A Click Chemistry Method to Determine Glycosaminoglycan Composition in Articular Cartilage

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INTRODUCTION: In articular cartilage, the most abundant proteoglycan is aggrecan, on which glycosaminoglycan (GAG) chains attach to attachment domains along its protein core. The two dominant GAG chains are chondroitin sulfate (CS) and keratan sulfate (KS) [1]. Also present in cartilage extracellular matrix is hyaluronan (HA), which unlike other GAGs never links to a protein covalently [2]. CS chains are composed of repeating disaccharide units of glucuronic acid and N-acetylgalactosamine (GalNAc). In contrast, both KS and HA GAGs have N-acetylglucosamine (GlcNAc) in their repeating disaccharide unit (Fig. 1 left) [3]. The composition of GAG chains in cartilage plays an essential role in the mechanical function of the tissue and the pathology of osteoarthritis. Here, we present a novel technique to separately quantify the synthesis rates of new CS and KS&HA GAG chains in articular cartilage, and further measure changes in the ratio of KS&HA to CS GAG chains in cartilage under inflammatory challenge.

METHODS: Cartilage explants from juvenile bovine knee joints (1-2 months old) and senior human tibial plateaus (avg. age 69.5 yr.) were harvested and cultured in chondrogenic medium. *GAG labeling by click chemistry*: New synthesis of CS and KS&HA GAG chains in cartilage was measured using a copper-free click chemistry-based assay [4]. Samples were cultured in media containing N-azidoacetyl-galactosamine-tetraacylated (GalNAz) or N-azidoacetylglucosamine-tetraacylated (GlcNAz), two azide-modified monosaccharide building blocks for the GAG disaccharide units. Chondrocytes can incorporate GalNAz into CS chains, and GlcNAz into KS&HA chains. A fluorescent dye, AZ488, was "clicked" to the azide groups on the newly synthesized GAG chains (Fig. 1). New GAG synthesis was visualized on a confocal microscope and quantified by enzymatically digesting the cartilage and normalizing the fluorescent intensity of the digested solution to the sample weight. *GAG Composition by Anatomic Location*: Bovine cartilage (4 mm diameter, 2 mm thick) was harvested from four different regions of the knee: medial and lateral condyles (MC and LC), patellar groove (PG), and tibial plateau (TP, non-loaded region). The top zone tissue was removed, leaving only middle zone cartilage. Samples were then bisected and labeled with either GalNAz or GlcNAz for 24 hrs using the click chemistry method (Fig. 3a) (n=6). *Response to Inflammation*: Bovine condylar cartilage (5 mm diameter) was split into the superficial zone (1 mm thick) and middle zone (2 mm thick). Samples were cultured for 3 days with and without pro-inflammatory cytokine IL-1β (10 ng/ml). On day 3, samples were labeled with either GalNAz or GlcNAz for 24 hrs (Fig. 4a) (n=6). *Senior Human Cartilage Response to Inflammation*: Human cartilage (5 mm diameter, 2 mm thick) was cut into quarters and labeled for 96 hrs with either GalNAz or GlcNAz, with and without the treatment of a pro-inflammatory human cytokine cocktail (5 ng/ml IL-1α, 5 ng/ml IL-1β, 50 ng/ml IL-6, and 20 ng/ml TNF-α) (Fig. 5a) (n=6).

RESULTS: Fluorescent confocal images show the juvenile bovine chondrocytes synthesized more GAG in 24 hrs than the senior human chondrocytes in 96 hrs (Fig. 2a,b). New GAGs formed a similar halo pattern surrounding the cells after both GalNAz and GlcNAz labeling. *GAG Composition by Anatomic Location*: The total amount of new GAG (GalNAz+GlcNAz) did not vary between anatomic regions. However, the ratio of KS&HA to CS in the medial condyle (0.21±0.04) was significantly lower than both the lateral condyle (0.30±0.05) and patellar groove (0.27±0.02) ($p < 0.05$) (Fig. 3c). *Response to Inflammation*: Three-day treatment with IL-1β significantly decreased all GAG synthesis, but CS synthesis decreased more than KS&HA synthesis ($p < 0.05$). Thus, in both superficial and middle zones, inflammation significantly increased the ratio of KS&HA to CS in newly synthesized GAGs ($p < 0.05$) (Fig. 3b,c). *Senior Human Cartilage Response to Inflammation*: Inflammatory cytokines reduced newly synthesized CS in human cartilage by 30% but did not affect KS&HA synthesis ($p < 0.05$), and thus increased the ratio of KS&HA to CS in newly synthesized GAG ($p < 0.05$) (Fig. 3b).

DISCUSSION: Both GalNAz and GlcNAz labeled GAGs have similar spatial distributions but differ in fluorescent intensity, which is consistent with the fact that aggrecan has both CS and KS attachment domains along the core protein, despite the different density of attached chains (Fig 1 middle). The ratio of KS&HA to CS GAG varied by anatomic location in the knee. The medial femoral condyle experiences the highest loading in the knee, and had the lowest amount of KS&HA compared to CS [5]. In both juvenile bovine and senior human cartilage, inflammation reduced CS synthesis more than KS&HA synthesis. **SIGNIFICANCE:** We present a novel method to quantify the synthesis rates of different types of GAG chains in articular cartilage under various mechanical and/or biochemical stimuli.

