calcified and paraffin embedded, for histological, morphometric, and immunohistochemical characterization. The degree of cartilage breakdown was assessed by using OARSI score by two independent investigators. Six animals/genotype were left ageing for 1 year to evaluate the development of spontaneous OA. To test the involvement to oxidative stress in Wnt16 modulation, organ cultures of articular cartilage explanted from femoral heads were treated with H2O2 in presence/absence of a NADPH oxidase inhibitor. Gene expression analysis for Wnt16 and cartilage markers performed afterwards.

Results: Wnt16 expression was upregulated at 2 and 7 days post-DMM and was associated with an increased expression of β -catenin. Modulation of the Wnt- β catenin pathway was confirmed *in vitro* on bovine articular chondrocytes stimulated with recombinant Wnt16. Wnt16-deficient mice developed more severe cartilage breakdown in comparison to WT littermate following DMM, whereas, if left unchallenged, did not develop spontaneous OA with age. Wnt16-deficient mice displayed a lower expression of lubricin, the absence of which is known to result in OA. Finally, we found that mechanical trauma to cartilage resulted in oxygen radical release, and this was required and sufficient to trigger WNT16 upregulation in chondrocytes. Indeed H2O2 alone was sufficient to up-regulate WNT16, whereas chemical blockade of NADPH oxidase inhibited WNT16 up-regulation induced by mechanical trauma.

Conclusions: We demonstrated for the first time that Wnt16 supports cartilage homeostasis following injury, and it is required to reduce degradation upon mechanical trauma. We also showed that oxidative stress, previously thought to play a pathogenic role in cartilage breakdown in the first phases of OA development, in our settings, drives Wnt16 up-regulation, suggesting a new homeostatic role for oxidative stress. Finally, we show that Wnt16 supports the expression of lubricin. Our results shed new light on the role of Wnt signalling in the articular