

Histological scoring of articular cartilage alone provides an incomplete picture of osteoarthritic disease progression

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Summary. *Purpose:* To ascertain whether molecular subcategories of disease progression exist within established histological grades of articular cartilage (AC). *Methods:* Based on H&E and safranin-O staining of AC sections obtained from 18 knee arthroplasty surgeries, 30 samples ranging from Mankin Scoring System grade 1 through 5 were identified. Immunohistochemical (IHC) analysis for collagen type II and aggrecan was performed on serial sections of the paraffin-embedded AC samples. Six AC samples from each of the five Mankin Scoring System grades were examined. *Results:* Significant IHC differences in collagen type II and aggrecan deposition were seen within AC samples from all five histological grades. The range of IHC differences in collagen type II and aggrecan increased with increasing histological grade. A change in the pattern of collagen type II deposition was observed in MG-3 AC that was consistent with a switch in collagen type II metabolism. *Conclusions:* IHC staining of collagen type II and aggrecan can identify differences within histological grades of AC that are consistent with the existence of molecular subcategories. These differences were detectable even within the lowest histological grades; therefore the use of IHC staining can further enhance and refine the scoring of AC deterioration in early osteoarthritis (OA). Furthermore, the changes seen in the deposition pattern for both aggrecan and collagen type II suggest that they could be used to monitor key molecular events in OA progression. These findings also underscore the need for the development of IHC scoring criteria.

Key words: Osteoarthritis, Cartilage assessment, Immunohistochemistry, Collagen, Aggrecan

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Introduction

Articular cartilage scoring systems

Over the past several decades, a variety of tools for the assessment of articular cartilage (AC) deterioration have been developed (Collins, 1949; Dougados et al., 1994; Mankin, 1971; Noyes and Stabler, 1989; Outerbridge, 2001; Pritzker et al., 2006) but, unfortunately, none were designed to assess molecular changes. Macroscopic assessment systems such as Collins (1949), Outerbridge (2001) and Société Française d'Arthroscopie (SFA) (Dougados et al., 1994), which are relatively rudimentary, continue to be used for arthroscopic assessment, where they provide a convenient yet somewhat inexact (Brismar et al., 2002) method for distinguishing between broad stages of disease progression. Although macroscopic assessment systems are widely used in clinical applications, the limited number of scoring categories that are available in these systems fails to account for many of the important changes occurring at the histological (Lorenz and Richter, 2006), cellular (Lippiello et al., 1977; Aigner et al., 1997; Nelson et al., 1998) and biochemical (Rizkalla et al., 1992) levels. For this reason, more rigorous scoring systems such as the Mankin Scoring System (Mankin, 1971), and Osteoarthritis Research Society International (OARSI) Histopathologic Grading System (Pritzker et al., 2006) were developed which permit the distinction of far more subtle changes during AC deterioration.

Histological scoring systems

The Mankin Scoring System, also known as the Histological/Histochemical Grading System (HHGS), utilizes a comprehensive 14 point scale for the assessment of AC deterioration (Mankin, 1971).

However, one criticism of the Mankin Scoring System is that it was developed using advanced osteoarthritic tissue and therefore lacks the ability to adequately distinguish between mild and moderate osteoarthritic (OA) samples (Ostergaard et al., 1999). The newly developed OARSI System was specifically designed to address this shortcoming and, hence, offers better resolution at this level of AC degeneration. Although the OARSI System does appear to offer linearity and greater resolution for early OA, the reliability and validity of this system has not been demonstrated on human AC. Aside from the above-mentioned deficiencies, the greatest shortcoming with the use of these scoring systems in the future may be that neither one has the ability to account for the molecular changes occurring during disease progression.

