Chondroinduction Is the Main Cartilage Repair Response to Microfracture and Microfracture With BST-CarGel

Results as Shown by ICRS-II Histological Scoring and a Novel Zonal Collagen Type Scoring Method of Human Clinical Biopsy Specimens

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Background: Current cartilage repair histological scoring systems are unable to explain the relationship between collagen type II deposition and overall repair quality.

Purpose/Hypothesis: The purpose of this study was to develop a novel zonal collagen type (ZCT) 5-point scoring system to measure chondroinduction in human clinical biopsy specimens collected after marrow stimulation. The hypothesis was that the ZCT scores would correlate with the International Cartilage Repair Society–II (ICRS-II) overall histological repair assessment score and glycosaminoglycan (GAG) content.

Study Design: Descriptive laboratory study.

Methods: After optimizing safranin O staining for GAG and immunostaining for human collagen type II and type I (Col2 and Col1, respectively), serial sections from clinical osteochondral repair biopsy specimens (13 months after microfracture or microfracture with BST-CarGel; n = 39 patients) were stained and 3 blinded readers performed histomorphometry for percentage of staining, ICRS-II histological scoring, polarized light microscopy (PLM) scoring, and 5-point ZCT scoring based on tidemark morphology, zonal distribution of Col2 and Col1, and Col1 percentage stain. Because 1 biopsy specimen was missing bone, 38 biopsy specimens were evaluated for ICRS-II, PLM, and ZCT scores.

Results: Chondroinduction was identified in 21 biopsy specimens as a Col2 matrix fused to bone that spanned the deep-middle-superficial zones (“full-thickness hyaline repair”), deep-middle zones, or deep zone (“stalled hyaline”) that was covered with a variable-thickness Col1-positive matrix, and was scored, respectively, as ZCT = 1 (n = 4 biopsy specimens), ZCT = 2 (n = 6) and ZCT = 3 (n = 11). Other biopsy specimens (n = 17) were fibrocartilage (n = 9; ZCT = 4), fibrous tissue (n = 4, ZCT = 5), or non-marrow derived (n = 4; ZCT = 0). Non-marrow derived tissue had a mean mature tidemark score of 84 out of 100 versus a regenerating tidemark score of 24 for all other biopsy specimens (P = .005). Both “stalled hyaline” repair and fibrocartilage had the same mean Col2 percentage stain; however, fibrocartilage was distinguished by heavy Col1 deposits in the deep zone, a 2-fold higher mean Col1 percentage stain (P = .001), and lower surface integrity (P = .03). ZCT scores correlated with GAG content and the ICRS-II overall assessment score, especially when combined with the PLM score for collagen organization (R = 0.82). Histological scores of the deep zone strongly predicted the ICRS-II overall assessment score (R = 0.99).

Conclusion: The ICRS-II overall repair assessment score and GAG content correlated with the extent of Col2 deposition free of fibrosis in the deep/middle zone rather than bulk accumulation of Col2.

Clinical Relevance: Biopsy tissue from the BST-CarGel randomized clinical trial (microfracture without and with BST-CarGel, as treatment groups were not unblinded) showed regenerated tissue consistent with a chondroinduction mechanism in at least half of the treated lesions.
Effective technologies are needed to help regenerate hyaline articular cartilage in severe chondral lesions, particularly in patients who are too young for arthroplasty. Microfracture is a cartilage repair therapy that starts with complete debridement of the damaged cartilage to expose the vascularized subchondral bone, which is a hard tissue with mineralized collagen type I (Col1) containing progenitor-rich bone marrow. Animal models have shown the potential for bone marrow stimulation to produce cartilage repair tissue containing collagen type II (Col2) and glycosaminoglycan (GAG). However, in microfractured human lesions, most repair tissue biopsy specimens retrieved at 1 or 2 years postoperatively in 2 different randomized controlled clinical trials consisted of either fibrous tissue or fibrocartilage, a mixture of Col1 and Col2 with a poor capacity to retain GAG. BST-CarGel is a chitosan-glycerol phosphate/autologous whole blood implant that is applied over CarGel (Piramal Life Sciences) is a chitosan-glycerol phosphate/autologous whole blood implant that is applied over CarGel (Piramal Life Sciences) is a chitosan-glycerol phosphate/autologous whole blood implant that is applied over CarGel (Piramal Life Sciences) is a chitosan-glycerol phosphate/autologous whole blood implant that is applied over CarGel (Piramal Life Sciences). At 13 months postoperatively, 39 volunteer biopsy specimens retrieved at 1 or 2 years postoperatively in 2 different randomized controlled clinical trials consisted of either fibrous tissue or fibrocartilage, a mixture of Col1 and Col2 with a poor capacity to retain GAG. BST-CarGel was reported to result in more filling of the cartilage repair matrix than microfracture alone. 

The purpose of this study was to evaluate the histological repair tissue quality of the 39 human biopsy tissues elicited by microfracture with and without BST-CarGel, analyzed here as a single group because the data to break the groups out by treatment were not available from the company.