REFERENCES: [1] Sharma+ 2007. [2] Garantziotis and Savani 2019. [3] Li+ 2012. [4] Porter+ 2022. [5] Becker+ 2013.

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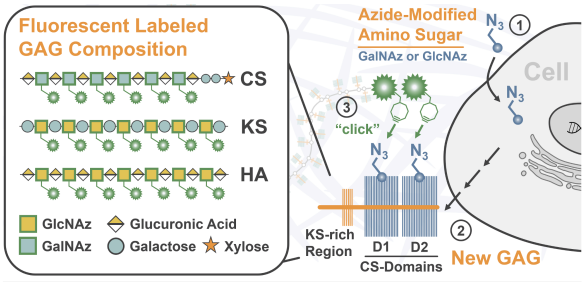

Fig. 1 Click chemistry method to quantify glycosaminoglycan composition. ① Cartilage samples are cultured for 24 hrs (bovine) or 96 hrs (human) with the azide modified monosaccharides GalNAz or GlcNAz. ② The modified monosaccharides are incorporated into newly synthesized CS or KS&HA GAG chains through chondrocyte's biosynthetic pathways. The new GAG chains bind to the proteoglycan core protein attachment domains. ③ A fluorescent dye is "clicked" onto the azide groups of the newly synthesized GAG chains in a copper-free click chemistry reaction.

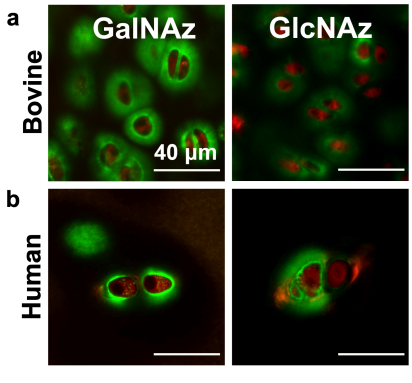

Fig. 2 Fluorescent labeled GAG chains. Newly synthesized CS (GalNAz) and KS&HA (GlcNAz) chains were labeled using the click chemistry method in (a) juvenile bovine and (b) human cartilage. The GAG chains (green) surrounding chondrocytes (red) were imaged with a confocal microscope.

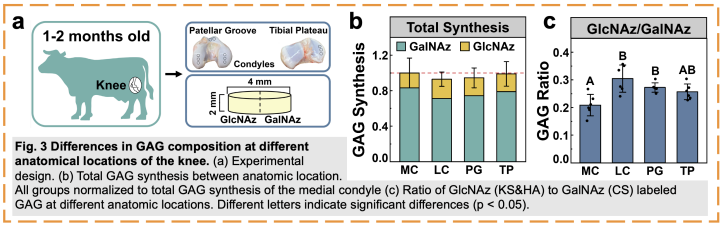

Fig. 3 Differences in GAG composition at different anatomical locations of the knee. (a) Experimental design. (b) Total GAG synthesis between anatomic location. All groups normalized to total GAG synthesis of the medial condyle (c) Ratio of GlcNAz (KS&HA) to GalNAz (CS) labeled GAG at different anatomic locations. Different letters indicate significant differences ($p < 0.05$).

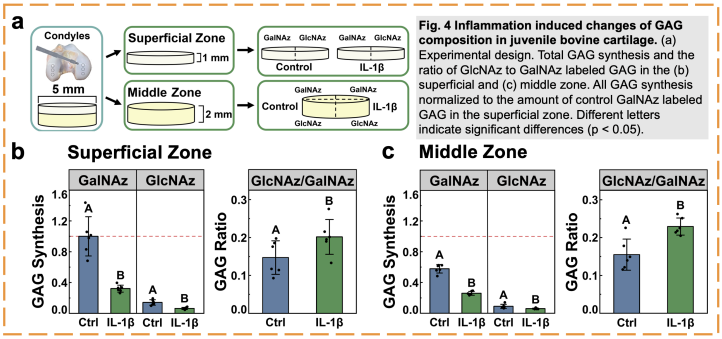

Fig. 4 Inflammation induced changes of GAG composition in juvenile bovine cartilage. (a) Experimental design. Total GAG synthesis and the ratio of GlcNAz to GalNAz labeled GAG in the (b) superficial and (c) middle zone. All GAG synthesis normalized to the amount of control GalNAz labeled GAG in the superficial zone. Different letters indicate significant differences ($p < 0.05$).

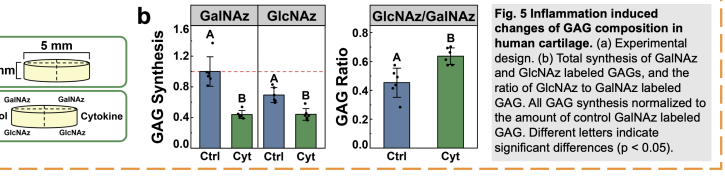

Fig. 5 Inflammation induced changes of GAG composition in human cartilage. (a) Experimental design. (b) Total synthesis of GalNAz and GlcNAz labeled GAGs, and the ratio of GlcNAz to GalNAz labeled GAG. All GAG synthesis normalized to the amount of control GalNAz labeled GAG. Different letters indicate significant differences ($p < 0.05$).