pathogenesis of various age-related diseases including osteoarthritis (OA). However, the mechanisms through which high AGE diets lead to cartilage breakdown are largely unknown. ADAMTS-5 (A Disintegrin and Metalloproteinase with Thrombospondin Motifs-5, aggrecanase-2) is critical for OA progression and syndecan-4 (transmembrane heparan sulfate proteoglycan) is a key regulator of ADAMTS-5 activation. Furthermore, transcriptional regulator nuclear factor kappaB (NF-kB), activated by pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), also plays a role in the activation of ADAMTS-5. In this study we test the following hypotheses: (1) high AGE diets cause cartilage degradation, at least in part, through activation of ADAMTS-5 in chondrocytes; and (2) NF-kB is a mediator of ADAMTS-5 activation for cartilage breakdown in AGE-related osteoarthritis.

Methods: Animal studies: Following an IACUC-approved protocol, C57BL6 mice (n = 12 per cohort) were fed with low or high AGE diets (low AGE diet + methylglyoxal, AGE-precursor which increases AGE formation in vivo) from birth until euthanasia at 12 or 20 months of age. Knee joints of the experimental mice were fixed in formalin and embedded in paraffin for histological evaluation of articular cartilage integrity and immunohistochemical staining. To assess whether high levels of dietary AGEs promote cartilage degradation, we performed Safranin O staining (to visualize glycosaminoglycans) and immunostaining for AGES and ADAMTS-5 in aged mice. Immunostaining of 5-7 μm thick sections was performed using polyclonal antibodies against ADAMTS-5, AGE, syndecan-4, followed by incubation with anti-rabbit secondary antibody and DAB visualization. In vitro studies: C28/I2 human chondrocytes were treated with 0.50, 100, 200, 400 μg/ml of AGE-BSA (Biovision) for 8 and 24 hours. In some experiments semi-confluent cells were treated with NF-κB inhibitor (JSH23, Santa Cruz), 20μM for 24 hr or anti-syndecan-4 antibodies (Santa Cruz), 2μg/ml for 72 hr. Total RNA was isolated and qRT-PCR was performed to analyze expression of a panel of relevant genes.

Statistical Analysis. Results are presented as mean +/- SD. Statistical analysis was carried out using a Student’s t-test with significance set at P < 0.05.

Results: Mice fed with a high AGE diet showed proteoglycan loss as indicated by reduced Safranin O staining in comparison with age-matched control mice fed with a low AGE diet. Immunostaining revealed that AGE levels in cartilage were significantly higher in the high AGE diet group compared to the low AGE group. The cartilages of high AGE diet-fed mice exhibited elevated levels of ADAMTS-5 and syndecan-4. In vitro treatment of C28/I2 human chondrocytes with AGEs mimicked the in vivo upregulation and activation of ADAMTS-5 and syndecan-4, in a time and AGE dose-dependent manner. Furthermore, inhibition of NF-κB activity resulted in suppressed levels of syndecan-4 and decreased ADAMTS-5 activity in the AGE-treated C28/I2 chondrocytes. Neutralization of syndecan-4 by anti-syndecan-4 antibodies in the AGE-treated chondrocytes suppressed activity of ADAMTS-5, but not NF-κB.

Conclusions: Our study indicates that NF-κB is a critical mediator of AGE-induced cartilage breakdown that activates ADAMTS-5 via suppression of syndecan-4. This pathway may provide a potential target for the prevention and therapeutic treatment of high AGE diet-induced OA.

220 AGGREGAN FRAGMENTS IN THE SYNOVIAL FLUID OF OSTEOARTHRITIS AND JUVENILE IDIOPATHIC ARTHRITIS PATIENTS REVEALS DIFFERENCES IN THE SPECIFICITY OF AGGREGANASE CLEAVAGE IN THE INTERGLOBULAR DOMAIN, BUT NOT IN THE CHONDROTIN-SULPHATE-RICH REGION

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Purpose: Aggrecan, an extracellular proteoglycan in cartilage, is degraded by proteolysis in joint injuries and arthritis. We compared the pattern and concentration of aggrecanase-generated aggrecan fragments in the synovial fluid (SF) between patients with juvenile idiopathic arthritis (JIA), osteoarthritis (OA), young (juvenile) knee injured patients and knee healthy reference (Ref) subjects.