Traditional cartilage histology scoring systems were originally developed using non-parametric measures (ie, +, ++, +++), and allow cartilage repair tissue to be scored as "hyaline" or "hyaline-like" (ie, +++) when only 50% or 60% of the matrix has a hyaline appearance. A hyaline or glassy appearance indicates high levels of GAG entrapped in a Col2-positive matrix, components that endow the tissue with a viscoelastic biomechanical behavior. However, when tissues are heterogeneous, as is usually the case for repair cartilage, these non-parametric scoring systems are often subjective, leading to low inter-reader agreement. To address this shortcoming, the International Cartilage Repair Society–II (ICRS-II) scoring system was developed to evaluate 13 histological features and a 14th feature, "overall" repair quality, using a visual analog scale (VAS). In the VAS system, the reader places a mark along a 100-mm line, where 100 represents the features of perfect cartilage; this is then converted to a continuous measure from 0 to 100. In a previous human cartilage repair biopsy study, the ICRS-II overall score was found to correlate with the percentage of GAG staining but not with the percentage of Col2 staining for reasons that remain unclear.

When evaluating articular cartilage repair tissue, it is useful to consider that in embryonic bone development, Col1 is a temporary scaffold, inside of which chondroinduction takes place. Chondroinduction occurs when mesenchymal stem cells condense and differentiate to chondrocytes that produce Col2 instead of Col1. After epiphyseal bone fills the central core of distal long bones, articular cartilage continues to grow from the bone extremities by appositional deposition of arcs of Col2 during the proliferation and hypertrophy of chondrocytes, resulting in an anisotropic structure with 3 zones: the radial deep zone, the nonoriented middle zone, and the superficial zone, where collagen runs parallel with the surface. All 3 cartilage zones contain pure Col2, with only a thin layer of Col1 at the surface. In repaired cartilage lesions, the presence of a Col2 matrix in the deep zone that is devoid, or nearly devoid, of Col1 deposits and fused to subchondral bone could be said to reflect prior events of chondroinduction at the distal end of the femur. The extent of chondroinduction elicited in marrow-derived repair is then related to the thickness of the Col2 matrix integrated with the subchondral bone; this tissue should also be devoid or nearly devoid of Col1. By contrast, fibrosis during the articular cartilage repair process leads to Col1 deposits in the cartilage repair matrix, and codeposited Col1 and Col2 are hallmarks of fibrocartilage, whose positive identification requires Col1 staining in addition to Col2

Keywords: knee articular cartilage; cartilage repair; cartilage resurfacing; tidemark; zonal collagen scoring system; human clinical biopsy
staining. Fibrosis without chondroinduction leads to fibrous repair tissue devoid of the Col2 matrix.

The tidemark is a cartilage structure that can be used as an indicator of the degree of articular cartilage maturity. In postnatal development, a thin and disconnected tidemark appears at the base of the deep zone that gradually evolves into a continuous structure with a trilaminar morphology. The tidemark separates the deep zone from a calcified cartilage layer that is fused to the underlying subchondral bone at the cartilage-bone interface. A mature tidemark is a hallmark of skeletally mature articular cartilage, whereas multiple tidemarks and loss of surface integrity reflect cartilage degeneration.

In this study, methods were developed to reproducibly stain and detect GAG as well as human Col1 and Col2. Stained histological sections were evaluated by 3 trained and blinded observers for the ICRS-II histological score, by histomorphometry for the percentage of staining, and by the polarized light microscopy (PLM) score for zone-appropriate collagen orientation. In addition, a novel 5-point zonal collagen type (ZCT) score was developed to discriminate biopsy specimens with different levels of chondroinduction from fibrocartilage and fibrous tissue. We tested the hypothesis that ZCT scores correlate with the ICRS-II overall assessment score and GAG content as measured by the percentage of safranin O (SafO) staining. In addition, we tested the hypothesis that, instead of fibrocartilage, most of the biopsy specimens from the trial would contain a pure Col2 deep zone with radial collagen fiber organization characteristic of the native articular cartilage.

**METHODS**

**Tissue Specimens**

All protocols involving human participants were approved by institutional committees. Inclusion criteria for participant recruitment (N = 80) were as follows: male or female patients aged 18 to 55 years with knee pain secondary to a chondral defect grade III or IV according to the Outerbridge score on the medial or femoral condyle up to 10 cm² (diagnosed clinically and on MRI), <5° varus or valgus, intact anterior cruciate ligament and healthy rim of the meniscus, failure of nonsurgical measures, and no signs of osteoarthritis (OA) outside the afflicted compartment; in addition, all patients agreed to stop all knee pain medication 7 days before surgery and to undergo the recommended physical therapy regimen. After arthroscopic inspection to corroborate the presence of a lesion, patients were randomized to receive BST-CarGel (n = 41) or undergo microfracture (n = 39), after which the lesion cartilage was debrided and exposed subchondral bone was microfractured arthroscopically; patients randomized to the BST-CarGel group underwent further miniarthroscopy, and liquid BST-CarGel mixed with autologous whole blood ex vivo was deposited on the microfracture site and allowed to solidify in situ. Osteochondral repair tissue biopsy specimens 13 months (mean) after microfracture or those microfractured and treated with BST-CarGel were obtained with 2 mm–diameter 11-gauge Jamshidi needles (Cardinal Health) by arthroscopic surgery in consenting patients (microfracture: n = 17; BST-CarGel: n = 22). Biopsy specimens were taken from the center of the lesion, perpendicular to the surface as much as possible, fixed in 10% normal buffered formalin, decalcified in 0.5 N HCl/0.1% glutaraldehyde for 30 hours, and paraffin embedded, and 5-μm serial osteochondral sections were generated. Biopsy specimens from cadaveric medial femoral condyles or surgical waste from consenting patients undergoing hip or knee total arthroplasty were histoprocessed similarly in paraffin as comparative normal and OA controls. Serial biopsy sections were systematically stained with hematoxylin and eosin (H&E) and SafO (S2255; Sigma-Aldrich) and were immunostained for Col2 and Col1.