Immunohistochemical OA progression

In the past two decades, great strides have been made towards understanding the immunohistochemistry underlying OA. In AC, the immunohistochemical (IHC) distribution of numerous extracellular matrix (ECM) components has been examined including; collagen type I (Miosge et al., 2004), collagen type II (Nelson et al., 1998), collagen type III (Aigner et al., 1993), collagen type V (Wachsmuth et al., 2006), collagen type VI (Hambach et al., 1998), collagen type IX (Eyre et al., 2004), collagen type X (von der et al., 1992), aggrecan (Lark et al., 1997), tenascin (Veje et al., 2003), biglycan (Miosge et al., 1994), decorin (Kavanagh and Ashhurst 1999), link (Hoedt-Schmidt et al., 1993), comp (Wagner et al., 2003) and fibronectin (Pfander et al., 1999). Mounting evidence suggests that the presence and distribution of many of these ECM components is altered during disease progression (Hollander et al., 1995; Pfander et al., 1999; Miosge et al., 2004; Aurich et al., 2005; Lorenz et al., 2005), yet, no IHC scoring system or module has been developed to describe and assess these changes. As the molecular understanding of OA progression continues to improve, so should the ability to classify these pathological alterations. Unfortunately, it remains unclear whether IHC analysis will yield information about OA progression beyond what is currently available through conventional histological methods. To determine if additional information about disease progression can be obtained from IHC analysis a range of 30 AC samples spanning Mankin grades (MG) 1-5 were examined for intra-grade discrepancies in collagen type II and aggrecan deposition.

Materials and methods

Tissue collection and processing

OA tissue from femoral condyles was obtained during total knee arthroplasty surgery. Osteochondral tissue was immediately placed in saline solution and

stored at 4°C for up to 24 hrs. From all tissue samples, one centimeter diameter cylindrical cores of AC and underlying bone (dowels) were obtained from load-bearing regions (Fig. 1) using a stainless steel coring tool. Dowels were collected from 18 OA patients (8 males and 10 females) that ranged in age from 56 to 86 years with a mean age of 68.4±8.5 years. For the purposes of this study, the cartilage portion of the dowel was considered as the study unit.

Histology

Osteochondral dowels were washed in PBS and the cartilage was removed from the underlying bone at the cartilage-bone junction thus eliminating the need for a lengthy decalcification step that would promote excessive leaching of proteoglycans. The AC was fixed in 4% formalin and embedded in paraffin. AC samples were sectioned at a 5 µm thickness and mounted on Superfrost Plus glass slides (#12-550-15 Fisher Scientific, Ottawa, ON). Immediately prior to staining, sections were deparaffinized using ProPar (Anatech Ltd, Battle Creek, MI) and rehydrated in a graded series of ethanol washes. Deparaffinized sections were stained with H&E to show overall tissue morphology and safranin-O staining to reveal the distribution of proteoglycans within the tissue. Safranin-O staining was achieved by incubating the sections in a 5% safranin-O solution for 5 minutes, followed by a 20 second wash in water. All sections were dehydrated through a series of graded ethanol washes for 20 seconds each, mounted and visualized at 50x magnification on a Leica DM/RE microscope. Photomicrographs of representative regions within the sections were taken using a CoolSnap CCD camera.

Histologic evaluation

Photomicrographs of the H&E and safranin-O stained sections were scored using the modified Mankin Scoring System (Table 1). Staining artifacts resulting from creasing or folding of the cartilage sections were not considered for analysis. Six AC samples from Mankin Grades 1-5 were identified resulting in 30 AC samples in the study. The tide mark criterion was omitted from the histological scoring because tissue processing made it impossible to evaluate.

Immunohistochemistry

Formalin-fixed 5 µm thick AC sections were deparaffinized and subjected to an antigen retrieval step consisting of a 60 minute digestion in 5 mg/ml pepsin, pH 2.0 (P-7000 Sigma-Aldrich, Oakville, ON). Sections were rinsed thoroughly in water to remove the pepsin and endogenous peroxidase activity was quenched using a 30 minute incubation in 3% H₂O₂ in methanol. All IHC analysis was performed using Vectastain Elite ABC kits (# 6102 and 6105, Vector Laboratories, Burlingame,

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CA) according to the manufacturer's directions. Primary antibodies [mouse anti-collagen type II (MAB 8887, Millipore, Billerica, MA) or rabbit anti-aggrecan (MAB 1220, R&D Systems Minneapolis, MN)] were applied at a 1:100 dilution in PBS to separate sections from each donor sample and incubated for 30 minutes in a humidified chamber. Sections were washed in PBS and biotinylated secondary antibodies were applied for 30 minutes followed by washing in PBS and incubation for 30 minutes in VectaStain ABC reagent. Sections were subsequently washed in PBS and exposed to metal-enhanced DAB (# 1856090, Thermo Scientific, Rockford, IL) for 5 minutes. The reaction was stopped by rinsing the sections thoroughly in water. Stained tissue sections were dehydrated using a series of graded ethanol washes, mounted and imaged as outlined above. Further tissue unmasking with citrate buffer or an equivalent solution was not required with the above-mentioned antibodies. Sections of human anterior cruciate ligament, stained with antibodies raised against collagen type II, were included as negative controls. Sections in which primary antibodies were omitted showed no significant signs of non-specific staining.