Methods: SF aggrecan fragments were purified by dissociative CsCl density gradient centrifugation, collecting SF-D1 fractions, from JIA (n = 12), OA (n = 4), juvenile knee injury (n = 9) and Ref (n = 11) subjects. SF-D1 pools from corresponding subject groups were also prepared. SF-D1 samples were de-acylated and analysed by quantitative Western blot, using ADAMTS5 digested human cartilage-A1D1 fragment as ARGs-standard and bovine cartilage-A1D1 as G3-standard, with antibodies against the aggrecanase-generated ARGs neoepitope (i.e. TEGERGARGS cleavage in the interglobular domain [IGD]) or against the aggrecan G3-domain. The SF concentration of sulfated glycosaminoglycans (sGAG) was measured by Alcian blue precipitation in JIA (n = 103 samples [from 40 patients, 0.4-21 years]), OA (n = 47, 16-89 years), juvenile knee injury (n = 7, 13-15 years) and Ref (n = 10, 19-58 years) subjects. For statistics (Mann-Whitney rank sum test) the data was expressed in pmol ARGs/ml SF, pmol chondroitin-sulphate-rich region 2 (CS2)-G3 fragments/ml SF and μg sGAG/ml SF.

Results: The SF-sGAG concentration of the JIA group was significantly lower compared with levels in the OA (P > 0.001), juvenile knee injury (P = 0.003) and Ref (P = 0.033) groups. By Western blot analysis, aggrecanase generated CS2-G3 fragments (i.e. RGRG-TG3, GLGS-G3, AGE-G3 and LGQR-G3 fragments of 100 - 250 kDa) were detected in the SF-D1 samples of JIA patients (Fig. 1), and a comparison between SF-D1 pools indicated only minor variations (1 - 2 fold) of the CS2-G3 fragment concentrations between JIA and the other subject groups. These results show that aggrecanases generate similar G3-containing fragments in OA and JIA patients, albeit in varying ratios. Surprisingly, very low (or no) aggrecanase generated ARGs fragments were detected in the SF-D1 samples of JIA patients (Fig. 1). The ARGs concentration of the JIA group was significantly lower compared with the OA (P = 0.027) and juvenile knee injury (P < 0.001) groups, but was not different from the Ref (P = 0.060) group.

Conclusions: The Western blot analysis of SF aggrecan fragments in the JIA group suggests that although the pattern of aggrecan fragments derived from the CS-rich region of aggrecan is similar in OA and JIA, there is negligible aggrecanase cleavage in the aggrecan IGD of JIA patients. This is despite the fact that aggrecan in young cartilage can be cleaved by aggrecanases in the IGD, as shown by the high level of ARGs fragments detected in the juvenile knee injury group.

221 GLUCOSAMINE REGULATES AUTOPHAGY IN VITRO AND IN VIVO

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Purpose: Declining joint health as exhibited by joint pain and dysfunction may ultimately manifest as osteoarthritis (OA), the most prevalent aging-
related musculoskeletal disorder. Autophagy is an essential cellular homeostasis mechanism that is responsible for removal of dysfunctional macromolecules and organelles. Aging-associated or experimental defects in autophagy contribute to organisal and tissue-specific aging while enhancement of autophagy may protect against certain aging related pathologies such as extended lifespan in model organisms. The objective of this study was to determine whether glucosamine (GlCN), a dietary supplement that is safe and has shown beneficial effects in cellular and animal models of joint health, can activate autophagy in vitro and in vivo.