**Histological Staining With H&E and SafO–Fast Green–Weigert Iron Hematoxylin**

The H&E-stained repair biopsy sections were provided by Piramal Life Sciences, and control sections were H&E stained manually with Harris hematoxylin (Fisher) and Eosin Y (Leica Surgipath). For SafO–fast green staining, sections were deparaffinized and rehydrated, followed by 8 minutes in Weigert iron hematoxylin (equal volumes part A and part B; Sigma-Aldrich), a 5-minute tap water rinse, 10 seconds in 10-fold diluted Weigert iron hematoxylin part B solution (FeCl₃), a 3-minute rinse in tap water, 1 minute in 0.04% wt/vol fast green (F-7252; Sigma-Aldrich), a 2-second rinse in 1% vol/vol acetic acid, 4 minutes in 0.2% wt/vol SafO and then directly into 3 washes of absolute ethanol, followed by toluene and Permount (Fisher) coverslapping. Control sections were included in each staining batch to verify the consistency in staining intensity.

**Immunohistochemistry**

Paraffin sections were rehydrated, submitted to antigen retrieval by placing them twice in 10 mM Tris, pH 10, at 60°C and allowing to cool to room temperature each time, and then either rinsed in phosphate-buffered saline (PBS) and incubated at room temperature for 30 minutes in 1 mg/mL pronase in PBS (P8811; Sigma-Aldrich) or rinsed in Tris-buffered saline (TBS) and incubated at room temperature for 30 minutes in 2.5 mg/mL trypsin in TBS (T8802; Sigma-Aldrich). Sections were rinsed in PBS and incubated for 30 minutes at 37°C in 25 mg/mL hyaluronidase in PBS (H-3506; Sigma-Aldrich). Sections were rinsed in PBS and incubated for 30 minutes at 37°C in 25 mg/mL trypsin in TBS (T8802; Sigma-Aldrich). Sections were rinsed in PBS and incubated for 30 minutes at 37°C in 25 mg/mL hyaluronidase in PBS (H-3506; Sigma-Aldrich). Sections were rinsed in PBS and incubated for 30 minutes at 37°C in 25 mg/mL hyaluronidase in PBS (H-3506; Sigma-Aldrich). Sections were blocked for 1 hour in 20% wt/vol normal goat serum/PBS/0.1% Triton X-100; then incubated in antibody dilution buffer (10% goat serum/PBS/0.1% Triton X-100) with the primary monoclonal antibody, either anti-Col1 (I-8H5; 1:100 dilution; 20 μg/mL; optimization studies: MP Biomedical Product #631702, Lots 6853-J, 1467K; biopsy staining: Calbiochem Product #CP17, Lot D0008056) or anti-Col2 (I6B3; 1:10 diluted hybridoma supernatant; Developmental Studies Hybridoma Bank); and rinsed with PBS and then secondary biotinylated goat anti-mouse antibody.
ICRS-II Histological Scoring

Three trained and blinded observers carried out ICRS-II histological scoring using 40× magnification digital scans (NanoZoomer Slide Scanner; Olympus) of stained histology sections, visualized with NDPview software (Hamamatsu Photonics). Each observer generated a VAS score for each parameter by placing a pen mark on a 100-mm line (100 representing features of perfect hyaline cartilage) that was measured using a National Institute of Standards and Technology traceable ruler. The ICRS-II scoring parameters are (1) tissue morphology (0 = fibrous/100 = hyaline), (2) matrix GAG staining, (3) zone-appropriate cell morphology, (4) chondrocyte clustering (0 = present/100 = none), (5) surface architecture, (6) basal integration, (7) extent of tidemark across the biopsy specimen width, (8) subchondral bone abnormalities, (9) inflammation, (10) abnormal calcification/ossification, (11) soft tissue vascularization, (12) surface/superficial assessment, (13) orientation transitional zone, and horizontally oriented transitional zone, and (14) overall assessment. Parameters 3, 4, 7, and 10 were scored with SafO-stained sections. A novel ZCT scoring system of repair tissue quality was developed based on 3 important features of chondroinduction that are not present in the ICRS-I or ICRS-II histological scoring systems: (1) tidemark morphology, (2) Col1/Col2 zonal distribution, and (3) Col1 %stain (Table 1). Three blinded readers used specific procedures and guidelines (Tables 1 and 2; see Appendix 2, available online), and a photomontage generated by a third party (see Appendix 3, available online) was used to accurately discriminate red pixels from other pixel colors, while saturation threshold was used to eliminate gray nonpositive staining. For each stain (SafO, Col1, and Col2), a constant set of HSV threshold limits was selected using the Threshold_Colour ImageJ plugin (a modification of Bob Dougherty’s BandPass2 filter), by consensus among 3 trained blinded readers, to ensure an unbiased evaluation. The percentage of staining (%stain), defined as (number of positively stained pixels in the soft repair tissue)/(total number of pixels in the soft repair tissue) × 100, was calculated with an in-house Matlab routine (MathWorks). The final %stain values were taken as the mean of 2 distinct serial sections evaluated per biopsy specimen.