Results

Mankin scoring system vs. IHC staining

The range of IHC differences within AC samples of the same MG increased with increasing amounts of cartilage deterioration. In general, MG-1 samples displayed the narrowest range of IHC staining patterns, while MG-5 displayed the broadest range. As a result, IHC techniques revealed marked differences between

AC samples that could not have been predicted on the basis of histologic staining alone. For example, samples classified as MG-1 displayed differences in the extent of staining for both collagen II (Fig. 2e,f) and aggrecan (Fig. 2g,h), despite similarities in structural integrity (Fig. 2a,b) and safranin-O staining (Fig. 2c,d). Sample 298-1 displayed a relatively minor loss of collagen type II and aggrecan staining from the superficial AC layer, while sample 300-1 showed an almost complete absence of both components from the corresponding region. The

Table 1. Summary of the modified scoring criteria for the Mankin classification system.

I. Structure		
a.	Normal	0
b.	Surface irregularities	1
c.	Pannus + irregularities	2
d.	Clefts to transitional	3
e.	Clefts to radial	4
f.	Clefts to calcified	5
g.	Disorganization	6
II. Cells		
a.	Normal	0
b.	Diffuse hypercellularity	1
c.	Cloning	2
d.	Hypocellularity	3
III. Safranin-O staining		
a.	Normal	0
b.	Slight reduction	1
c.	Moderate reduction	2
d.	Severe reduction	3
e.	No dye noted	4

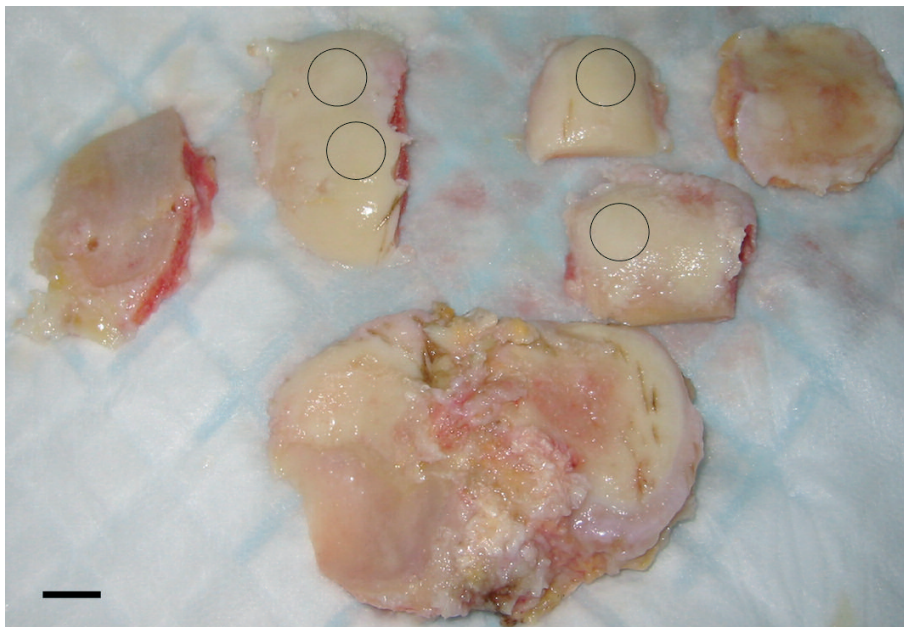


Fig. 1. Image showing typical OA tissue obtained from patients undergoing total knee arthroplasty. Circles indicate the potential sites collection of 1 cm diameter dowel in this specimen. Bar: 1 cm.