Methods: Chondrocytes from normal human articular cartilage were treated with GlCN (0.10-1.1 mM). Autophagy and mTOR activation was analyzed by Western blotting and quantification of LC3, a main marker of autophagy activation and phosphorylation levels of ribosomal protein S6 (pS6), a downstream target of mTOR. Transgenic reporter mice with fluorescent protein fused to the autophagy marker LC3 (GFP-LC3 mice) were treated with GlCN (250 mg/kg weight/day) for 7 days. Autophagy activation and mTOR signaling were analyzed by confocal microscopy and immunohistochemistry.

Results: In vitro studies: we investigated the effect of GlCN on autophagy activation by quantification of the levels of LC3-II in normal human chondrocytes. The results indicated an increase of LC3-II expression after GlCN treatment at 4 and 24 hours. To determine whether GlCN regulates the mTOR signaling pathway, we evaluated the pS6. The results indicated a reduction of pS6 after GlCN treatment in a time dependent manner at 4 and 24 hours, indicating that autophagy activation by GlCN, associated with modulation of a second main signaling event that is mediated by mTOR inhibition. In vivo studies: we analyzed in vivo effect of GlCN on autophagy using GFP-LC3 mice. Oral administration of GlCN led to a marked activation of autophagy in liver. In addition, in knee articular cartilage and menisci, there was a decrease in pS6 expression.

Conclusions: GlCN modulates several components of the mTOR and autophagy pathway in vitro and in vivo. These findings suggest that GlCN is an effective autophagy activator and motivate future studies on its efficacy in modifying aging-related cellular changes and supporting joint health.

222 IDENTIFICATION OF AN EXTRACELLULAR O-N-ACETYLGLYCOSYLASE RESPONSIBLE FOR AN INCREASED LEVEL OF O-LINKED N-ACETYLGLUCOSAMINE MODIFIED PROTEINS IN THE CARTILAGE OF PATIENTS WITH OSTEARTHROSIS.

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Purpose: The modification of serine/threonine residues on cytoplasmic and nuclear proteins by O-linked N-acetylglucosamine (O-GlcNAc) is a post-translational modification that has been implicated in the regulation of many different signal transduction pathways. Although O-GlcNAc modification has been involved in the pathophysiology of different degenerative and age-related diseases, such as Alzheimer or diabetes, its role in OA remains unknown. O-GlcNAc transferase (OGT) and O-GlcNAcase are the intracellular enzymes responsible for the level of O-GlcNAcylated proteins. The goals of this study were: (1) to determine the amount O-GlcNAcylated proteins in the cartilage of OA patients in comparison to healthy cartilage and menisci, (2) to localize the presence of OGT in the cartilage of these patients.

Methods: Human OA cartilage was isolated from the knee of patients during joint replacement surgery (n = 8), while healthy cartilage was obtained from the knee of age and sex-matched healthy donors (n = 8). To carry out further protein expression studies, chondrocytes were isolated by sequential protease digestion. Additional cartilage fragments were immediately frozen or fixed to be embedded in paraffin. The level of O-GlcNAcylated, OGT and O-GlcNAcase expression were assessed by western blot analysis employing specific antibodies. Furthermore OGT expression was localized by immunohistochemical techniques.

Results: The amount of O-GlcNAcylated proteins was significantly increased in human OA cartilage compared to that of healthy cartilage (approx. 4.5 fold, p < 0.05). So, human knee OA was characterized by the accumulation of different O-GlcNAc modified proteins. We also observed an increased expression of the short and long isoforms of OGT in the cartilage of OA patients in comparison with healthy donors (short OGT: 0.19±0.03 vs. 0.002±0.003, p < 0.05; long OGT: 0.10±0.03 vs. 0.049±0.008, p < 0.05). A prevalence of the short isoform was found in the analysis of OGT in human total cartilage, while a predominant expression of the long isoform was noted in OA chondrocytes (short OGT: 0.21±0.04 vs. 0.014±0.008, p < 0.05; long OGT: 0.48±0.09 vs. 0.13±0.07, p < 0.05). Unexpectedly, immunohistochemical studies revealed the presence of OGT both in the cartilage matrix and within cells. We observed a clear increase in the pericellular OGT signal in OA patients and an in the chondrocyte clones, while a diffuse OGT presence was noted in healthy donors. Regarding O-GlcNAcase presence, we observed a decrease in the expression of the long isoform of this enzyme in the OA cartilage and an increased presence of the short isoform in comparison to healthy cartilage samples (long O-GlcNAcase: 0.05±0.02 vs. 0.214±0.07, p < 0.05; short O-GlcNAcase: 2.59±0.30 vs. 0.04±0.035, p < 0.05).