PLM Scores

Unstained, permanent-mounted sections were evaluated for collagen organization by PLM scoring as previously described by 3 blinded and trained readers, with an ordinal scale of 0 (no organization), 1 (birefringent vertical fibers characteristic of the deep zone and perpendicular to the cartilage-bone interface present in up to 50% of the tissue thickness), 2 (vertical fibers corresponding to >50% of the tissue thickness without or with a second birefringent zone above), 3 (deep-zone vertical fibers and the presence of zones approximating superficial and transitional zones), 4 (vertically oriented deep zone, randomly oriented transitional zone, and horizontally oriented superficial zone of approximate zonal proportions), and 5 (3 zonal layers with desired fiber orientations in the deep, tangential, and superficial zones along with desired zonal proportions).

ZCT Scores

A novel ZCT scoring system of repair tissue quality was developed based on 3 important features of chondroinduction that are not present in the ICRS-I or ICRS-II histological scoring systems: (1) tidemark morphology, (2) Col1/Col2 zonal distribution, and (3) Col1 %stain (Table 1). Three blinded readers used specific procedures and guidelines (Tables 1 and 2; see Appendix 2, available online), and a photomontage generated by a third party of each biopsy specimen showing low-magnification images of SafO-, Col1-, and Col2-stained sections with a calibration bar as well as a 40× magnification H&E-stained image showing representative tidemark morphology, to fill in a score sheet (see Appendix 3, available online). The biopsy specimen was first scored for tidemark morphology (feature 1). A fully mature tidemark has a trilaminar morphology consisting of a distal, central, and proximal lamina. Native cartilage contains a mature tidemark, while degrading native cartilage often contains multiple tidemarks. Biopsy specimens containing a complete/near-complete trilaminar tidemark or multiple tidemarks and uniform Col2 staining with a hyaline matrix were identified as tissues not derived from bone marrow stimulation repair (category “0”) because an ideal hyaline matrix with a mature tidemark is unlikely to represent repair tissue at 13 months after treatment, according to several lines of experimental evidence. First, a complete trilaminar tidemark morphology takes more than 6 years.
TABLE 1

ZCT Scoring Criteria*

<table>
<thead>
<tr>
<th>ZCT Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0: non–marrow derived</td>
<td>This tissue has a mature tidemark, a strong basophilic line, or a double</td>
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<tr>
<td></td>
<td>line (trilaminar morphology) at the base of the deep zone that is visible</td>
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<td></td>
<td>nearly the entire width of the biopsy specimen or duplicate tidemarks and</td>
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<td></td>
<td>a 100% Col2+ matrix with Col1 limited to the surface. Some biopsy specimens</td>
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<td></td>
<td>with a degenerated matrix may stain faintly for Col1 throughout. Surface</td>
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<td></td>
<td>disruption or features of osteoarthritis may be present (cell cloning,</td>
</tr>
<tr>
<td></td>
<td>surface degradation). To summarize, if the tissue has a mature tidemark</td>
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<tr>
<td></td>
<td>and 100% Col2+, the score is 0.</td>
</tr>
<tr>
<td>1: full-thickness hyaline</td>
<td>Tidemark: none or incomplete. In this tissue, all 3 zones are Col2+, while</td>
</tr>
<tr>
<td></td>
<td>Col1, if present, is limited to the articular surface. Place a check mark</td>
</tr>
<tr>
<td></td>
<td>in the row of Col2 for deep, middle, and superficial; and for Col1, place</td>
</tr>
<tr>
<td></td>
<td>a check mark below “surface” (if present). Less than 15% of the tissue is</td>
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<tr>
<td></td>
<td>Col1 positive.</td>
</tr>
<tr>
<td>2: almost full-thickness hyaline</td>
<td>Tidemark: none or incomplete. This tissue is similar to ZCT 1, except that</td>
</tr>
<tr>
<td></td>
<td>Col2 is missing from most of the superficial zone. Less than 15% is Col1</td>
</tr>
<tr>
<td></td>
<td>positive.</td>
</tr>
<tr>
<td>3: stalled hyaline</td>
<td>Tidemark: none or incomplete. This tissue has Col2+ deep and middle zones</td>
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<tr>
<td></td>
<td>that are devoid of Col1 at the cartilage-bone interface. Col1 is absent or</td>
</tr>
<tr>
<td></td>
<td>only present as occasional punctate deposits in the deep zone. A uniform or</td>
</tr>
<tr>
<td></td>
<td>strong Col1 stain (16%-50% Col1 stain) may be present in the middle and</td>
</tr>
<tr>
<td></td>
<td>superficial zones.</td>
</tr>
<tr>
<td>4: fibrocartilage</td>
<td>Tidemark: none or incomplete. Strong Col2 and Col1 stains are overlapping in</td>
</tr>
<tr>
<td></td>
<td>the deep or middle zone (ie, more than just a small patch or focal deposit).</td>
</tr>
<tr>
<td></td>
<td>Col1 is present in the deep zone, and the Col1 percentage stain is &gt;20%.</td>
</tr>
<tr>
<td></td>
<td>This is fibrocartilage.</td>
</tr>
<tr>
<td>5: fibrous</td>
<td>No tidemark is visible. Col2 is absent or only present as punctate deposits.</td>
</tr>
<tr>
<td></td>
<td>The Col1 percentage stain is variable.</td>
</tr>
</tbody>
</table>