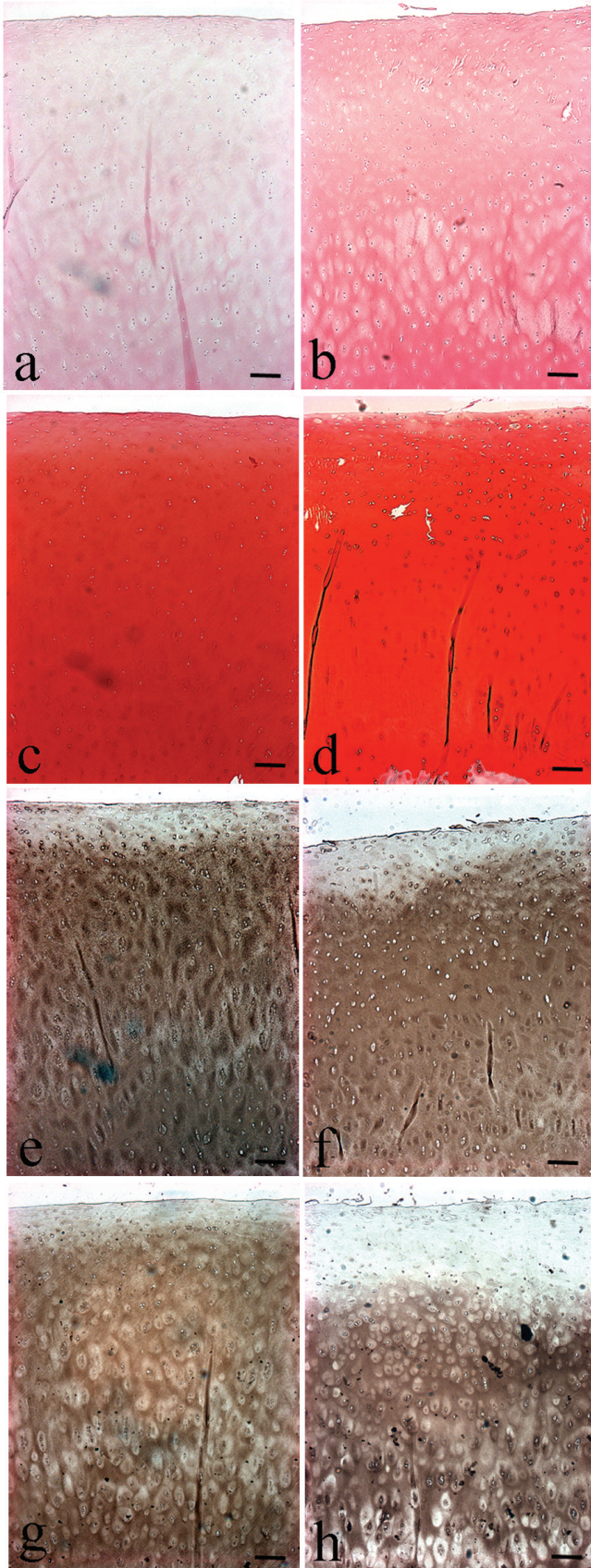


Fig. 2. Micrograph of MG-1 graded OA sections of AC (298-1 and 300-1) showing histological staining for H&E (a, b) and safranin-O (c, d) as well as IHC staining for collagen type II (e, f) and aggrecan (g, h). Note the differences in the distribution of staining for collagen type II and aggrecan within each pair. Bar: 100 μ m.

