Sliding Motion Improves Superficial Zone Properties of Engineered Cartilage Assessed by Friction Force and Indentation-Type Atomic Force Microscopy

1Loparic, M; 1Aebi, U; 1Stolz, M; 1Peter, R; 1Alini, M; +3Grad, S
1M.E. Müller Institute for Structural Biology, Biozentrum University of Basel, Switzerland; 2Bioengineering Sciences Research Group, School of Engineering Sciences, University of Southampton, UK; +3AO Research Institute Davos, Switzerland

INTRODUCTION: The structure and properties of the superficial zone in articular cartilage (AC) are of crucial importance for gliding and weight bearing of the joints. AC engineering aims to produce grafts with an intact and functional surface, similar to that of authentic AC. Here, we introduce two methods based on atomic force microscopy (AFM) to obtain information about the quality and function of engineered AC graft surfaces, to complement biochemical and structural analyses. First, we measured the sliding properties by friction force (FF) AFM. Next, the tensile properties of the graft surfaces were measured by indentation-type (IT) AFM at two different length scales by employing micrometer- and nanometer-size tips [1,2]. The sliding and tensile properties of the engineered AC grafts were then compared and complemented with their immunostaining patterns, scanning electron microscopy (SEM) images and glycosaminoglycan (GAG) content.

METHODS: Culturing and stimulation of constructs: Grafts were produced and mechanically stimulated as described [3-5]. Briefly, bovine articular chondrocytes (5x10^6 cells/scaffold) were seeded into porous polyurethane scaffolds (d = 8 mm; h = 4 mm). A ceramic ball (d = 32 mm) was pressed onto the scaffold to provide a preload of 0.6 mm in the center of the construct. Constructs of loading group 1 (LG1) were dynamically compressed ±0.2 mm with a frequency of 1 Hz. For loading group 2 (LG2), the ball additionally oscillated, i.e. about an axis perpendicular to the scaffold axis over the scaffold surface at ±2.5° and 1 Hz. Controls were not loaded. After 3 weeks of loading (1h/day), friction and stiffness measurements were performed at the surface of the constructs. Measuring friction and stiffness by AFM: For friction measurements, borosilicate spherical tips were coated with PEG4 undecanethiol [6,7] and glued onto tipless cantilevers (nominal spring constant k=0.6 N/m). The coefficient of friction (COF) was determined by measuring the friction force as a function of normal load. The friction force in turn was obtained from the lateral deflection of the tip when scanning over a 40x40-µm area in the direction perpendicular to the cantilever long axis, at a velocity of 40 µm/s. Friction measurements were performed in 3 different regions for each sample and repeated for 5 incremental values of normal force ranging from 10 to 50 nN. Stiffness measurements were based on recording the elastic response of the engineered cartilage using the AFM tip as either a micro- or a nanoscale indenter. Load-displacement curves were recorded at two different trigger deflections (maximal loads) for two types of indenter tips: at 50 nm for microspheres (radius 7.5 µm, k = 0.35 N/m), and at 30 nm for sharp pyramidal tips (radius 20 nm, k = 0.06 N/m). The stiffness values were measured at a cyclic loading frequency of 3 Hz and at 8 different sites for each sample surface. Immunostaining: Grafts were analyzed by immunostaining for collagen type I, type II, aggrecan, and lubricin (PRG4, superficial zone protein SZP). Scanning electron microscopy (SEM): Samples were prepared as described before [2]. Briefly, the proteoglycans from grafts were extracted in 100 mM Sorensen's buffer (pH 7.2) containing 1 mg/ml bovine hyaluronidase. Specimens were chemically fixed, dehydrated, critical point dried and sputtered with platinum before being examined by the SEM. GAG analysis: Sulfated GAG concentrations were determined by the dimethylmethylene-blue method. Statistical analysis: ANOVA was performed using SPSS, with α=0.05.

RESULTS: Constructs loaded both axially and radially (LG2) yielded the lowest COF (0.136 ±0.038), followed by the axially loaded (LG1) constructs (0.264 ±0.051), and unloaded controls (0.618 ± 0.1) (Fig. 1a). Both averaged micro- and nanostiffness were highest for the LG2 group (53.6±32.8 kPa & 39.7e±16.9 kPa), followed by LG1 (33.8±8.2 kPa & 24±15.4 kPa) and controls (13.4±2.8 kPa & 3±1.4 kPa) (Fig. 1b). Strong immunoreactivity for lubricin was observed at the top surface of LG2 (Fig. 2). Some positive cells were also visible in deeper zones. In LG1 and in unloaded controls, the cells at the surface were mostly negative for lubricin. Type II collagen staining was most intense in the LG2 constructs. Type I collagen, while appearing most intense in controls, was most distinctly visible, i.e. as a thin layer, at the surface of loaded LG2 constructs. Aggrecan staining was more prominent at surface of loaded (LG1 and LG2) than unloaded constructs, indicating increased proteoglycan accumulation. SEM images of the grafts (Fig. 3) exhibited a randomly oriented collagen fibril meshwork similar to normal cartilage. However, collagen fibrils in controls (Fig. 3a) appeared more “loose” and tangled compared to those in loaded samples. In LG1 (Fig. 3b) we observed parallel and strained collagen fibrils, whereas in LG2 (Fig. 3c) the fibrils appeared strained, crossing each other nearly perpendicularly. The amount of GAG remaining in the constructs was slightly higher in loaded (LG1: 821±261 µg; LG2: 805±235 µg) compared to control scaffolds (658±315 µg). The total GAGs synthesized, including the GAGs released, were significantly increased in loaded (LG1: 2.90±0.41 mg; LG2: 2.96±0.46 mg) compared to unloaded scaffolds (2.23±0.54 mg) (mean±sd; n=8).

DISCUSSION: Axial loading (LG1 and LG2) increases the amount of GAGs, whereas simultaneous axial loading and sliding motion (LG2) also upregulates lubricin expression at the surface, thus resulting in lower friction of LG2 compared to control grafts (low GAG, no lubricin) and LG1 (high GAG, low lubricin). SEM images reveal the orientation of the collagen fibrils, which appears consistent with our mechanical data. In contrast, quantitative immunostaining of the grafts is difficult, and SEM images do not provide direct information about their mechanical properties. Therefore, testing of the functional performance of AC should be based on mechanical testing, including friction analysis (gliding) and a multiscale mechanical testing (load-bearing) [2]. In conclusion, this study underlines the importance of an LG2-type biomechanical stimulus for the (re-)generation and maintenance of a functional AC surface. Most importantly, this study proposes and validates a quantitative AFM-based functional analysis of graft material to evaluate the quality of engineered articular cartilage surfaces.