*See Appendix 3 for the ZCT score sheet with features 1 (tidemark), 2 (zonal collagen), and 3 (% Col1). Col1, collagen type I; Col2, collagen type II; Col2+, Col2-positive matrix; ZCT, zonal collagen type.

Statistical Analyses

Interrater reliability of the ZCT scoring system was calculated for 3 blinded readers against scores of a fourth reader using the population intraclass correlation coefficient (ICC) in variance estimation and precision (Statistica v10; Statsoft). The general linear model (GLM) (Statistica v12; Statsoft) was used to evaluate the ZCT score as a categorical predictor of differences in the mean Col1, Col2, and SafO %stain; tidemark (ICRS-II parameter 7); surface architecture (ICRS-II parameter 5); overall assessment (ICRS-II parameter 14); and PLM score using unequal N honest significant difference post hoc analysis (n = 39 for %stain and n = 38 for ICRS-II parameters, ZCT scores, and PLM scores because 1 biopsy specimen was missing bone and could not be scored for these parameters). Regression coefficients in the GLM were determined using the ZCT score as a categorical predictor in each of the models for Col1, Col2, and SafO %stain; ICRS-II scores for middle/deep zone, surface, tidemark; and ICRS-II overall score. The mean PLM score of 3 readers was also evaluated as a continuous copredictor with the ZCT score as the covariate of SafO %stain and the ICRS-II overall assessment score. P < .05 was considered significant.

RESULTS

Reproducible Detection Methods of GAG, Col1, and Col2 in Human Repair Cartilage

SafO (red) binds to GAG, and fast green stains collagen in osteochondral sections. A faint red stain could arise from low GAG content or inconsistent SafO dye retention. We discovered that SafO staining becomes variably depleted during graded water/alcohol rinses that are commonly used to dehydrate sections for permanent mounting.
TABLE 2
ZCT Scoring System and Biopsy Specimen Classification

<table>
<thead>
<tr>
<th>ZCT Category</th>
<th>1. Tidemark</th>
<th>2. Cartilage Zone (Strong Uniform Stain)</th>
<th>3. % Col1</th>
<th>Incidence (per n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0: non–marrow derived</td>
<td>Mature or osteoarthritic features</td>
<td>Col2</td>
<td>&lt;15</td>
<td>Variable</td>
</tr>
<tr>
<td>1: full-thickness hyaline</td>
<td>Incomplete</td>
<td>Col2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2: almost full-thickness hyaline</td>
<td>Incomplete</td>
<td>Col2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3: stalled hyaline</td>
<td>Incomplete</td>
<td>Col2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4: fibrocartilage</td>
<td>Incomplete</td>
<td>Col2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5: fibrous</td>
<td>No tidemark</td>
<td>Col2</td>
<td>negligible</td>
<td>Variable</td>
</tr>
</tbody>
</table>

*aCol1, collagen type I; Col2, collagen type II; ZCT, zonal collagen type.

For tissues with >20% Col1 staining, ZCT parameter 2 (notably Col1 staining in the deep zone) is needed to distinguish between stalled hyaline and fibrocartilage.

Zonal Collagen Typing Suggests a Chondroinduction Repair Mechanism in 21 Biopsy Specimens

The ZCT scoring system was reproducible among 3 blinded readers, with a mean estimated ICC of 92.6% (reader 1: 94.7%, reader 2: 84.1%, and reader 3: 98.9%). Biopsy cartilage repair tissues showed distinct zonal collagen patterns. Col2-positive tissues were often stained by SafO, but not by Col1. From being present in all 3 cartilage zones (full-thickness hyaline) to absent (fibrocartilage) (Figure 2 and Table 2). Col1 was detected in bone and ranged from being completely absent to intense deposition throughout in soft repair tissues (Figure 2 and Table 2). Among 8 biopsy specimens scored as full-thickness hyaline, 4 contained a fully mature tidemark that resembled a native cartilage tidemark or duplicated tidemarks, and occasional signs of OA and surface disruption, and were classified as non–marrow derived (ZCT = 0) (Figures 2A, 2B, 3A, and Table 2). Such biopsy specimens could potentially arise from inadvertent biopsy specimen retrieval outside of treated lesions filled with perfect macroscopic repair, although it was recorded at the time of biopsy specimen retrieval that lesion borders were macroscopically visible in all repair sites (A. Restrepo, personal communication). Such biopsy specimens could also represent areas where the native tidemark was incompletely debrided during the microfracture procedure (Figure 3, A and B), given that complete removal of the calcified layer is known to vary according to the surgeon, technique, and surgical instruments used. Aside from these 4 biopsy specimens, only a partial tidemark (thin, disconnected basophilic blue line) (Figure 3C) or no tidemark with subchondral bone remodeling and endochondral ossification was seen (Figure 3D). Fibrous repair (ZCT = 5) had no histological evidence of subchondral bone remodeling or tidemark formation, meaning that these tissues had no cartilage calcification at the cartilage-bone interface (Table 2).