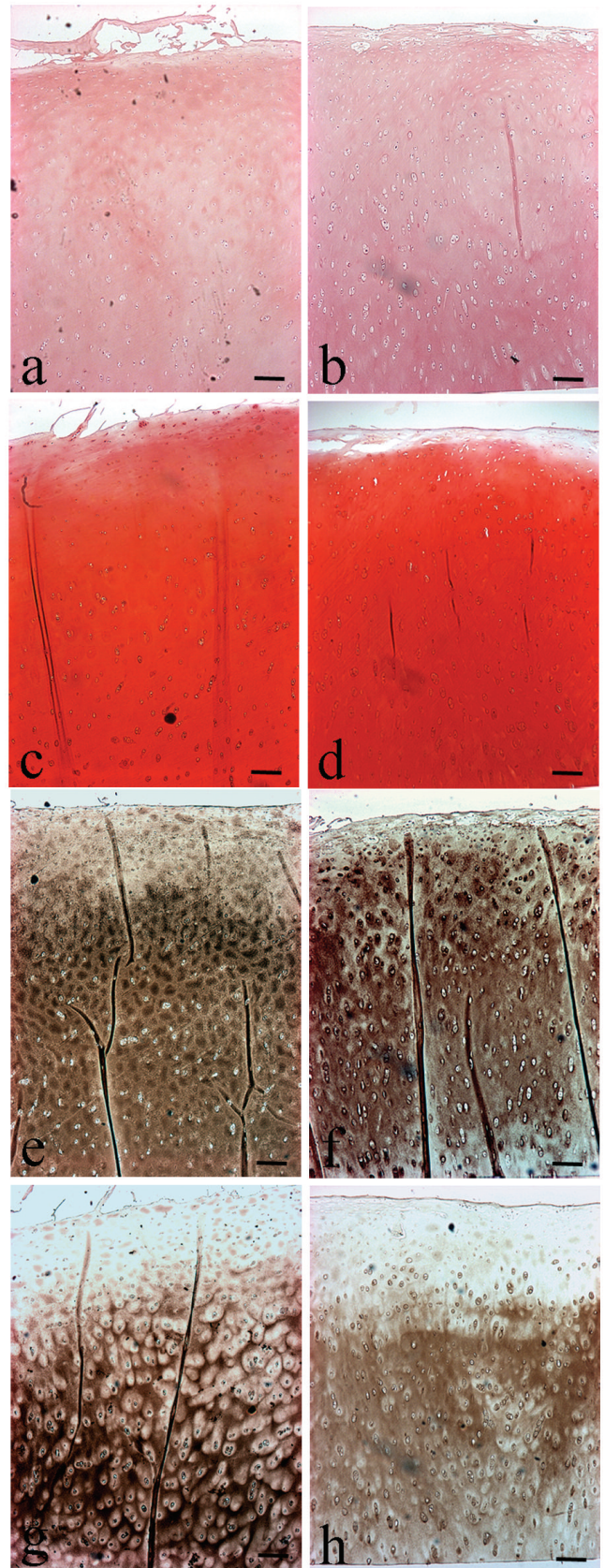
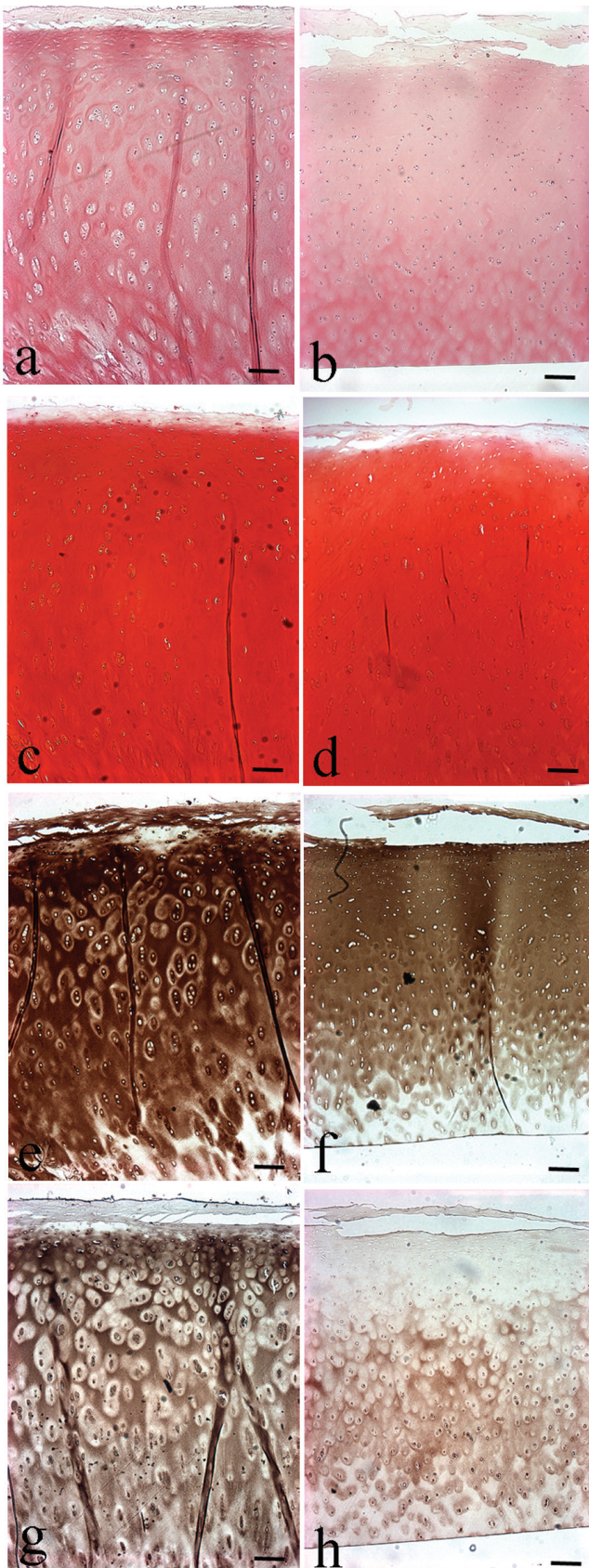