Conclusions: These results demonstrate that OA could be associated with a deregulation in the hexosamine biosynthesis pathway that could lead to an accumulation of O-GlcNAcylated proteins in the cartilage, mainly due to an increased OGT presence. Our data would link OA pathophysiology to alterations in key glucose sensor pathways, and support the hypothesis that O-GlcNAc modification may play an important role in the development of chronic and age-related diseases.

223 P21 REGULATES MMP-13 EXPRESSION AND DECREASED AGGREGAN EXPRESSION VIA STAT3/SDF-1 PATHWAY.


[Purpose]The cyclin-dependent kinase inhibitor p21 was initially identified as a potent inhibitor of cell cycle progression. Recently, p21 was identified as not just a CKI, but also an important transcriptional regulator. p21 has been shown to regulate the activity of NF-κB, c-Myc, C/EBP, EZF and STAT3. Olive et al. reported that p21 activity was essential for the regulation of cell proliferation and inflammation after arterial injury in local vascular cells. Further, p21 regulated the expression of SDF-1 and MMP-13. These molecules are believed to be onset of osteoarthritis (OA) in articular cartilage. We previously presented that p21 mRNA expression was down-regulated in OA chondrocytes compared with normal chondrocytes. In this study, we evaluated the function of p21 in response to cyclical stretch stress.

[Methods]Cyclical stretch stress was introduced to human normal chondrocytes (NHAC-kn; cell line derived from human normal chondrocyte) for 24h (0.25 Hz) by using STREX (Osaka Japan). Chondrocytes were treated with p21 siRNA by lipofection method, and cyclical stretch stress was introduced. Col2, aggrecan, MMPs, SDF-1 mRNA expressions were analyzed by realtime PCR and phosphorylation of STAT3 was analyzed by western blotting. Pull down assay was performed to confirm binding p21 and STAT3. Chondrocytes were treated with p21 siRNA and cyclical stretch stress was introduced to chondrocytes with treatment of STAT3 specific inhibitor. Aggrecan, MMP13, SDF-1 mRNA expressions were analyzed by realtime PCR. Finally, soluble glycosaminoglycan concentrations (sGAG)/cell were analyzed after cyclical stretch stress.

[Results]Expression levels of p21, col2 and aggrecan were increased at 5% stretch stress, but decreased at 10% stress. Expression levels of MMP3, 13 were increased at 5% stress (fig 1). The expression levels of MMP-13 were increased by p21sirNA transfection after 5% stress (fig 2). However, the expression levels of aggrecan were decreased (fig 2). Phosphorylation of STAT 3 was up-regulated by down-regulation of p21 expression (Fig3a). SDF-1 expression was up-regulated by down-regulation of p21 expression (Fig3b). Pull down assay showed that p21 bound to STAT3 (Fig3c). MMP13 and SDF-1 expressions were up-regulated by p21 down-regulation, but decreased by STAT3 inhibitor (Fig4). Cell number was increased by p21 down-regulation. Total sGAG and sGAG/cell were decreased (Fig5). [Conclusion]Down-regulation of p21 increased MMP-13 expression and decreased aggrecan expression via STAT3/SDF-1 pathway. Cell proliferation was increased by p21 down-regulation, but total matrix syntheses were decreased. P21 may contribute to maintain homeostasis in normal cartilage.