Among 34 biopsy specimens classified as marrow derived, 4 were scored as full-thickness hyaline cartilage repair (ZCT = 1), 6 almost full-thickness hyaline (ZCT = 2), 11 stalled hyaline repair (ZCT = 3), 9 fibrocartilage (ZCT = 4), and 4 fibrous tissue (ZCT = 5) (Table 2, incidence column). Repair tissues with pure Col2 contained cells with a round or semilunar chondrocyte morphology and intense SafO staining (Figure 4, B and C). Areas of mixed Col1 and Col2 contained chondrocytes, mesenchymal stromal cells with a slight elongated shape, and a pale SafO-stained matrix (Figure 4, D and E). Fibrous repair contained mainly...
stromal cells and had negligible Safo staining (Figure 4F). To summarize, 62% of all marrow-derived biopsy specimens (21/34) presented a ZCT score of 1 to 3 and contained a Col2-positive deep zone free of Col1, suggesting a chondroinduction mechanism underlying the repair process.

ZCT Score–based Histological Profiles

As ZCT scores increased from 1 to 5 (ie, high-quality full-thickness hyaline to low-quality fibrous repair), the mean Col2 %stain, Safo %stain, and ICRS-II overall score progressively declined (Figure 5, A and B). In parallel, the mean Col1 %stain incrementally increased for scores 1 to 4 (Figure 5A). Fibrous tissue (ZCT = 5) showed a lower mean Col1 %stain and negligible Col2 staining (P < .005 vs ZCT 1-3) (Figure 5A), suggesting that fibrous tissues contain other matrix species. Although “stalled hyaline” (ZCT = 3) and fibrocartilage repair (ZCT = 4) contained a similar mean approximately 66% Col2 stain (Figure 5A), compared with “stalled hyaline,” fibrocartilage had deep-zone Col1, a 2-fold higher mean Col1 %stain (27% vs 62%, respectively; P = .001), a lower mean Safo %stain, a lower ICRS-II overall assessment score, and a lower mean surface score (P = .03) (Figure 5, A-C). These data suggest that fibrocartilage repair tissue not only had less GAG but was already degrading at the surface at 13 months postoperatively. Altogether, these data suggest that deposition of a Col2 matrix devoid of Col1 in the deep zone is more important than a bulk Col2 %stain in determining GAG accumulation, ICRS-II overall score, and tissue durability.

Non–marrow derived biopsy specimens (ZCT = 0) were distinguished by a higher mean ICRS-II tidemark score compared with the other biopsy specimen ZCT categories (84 vs 32, respectively; P < .05) (Figure 5D). These biopsy specimens also had significant surface disruption, a lower ICRS-II overall score, and a mean PLM score of 2.5 out of 5 (Figure 5, B and C). Mean PLM scores ranged from 1.6 to 2.6 for ZCT scores 1, 2, and 3, suggesting the presence of radial deep-zone fibers in most biopsy specimens with features of chondroinduction. These data are consistent with the notion that a radial deep zone is the first to regenerate and last to degenerate in hyaline articular cartilage.

Global histological features of all repair biopsy specimens from the study (n = 34, excluding non–marrow derived and 1 biopsy specimen missing bone) were mean ICRS-II overall score of 51 ± 27, ICRS-II surface integrity score of 75 ± 22, ICRS-II tidemark score of 24 ± 22, 56% ± 32% Safo stain, 65% ± 30% Col2 stain, and 32% ± 25% Col1 stain. The ICRS-II overall score was more strongly predicted by the Safo %stain (R = 0.89, R² = 0.79; P < .0001) than the Col2 %stain (R = 0.66, R² = 0.44; P < .0001). The ZCT score showed a good linear correlation with the Safo %stain and ICRS-II overall score (R = 0.80, R² = 0.64 and R = 0.73, R² = 0.53, respectively; P < .0001) (see Appendix 4, panel A, available online) and was a stronger predictor of both the Safo %stain and ICRS-II overall score when combined with the PLM score.

