Appendix

Methods

Tissue Division for Repair-Site Analysis

Intact hindlimbs were received on wet ice within twenty-four hours after the animal was killed. Knees were harvested, photographed, and examined grossly for cartilage fill and integration. Next, osteochondral cores (15 mm in diameter and approximately 8 to 10 mm in height) were isolated to encompass the experimental site, using a custom coring bit under irrigation with cold phosphate-buffered saline solution (PBS) supplemented with antibiotics and antimycotics (PSF [100 U/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Fungizone (amphotericin B)]). Following biomechanical testing, osteochondral slabs (2 mm in width), cut centrally from proximal to distal, were isolated. Some of the remaining cartilage, corresponding to approximately 25% of the defect repair, was removed from the bone and analyzed for chondrocyte biosynthesis. Osteochondral slabs were analyzed for cellularity, subsequently fixed in 10% neutral buffered formalin containing 1% cetylpyridinium chloride, and analyzed for matrix content and osteochondral structure.

Cartilage Metabolism

Chondrocyte glycosaminoglycan biosynthesis was assessed as ^{35}S-sulfate-incorporation. For samples with sufficient tissue (eight nonoperative control samples, two frozen allograft samples, and four fresh allograft samples), full-thickness cartilage was incubated in tissue culture medium (low-glucose Dulbecco modified Eagle medium, 10% fetal bovine serum, and PSF) containing 5 µCi/mL ^{35}S-sodium sulfate (PerkinElmer Life Sciences, Boston, Massachusetts) for forty-eight hours at 37°C in a 5% CO_2/95% O_2 humidified incubator. ^{35}S-sulfate-incorporation was expressed as counts per minute divided by milligrams per dry weight [CPM/mg][5].

Matrix Fixed-Charge Density by HE-µCT

Matrix fixed-charge density in cartilage was assessed by Hexabrix-enhanced microcomputed tomography (HE-µCT). Hexabrix, an ionic contrast agent, distributes inversely to the fixed charge density in soft tissues, and is therefore a sensitive (inverse) indicator of proteoglycan content[26]. Fixed osteochondral slabs were incubated for approximately one day in a Hexabrix (20%, by volume in PBS) solution, and then imaged in three dimensions with use of µCT (eXplore Locus MicroCT; GE Healthcare): 80 kV, voxel = $(45\ \mu m)^3$. In a sagittal slice, including the indentation site, from three-dimensional µCT datasets, cartilage and bone contours were determined by applying thresholds[27]. HE-µCT gray value (i.e., x-ray attenuation) was calculated within a region of segmented cartilage (2 mm in width) surrounding the indentation site for the overall cartilage thickness, as well as zones of cartilage, excluding two pixels at the articular cartilage surface and bone interface to minimize partial volume effects; superficial,

middle, and deep zones were defined as the top 90 μm, the next 35%, and the remaining approximately 50% of the cartilage thickness, respectively. The spatial variation in matrix fixed-charge density was illustrated with color maps representing 5% to 95% HE-μCT gray value within the cartilage.

μCT Structural Properties

Cartilage and bone structure were assessed with use of μCT, quantifying cartilage thickness, fill, surface location, and bone-cartilage interface location. Cartilage thickness was measured as the vertical height from the cartilage surface to the calcified interface, and averaged at the three indentation sites. Cartilage fill was determined as the percent area of operatively treated (OP) cartilage that was within the nonoperative control (Non-OP) repair site. Cartilage surface and bone-cartilage interface locations were determined as the average vertical height between Non-OP and OP cartilage and bone contours, respectively, with positive or negative values indicating elevation or depression, respectively. Pairs of OP and Non-OP surfaces were registered with use of custom MATLAB code[57], and parameters were evaluated within a 2.5-mm radius of the repair center. The repair center was determined as the midpoint between visually identified discontinuities in the bone, or the corresponding Non-OP location.

Histological Processing

Osteochondral slabs, previously fixed, were decalcified, embedded in paraffin, sectioned at 7 μm, deparaffinized, stained with safranin-O or hematoxylin and eosin, and digitized at ×20 magnification (Aperio ScanScope, Aperio Technologies, Vista, California). The histopathology score had a 0 to 15 scale, with high scores corresponding to degeneration. In addition, surface irregularity and safranin-O staining components of the histopathology score were determined separately to assess surface-specific structural degeneration[22] and relative matrix content, respectively.

Results

Cartilage Metabolism

Adequate tissue for glycosaminoglycan synthesis evaluation was only available for two frozen allografts, but with that limitation, biosynthesis of glycosaminoglycans by chondrocytes in cartilage varied with allograft treatment ($p < 0.05$). ^{35}S-sulfate-incorporation in nonoperative control specimens was $23.2 \pm 9.3 \times 10^3$ CPM/mg, and was similar to that in fresh allograft specimens ($p = 0.8$), which was $20.2 \pm 2.6 \times 10^3$ CPM/mg. ^{35}S-sulfate-incorporation was approximately 95% lower in frozen allograft specimens than in nonoperative control specimens (95% CI, 95% to 97%) and fresh allograft specimens (95% CI, 95% to 96%) ($p < 0.01$ versus nonoperative control specimens, $p < 0.05$ versus fresh allograft), and was $0.9 \pm 0.1 \times 10^3$ CPM/mg.