Fig. 3. Micrograph of MG-3 graded OA sections of AC (268-2 and 272-3) showing histological staining for H&E (a, b) and safranin-O (c, d) as well as IHC staining for collagen type II (e, f) and aggrecan (g, h). Note the differences in the distribution of staining for collagen type II and aggrecan within each pair. Bar: 100 μ m.

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loss of aggrecan in sample 300-1 also extended deeper into the AC than in sample 298-1. Similar differences in collagen type II and aggrecan deposition were observed among AC samples classified as MG-2 (data not shown).

A broader range of IHC staining patterns was observed within MG-3 graded AC samples compared to either MG-1 or MG-2 samples. For example, samples 268-2 and 272-3 displayed significant differences in both the distribution pattern and extent of collagen type II and aggrecan staining despite comparable levels of disruption in the superficial layer (Fig. 3a,b) and similar losses in proteoglycan staining (Fig. 3c,d). Punctuate staining for collagen type II was observed in the superficial and transitional layers of 272-3 (Fig. 3f) whereas the corresponding region in samples 268-2 (Fig. 3e) was virtually devoid of staining. Sample 268-2 also exhibited a far more profound loss of aggrecan staining in the territorial regions of the transitional and deep layers (Fig. 3g) when compared to sample 272-3 (Fig. 3h). Interestingly, these marked IHC differences in aggrecan deposition were observed in AC samples with histologically comparable levels of proteoglycan staining. MG-3 and MG-4 samples (data not shown) displayed a similar range of IHC differences for both collagen type II and aggrecan.

The most visually striking IHC differences were observed within MG-5 AC samples. For example, samples 272-1 and 225-1 displayed profoundly different staining patterns for both collagen type II and aggrecan. Collagen type II staining in sample 225-1 (Fig. 4f) was relatively homogeneous in the upper half of the AC section, while sample 272-1 exhibited a marked decrease in staining throughout the territorial region (Fig. 4e). In sample 272-1, aggrecan staining in the interterritorial region was very intense from the superficial to the deep layer (Fig. 4g). Conversely, the superficial and transitional layers in sample 225-1 were nearly devoid of aggrecan staining (Fig. 4h). In addition to displaying the most visually striking IHC differences, MG-5 samples also displayed some of the most divergent staining of any grade examined, with 4 of 6 samples showing IHC staining patterns which were unique within the group.

Discussion

This was a descriptive study that summarized the IHC findings for 30 OA AC samples, spanning MG 1-5. IHC differences in collagen type II and aggrecan between AC samples of the same MG were examined. This study provided evidence that molecular differences exist within histological grades that could constitute novel subcategories within the established histological

Fig. 4. Micrograph of MG-5 graded OA sections of AC (272-1 and 225-1) showing histological staining for H&E (a, b) and safranin-O (c, d) as well as IHC staining for collagen type II (e, f) and aggrecan (g, h). Note the differences in the distribution of staining for collagen type II and aggrecan within each pair. Bar: 100 μ m.

progression of OA.

