INTRODUCTION: Aggrecan provides the compressive stiffness of cartilage via electrostatic repulsion between the negatively charged glycosaminoglycans (GAGs) in the chondroitin sulfate (CS) brush region [1] and by the resistance of the CS-GAG region to fluid flow [2]. Variations in aggrecan structure are known to exist as a function of age, disease, and species [3,4]. We previously reported the resolution of cartilage aggrecan components and visualization of its individual GAG chains via atomic force microscopy (AFM) [5]. Here, we extend these initial studies and use a combination of AFM, biochemical, and polymer statistical methodologies (1) to better understand the dependence of aggrecan structure and stiffness on the properties of its constituent GAG chains, and (2) to give a detailed quantitative comparison between fetal and mature aggrecan species.

METHODS: Aggrecan (A1A1D1D1) was isolated from bovine fetal epiphyseal and mature nasal cartilage using density-gradient centrifugation [5]. Olympus AC240TS2 rectangular Si cantilevers (k = 2 N/m, tip radius <10 nm) were used for tapping mode AFM (TM AFM) in air. SigmaScan Pro image analysis software (SPSS Science) was used to quantify dimensional measurements directly from the images. Using the worm-like chain model, the persistence lengths $L_p$ of aggrecan and constituent GAG chains were calculated. $L_p$, a measure of molecular stiffness, was estimated from the variance of $\theta$, the angle formed by two consecutive vectors $l$ from the molecule trace, at several increments of $l$ (Fig. 2). To test for differences between $L_p$ of fetal and mature aggrecan and GAG, a linear mixed effects analysis (LME) was used (Insightful Corp., CA). Significance in population means for trace and end-to-end lengths were quantified using the Student’s t-test. Superox 6 Chromatography [6], fluorophore assisted carbohydrate electrophoresis (FACE) [7], and Western analyses were used to biochemically characterize aggrecan samples and their constituent GAG chains.

RESULTS: Structural Details of Aggrecan Monolayers. High density monolayers revealed the flexibility of the aggrecan as well as interdigitation of the GAG chains (Fig. 1A). Based on known GAG content, the density of aggrecan in native cartilage is ~65 molecules/μm². For low density monolayers, aggrecan monomers displayed various degrees of GAG extension (Fig. 1B). Compared to fetal aggrecan, the size and structure of mature aggrecan appeared more dimensionally heterogeneous, as manifest in the distributions of the aggrecan and GAG contour and end-to-end lengths.

Measurements and Statistical Analysis. Core protein: The extended trace lengths, $L_c$, and end-to-end lengths, $R_e$, were measured directly from the images. For the smaller core protein length ($L_c=27 \pm 73$ nm, $R_e=257 \pm 87$ nm) all were both longer with $L_c$ significantly larger ($p<0.0001$) than that of mature aggrecan ($352 \pm 88$ nm and $226 \pm 81$ nm, respectively, $n=141$). The average extension of the core protein, defined as $R_e/L_c$, was 65% and 64% for fetal and mature, respectively. GAG: Similarly, fetal aggrecan GAG chain trace length ($41 \pm 7$ nm, $n=102$) and end-to-end ($32 \pm 8$ nm) length were both significantly larger ($p<0.0001$) than that of mature aggrecan ($32 \pm 5$ and $27 \pm 7$ nm, respectively, $n=49$). GAG-GAG spacing along the core protein was smaller in fetal (3.2 ± 0.8 nm, $n=102$) compared to mature aggrecan (4.4 ± 1.2 nm, $n=40$). The resulting average GAG chain extensions were 78% (fetal) and 80% (mature). For the molecules in which the CS-brush region was well defined and distinguishable from the N-terminal bare core protein region, the contour length of each of these regions was measured separately. $L_c$ of the bare N-terminal region was found to be 93 ± 14 nm and 81 ± 17 nm for fetal and mature aggrecan, respectively. A greater difference in $L_c$ was found for the CS-brush region, 327 ± 43 nm (fetal) and 268 ± 73 nm (mature).

Persistence Length ($L_p$) of Core Protein and GAG Chain. The $L_p$ of fetal aggrecan ($L_p=110$ nm) was significantly different than that of mature aggrecan ($L_p=82$ nm) based on 95% confidence intervals. The mean $L_p$ values for fetal epiphyseal and mature nasal GAG were 21 nm and 14 nm, respectively, but were not significantly different.

Biochemical Characterization. The average chain length of GAGs from fetal aggrecan was calculated from Superox 6 chromatograms to ~50 disaccharides ($L_s=60$ nm), and was longer than that from mature aggrecan (~42 disaccharides, $L_s=50$ nm). The shorter values of $L_s$ for both fetal and mature obtained by AFM may reflect inherent differences in the methods.

FACE gel analyses of aggrecan GAG chains revealed that the fetal epiphyseal aggrecan had a CS:KS ratio three times higher than that of the mature nasal GAG. The chondroitin-4-sulfate disaccharide (C4S) amount was higher than the chondroitin-6-sulfate (C6S) for the mature aggrecan, whereas the C4S and C6S contents were essentially equal in the fetal aggrecan.

DISCUSSION: Fetal aggrecan showed a narrower distribution of molecules having longer $L_p$, whereas mature aggrecan showed a slightly shorter and broader distribution of $L_p$. In conjunction with Western analyses of both fetal and mature species [5], the trace length measurements from AFM images can be interpreted as that of predominantly full-length core protein stretched to varying degrees depending upon the conformation of the CS-brush region. Measurements of structural differences suggest that mature aggrecan has a higher glycosylation of the core protein in the Nterminal region and as a shorter CS region. It is interesting to note that the average extension values ($R_e/L_p$) calculated for fetal and mature aggrecan (both ~65%) as well as the CS-GAG chains (both ~78%) indicated that monomers and GAG chains preferred an extended arrangement. In addition, the equal extension values for both fetal and mature aggrecan suggest that the combination of GAG lengths, GAG spacing, and core protein length scaled similarly for both populations. Thus, inter- and intra-molecular GAG-GAG electrostatic double layer interactions appear to dramatically affect the structure and stiffness of the monomer. The spacing between GAGs along the mature aggrecan was 72% greater than that of the fetal monomer. The difference may be attributed to the number increase in shorter KS chains in the CS-GAG brush-region in the mature aggrecan compared to the fetal aggrecan as demonstrated by the FACE compositional analyses. The consequent reduced electrostatic repulsion between GAGs may allow for small, sub-nm collapsed regions of the core protein which are below the resolution of AFM. This may result in a shorter overall contour length leading to a smaller calculated stiffness value for the mature aggrecan. These observations were reflected in the smaller calculated $L_p$ of mature aggrecan from the linear relationship between $\theta$ and $l$ (Fig. 2).

The near-zero kurtosis of $\theta$ supported the WLC assumption of equilibrated molecules on the surface.


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Figure 1. (A) Dense monolayer of fetal epiphyseal aggrecan imaged via tapping mode AFM images in ambient conditions. (B) Fetal aggrecan showing the core protein trace length, $L_c$, and the end-to-end distance, $R_e$.

Figure 2. $\theta^*$ vs. vector length $l$(nm) for mature nasal (n=15) and fetal epiphyseal (n=15) aggrecan monomers measured from AFM images and used to calculate the persistence length $L_p$. 