**Figure 1.** Optimization of (A) safranin O (SafO)–fast green histostaining, (B, C) collagen immunostaining, and (D) optimized staining results in an example repair biopsy specimen. SafO staining was variably lost when followed by water-ethanol baths for dehydration (A1) and was retained when excess SafO was rinsed with absolute ethanol (A2). Glycosaminoglycan (GAG) stained by SafO in osteoarthritic cartilage (B1, C1) was more efficiently cleared by pronase (B2) than trypsin (C2), leading to more complete immunostaining by collagen type II (Col2) (B3 vs C3, arrows). Optimized staining in a repair biopsy specimen for SafO (D1), and pronase-hyaluronidase pretreatment followed by Col2 (D2), collagen type I (Col1) (D3), or no primary antibody (D4). Arrows (B, C) show different GAG depletion and Col2 detection. All scale bars: 1 mm.
The ICRS-II middle/deep zone score was the strongest predictor of the SafO %stain and ICRS-II overall score ($R = 0.90$, $R^2 = 0.81$ and $R = 0.99$, $R^2 = 0.98$, respectively; $P < .0001$) (Appendix 4, panel B). This effect is partly attributed to the fact that the middle/deep zone normally comprises over half of the cartilage thickness. In SafO-stained histology sections, an ideal hyaline deep zone with no visible fibers was observed in only 1 marrow-derived repair biopsy specimen classified as stalled hyaline repair (ZCT = 3) (Figure 6, A-C). Other full-thickness hyaline repair cartilage had visible fibers in the deep-zone matrix (ZCT = 1) (Figure 6, D-F).

DISCUSSION

This study showed that approximately 60% of marrow-derived repair tissues arising from microfracture with and without BST-CarGel (taken together) contained a pure Col2 matrix fused to bone, which in most cases was accompanied by radial deep-zone collagen architecture. These observations are consistent with a mechanism involving chondroinduction of bone marrow–derived progenitors, followed by coordinated appositional growth from the subchondral bone plate. Stratification of biopsy specimen histological features by the novel ZCT score permitted the observation that 10 out of 21 biopsy specimens with features of chondroinduction matured at 13 months postoperatively to full-thickness or near–full-thickness hyaline repair with good surface integrity, representing a frequency of approximately 1 out of 2 (ie, 10 biopsy specimens with ZCT scores of 1 and 2 among 21 biopsy specimens with ZCT scores of 1, 2, and 3). This study also reports that repair tissues with both Col1 and Col2 can have mixed or zonal organization, and when the organization is zonal, Col1 localizes to the articulating layer. This study supports the hypothesis that ZCT scores are predictive of GAG accumulation and of the ICRS-II overall histological assessment score, especially when combined with PLM scores.

The mean ICRS-II overall score of 51 for the blinded repair tissues analyzed here (microfracture and microfracture + BST-CarGel) is comparable with that in a previous randomized controlled trial reporting an ICRS-II overall score of approximately 46 for microfracture and approximately 55 for characterized chondrocyte implantation (CCI) repair tissues at 12 months postoperatively. The hyaline-like repair observed in this study is better than the predominantly fibrous repair observed in biopsy specimens taken 24 months
Figure 3. Example cartilage-bone interfaces (hematoxylin and eosin stain) and tidemark morphologies for (A) a biopsy specimen classified as non–marrow derived (ZCT = 0), (B) osteoarthritic (OA) cartilage from total knee arthroplasty, and (C, D) 2 biopsy specimens classified as full-thickness hyaline repair (ZCT = 1). Dashed arrows (A, B) show a fully mature native tidemark with a trilaminar structure with a proximal (p), central (c), and distal (d) lamina and duplicated tidemark (A, open arrows). (C) Arrows show a regenerating partial tidemark in marrow-derived repair cartilage (RC). (D) Remodeling repair bone with endochondral ossification (EO) and no visible tidemark. CC, calcified cartilage; ZCT, zonal collagen type. All scale bars: 200 μm.

Figure 4. Cell morphology typical of chondrocytes (arrows) or stromal cells (open triangles) detected in a safranin O and fast green–stained extracellular matrix of the middle zone in (A) a pure Col2+ matrix from non–marrow derived tissue (ZCT = 0), (B, C) a pure Col2+ matrix of full-thickness hyaline repair from 2 distinct biopsy specimens (ZCT = 1), (D) a Col1+/Col2+ zone of stalled/emerging hyaline (ZCT = 3), (E) Col1+/Col2+ fibrocartilage (ZCT = 4), and (F) fibrous repair devoid of Col2 (ZCT = 5). Col1, collagen type I; Col2, collagen type II; ZCT, zonal collagen type. All scale bars: 50 μm.
after microfracture or autologous chondrocyte implantation. BST-CarGel was previously shown to improve hyaline repair tissue quality in animal models and to improve MRI-measured lesion fill and T2 relaxation time compared with microfracture only at both 1 and 5 years postoperatively; however, microfracture with and without BST-CarGel led to the same improvement in patient-reported outcomes from baseline at 1 and 5 years postoperatively. In this study, we did not have access to the unblinded clinical data; therefore, the clinical significance of the data in this study cannot be fully analyzed and interpreted. Another limitation is that 39 of 80 patients volunteered biopsy specimens, and the profiles could potentially change if the entire group had provided biopsy specimens. Biopsy specimen analyses are also limited because they represent only a small area of the treated lesion, but only biopsy specimens can provide histological structural information. Note that an ICRS-II overall score of 50 could qualify as “predominately hyaline” or “hyaline-like” by many traditional scoring standards; therefore, the ICRS-II overall assessment score alone is difficult to interpret without also performing zonal collagen typing and taking tidemark morphology into account.