Traditional histological scoring of AC failed to reveal differences in disease progression within histological grades that were detected at the IHC level. Even among AC samples representing the earliest stage of OA (MG-1), differences were observed in the extent of collagen type II and aggrecan staining. Because OA related changes begin at the articular surface and proceed deeper with disease progression (Hollander et al., 1995), the greater loss of aggrecan from the transitional layer of sample 300-1 indicated that it represented a more advanced stage of OA than sample 298-1. The distinction between these MG-1 graded samples at the IHC level suggested that a more detailed description and categorization of early OA progression is possible and that molecular subcategories may exist within each of the histological grades. Furthermore, the IHC detection of such early molecular changes also supported and extended the findings of a recent study that showed molecular changes in OA occurred well before any signs of overt structural had damage emerged (Lorenzo et al., 2004).

When MG-3 samples of AC were examined, significant differences in the depth and distribution of staining were observed for both collagen type II and aggrecan. Within these samples, changes in collagen type II distribution were observed that were consistent with the change in collagen type II metabolism reported elsewhere (Aigner et al., 1997). While the progressive loss of collagen type II from the superficial and transitional layers is characteristic of the earliest stages of AC deterioration, MG-3 appeared to represent a turning point in the expression of collagen II. Interestingly, the appearance of collagen type II throughout the transitional layer was observed in some MG-3 samples, but not in others. For example, the punctate, territorial collagen type II staining seen in the transitional layer in Fig. 3f, which appeared to mark a return of collagen type II to this region, was not observed in Fig. 3e. Judging from the absence of collagen type II in the superficial layer of sample 268-2, it represented an earlier catabolic phase in OA progression, while the punctate territorial staining seen in the superficial layer of 272-3 suggested that it may have recently entered an anabolic phase. This pronounced difference in the distribution of collagen staining suggested that IHC techniques can detect evidence of a change in metabolism within samples of the same histological grade. The IHC variability observed within MG-3 samples provided further evidence for the existence of subcategories within distinct histological grades.

In samples graded higher than MG-3 considerable IHC variability in collagen II and aggrecan deposition was observed between samples of the same histological grade. For example, MG-5 samples such as 272-1 and 225-1 displayed differences in the staining pattern and overall distribution of both of these extracellular matrix components. Given the divergent patterns of staining for

collagen type II and aggrecan among MG-5 samples, it was difficult to conceive how these could have represented subsequent subcategories within a simple model of linear cartilage deterioration. Since these samples have been collected from a variety of different patients with varying degrees of joint involvement and because AC is known to undergo changes in metabolism with disease progression, it was likely that the complex array of staining patterns observed in MG-5 AC arose as the result of these factors. In fact, this assumption was supported in part by the histological observations leading to the MG itself. For example, while both samples (272-1 and 225-1) obtained a score of MG-5, they arrived at this grade based on the additive scoring of such diverse histological characteristics as tissue structure, extent of safranin-O staining and cellularity. Sample 272-1 received component scores of: 2 for structure; 1 for safranin-O staining; 2 for cellularity, while sample 225-1 received scores of: 3 for structure; 2 for safranin-O staining; 0 for cellularity. Despite being the same MG, the differences in the individual component scores suggested that these samples had undergone significantly different cellular and proteolytic processes leading up to the point of surgery. Clearly all three of the characteristics, on which current histological scoring systems are based, are not associated with IHC staining to the same degree. As a result, the MG alone should not be relied upon to predict the depth or distribution of ECM components such collagen type II and aggrecan in more advanced OA samples.

Taken together these findings suggested that in addition to the more detailed description of OA progression afforded by IHC staining, staining for aggrecan may represent a sensitive indicator of very early disease progression whereas collagen type II staining may serve as an indicator of metabolic status of AC. Finally, we concluded that the use of the Mankin Scoring System can be misleading because AC samples of the same histologic grade can display significantly different molecular profiles and histological staining alone lacks the appropriate resolution to distinguish between them. The IHC disparities seen in MG-1 through MG-5 underscored the need for further molecular characterization in early AC degeneration. Additionally, because no IHC scoring system exists for AC, either a new scoring system would need to be developed or an IHC module would need to be incorporated into an existing scoring system to enhance the understanding of early AC degeneration. It is conceivable that once developed, an IHC scoring system for human AC could not only be readily adapted to animal models of human OA but perhaps also modified to assess the quality of tissue-engineered AC.

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