The ZCT score developed in this study constitutes a hybrid evaluation that combines histomorphometry and histological scoring. Hybrid scoring is appropriate for articular cartilage repair because this approach addresses the anisotropic growth mechanism of repair tissue and simplifies data interpretation. We provide detailed methodologies for Safranin O staining and collagen immunodetection to facilitate use of the hybrid scoring methodology by others. The ZCT score could be useful in revealing chondroinduction mechanisms elicited by other therapies that cannot be deduced from the Safranin O stain and ICRS-II scoring alone. The ZCT score may require further refinements for scoring tissues generated by other approaches and would require the availability of biopsy specimens retrieved at the same postoperative time point from other repair procedures. The ZCT scoring instrument would need further validation before using as an outcome measure in trials evaluating cell-based therapies in which microfracture is imposed as the control arm.

In this study, 2 out of 3 biopsy specimens showing evidence of chondroinduction also displayed Col1 deposition in the superficial or superficial and middle zones (ZCT scores of 2 and 3). The reproducible observation of such ZCT organization (44% of all biopsy specimens) suggests that hyaline cartilage repair arising from bone marrow stimulation may recapitulate some features of blastema-to-anlage developmental stages.

Figure 5. Differences due to zonal collagen type (ZCT) category in the (A) mean percentage of collagen type I (Col1) and collagen type II (Col2), (B) International Cartilage Repair Society–II (ICRS-II) overall score and percentage of safranin O (SafO), (C) ICRS-II surface architecture and polarized light microscopy (PLM) scores, and (D) tidemark. A ZCT score of 0 was distinguished by a significantly higher tidemark score and lower ICRS-II surface integrity and ICRS-II overall scores. Chondroinduction for ZCT scores 1, 2, and 3 (21/39 biopsy specimens) was indicated by significantly higher Col2 versus Col1, >60% SafO stain, deep-zone radial collagen architecture by a PLM score >1.6, high surface architecture, and approximately 24% incidence of tidemark formation at 13 months postoperatively. ZCT scoring: 0 = non–marrow derived, 1 = full-thickness hyaline, 2 = almost full-thickness hyaline, 3 = stalled hyaline, 4 = fibrocartilage, and 5 = fibrous tissue. The number of samples in each ZCT category was as follows: 0: n = 4; 1: n = 4; 2: n = 6; 3: n = 11; 4: n = 9; and 5: n = 4. Data are shown as mean ± SD. F, fibrous; FC, fibrocartilage; VAS, visual analog scale.
This notion is compatible with findings in skeletally mature rabbit models of osteochondral repair, where superficial Col1 was mutually exclusive of a Col2-positive deep zone. Although it was suggested that human cartilage repair tissues can continuously mature during several years postoperatively, it remains to be shown whether stalled hyaline repair can mature to full-thickness hyaline repair with time and whether a hyaline deep zone is sufficient to improve clinical outcomes. Heavy deposition of middle-zone Col1, or fibrosis, during 13 months after treatment may either delay or indefinitely inhibit hyaline repair maturation. Insights into the causes of fibrosis could lead to postoperative therapies that suppress excessive Col1 deposition and facilitate full-thickness hyaline repair that is more likely to resist mechanical wear.

The stability of the cartilage-bone interface is another important feature of tissue durability, especially in high-demand sports. Although rarely quantified in cartilage repair studies of human repair tissues, a mean tidemark formation of 20% or 30% at 13 months after microfracture or CCI, respectively, was observed previously and was reported as a collective mean 20% tidemark for all 86 biopsy specimens in a subsequent study. Here, a similar partial tidemark formation was observed at 13 months after marrow stimulation. Tidemark formation is favored in a pure Col2+ deep zone with signs of endochondral ossification. Repair cartilage arising from chondroinduction therefore has a greater probability of advancing over time to form a stable, calcified cartilage-bone interface. Regeneration of a functional tidemark may be one of the most challenging anatomic features to achieve in cartilage repair.

This study was limited by the inability to separate the 2 groups. It is therefore not possible to know whether successful biopsies occurred mainly in the control group or the experimental group or equally in both groups. The inability to analyze the effect of treatment on the ZCT score, or to perform clinical correlations with histology features, makes it hard to gauge the clinical implications of these findings. Perhaps the most remarkable finding in this study is that microfracture-based repair procedures in contained focal lesions (without any added cells, growth factors, or pharmaceuticals) have the potential to produce bone marrow–derived repair tissue with a full-thickness hyaline-like character.

CONCLUSION

We found marrow stimulation to elicit hyaline-like repair cartilage tissue in the deep zone of 54% of treated lesions, among which full-thickness hyaline repair was observed in 11%. This finding represents an important advance, compared with previous studies, in quantifying histological tissue quality produced by microfracture therapy. The reproducibility and utility of our novel zonal collagen typing system for human cartilage repair histological assessment were verified. This study also provides a new ZCT scoring approach for evaluating articular cartilage repair endpoints in clinical trials and can reveal chondroinduction mechanisms.

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