OsteoArthritis and Cartilage (2007) **15,** 1042–1052

doi:10.1016/j.joca.2007.03.005

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Age- and site-associated biomechanical weakening of human articular cartilage of the femoral condyle[1]

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Summary

Objective: To determine the time sequence of biochemical and structural events associated with, and hypothesized to underlie, age-associated tensile weakening of macroscopically normal adult human articular cartilage of the knee.

Methods: Macroscopically normal human articular cartilage of the lateral and medial femoral condyles (LFC and MFC) from Young (21–39 yrs), Middle (40–59 yrs), and Old (≥60 yrs) age donors were analyzed for tensile properties, surface wear, and cell and matrix composition.

Results: Variations in tensile, compositional, and surface structural properties were indicative of early, intermediate, and late stages of age-associated cartilage deterioration, occurring at an earlier age in the MFC than the LFC. Differences between Young and Middle age groups (indicative of early-to-intermediate stage changes) included decreased mechanical function in the superficial zone, with a loss of (or low) tensile integrity, and surface wear, with faint striations and mild staining on the articular surface after application of India ink. Differences between Middle and Old age groups (indicative of intermediate-to-late stage changes) included maintenance of moderate level biomechanical function, a decrease in cellularity, and a decrease in matrix glycosaminoglycan content. Tissue fluorescence increased steadily with age.

Conclusions: Many of these age-associated differences are identical to those regarded as pathological features of cartilage degeneration in early osteoarthritis. These findings provide evidence for the roles of mechanical wear, cell death, and enzymatic degradation in mediating the progression through successive and distinguishable stages of early cartilage deterioration.

Key words: Cartilage mechanics, Tensile weakening, Aging, Matrix degradation.

Introduction

With aging, the tensile properties of macroscopically normal adult human knee articular cartilage diminish markedly[1,2]. In particular, tensile strength and stiffness in the superficial layer of human femoral condylar cartilage diminish by ~65% between ~24 yrs of age and ~90 yrs of age. In the deeper layers of cartilage, tensile strength also decreases with age in the adult, with strength values being ~50% of those of superficial layer cartilage at the same ages. Such age-related weakening of cartilage may predispose the knee joint to development of pain, dysfunction, and the classical histopathological features of osteoarthritis (OA). However, it is unknown how the age-related biomechanical changes vary between sites within the knee joint and what the structural or compositional basis for these changes are.

Biomechanical weakening of articular cartilage with age may be due to alterations in cellularity or matrix properties, and reflect (1) consequences of cell death, (2) mechanical wear at the surface, (3) degradation of extracellular matrix components, or (4) modification of the collagen network through crosslinks. Mechanical weakening with age may arise from cell death and reflect an inability of the remaining cells to synthesize and remodel matrix components to maintain tissue homeostasis. However, whether cartilage undergoes an age-associated decrease in cellularity is controversial. The cell density, as determined from cell counting in histological sections, of macroscopically normal human articular cartilage of the femoral condyles has been shown to decrease with age[1,2], but this may be dependent on anatomical location and depth from the articular surface[3]. Further, it is unclear if this decrease in cellularity is due to cell death or if the presence of empty lacunae is due to cell loss during histological preparation[4]. Alternatively, cartilage weakening may relate directly to mechanical wear[5], with both cartilage weakening and mechanical wear being a manifestation of disruption of the collagen network, especially at or near the articular surface[6]. In addition, mechanical weakening may be due to degradation of extracellular matrix components.

[1]This work was supported by the Arthritis Foundation, HHMI Professor's Award (to UCSD for RLS) NIH, and NSF.

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Received 22 August 2006; revision accepted 3 March 2007.

Chondrocyte and synoviocyte production of pro-inflammatory cytokines and proteases[7,8] may cause cleavage and denaturation of the collagen network[9,10], fragmentation and loss of proteoglycans[11,12], and tissue weakening. However, these may be manifestations of more advanced stages of osteoarthritic degeneration. Finally, modifications to the collagen network such as the accumulation with age of products of non-enzymatic glycation, including pentosidine[13,14], have been associated with a decrease in instantaneous deformation and increased collagen stiffening[13]. This could give rise to a more brittle and fragile collagen network and contribute to age-associated tensile weakening[15]. These four postulated mechanisms of biomechanical weakening with age may, alone or in combination, underlie progressive cartilage degeneration and, ultimately, end-stage OA.

The site-specific pattern and severity of changes in cartilage properties in the knee joint may give insight into the mechanism of age-related cartilage weakening. The compositional, structural, and functional properties of articular cartilage appear to be modulated by the extent and pattern of joint loading across the knee joint. Areas of high weight-bearing are associated with a relatively high concentration of aggrecanase cleavage products[16] and low equilibrium tensile modulus[17] compared to areas loaded more intermittently. In addition, the medial compartment of the knee is subjected to higher joint forces than the lateral compartment during the stance phase of normal gait[18,19]. This loading pattern may cause early degenerative changes to be more prevalent in the medial femoral condyle (MFC) than the lateral femoral condyle (LFC)[20]. When degenerative changes are present, they are usually more severe in the MFC than the LFC[21] and include a decrease in cartilage thickness of the MFC, but not the LFC of patients with knee OA[22]. Functionally, the cartilage of the MFC also has a lower indentation stiffness than that of the LFC[23,24]. Overall cartilage function, composition, and structure appear to change progressively in early degeneration and OA[10,17,25,26]. Thus, comparison of the MFC and LFC cartilage with respect to age-associated differences in the function, composition, and structure may provide insight into the mechanisms of age- and OA disease-associated degeneration.

The hypothesis underlying this study was that human articular cartilage, devoid of gross erosion, exhibits tensile softening with age in a site-associated manner due to changes in composition and structure, reflecting sequential stages of early cartilage degeneration. As a first step to test a causal link between specific changes and biomechanical weakening, this study sought to establish the extent of variations in the relevant properties of individual human tissue samples that were macroscopically normal with age. Once principal age-associated changes are established, further study with experimentally controlled manipulations of those properties could test the role of specific processes as a pathogenic mechanism resulting in cartilage degeneration. Thus, the objectives were to analyze, in macroscopically normal and structurally characterized human articular cartilage from adults of young, middle, and old age and taken from different depths at the LFC and MFC sites, (1) tensile biomechanical properties, (2) density of cells, (3) content of extracellular matrix components, and (4) fluorescence indicative of non-enzymatic glycation products. The results were interpreted in terms of the mechanisms of cartilage weakening, as postulated above, and early, intermediate, and late adult stages of age-associated degeneration.

Materials and methods

SAMPLE SELECTION AND PREPARATION

Samples were selected from 31 human cadaveric donors distributed among three adult age groups, Young (20–39 yrs), Middle (40–59 yrs), and Old (≥60 yrs). Donor tissue was obtained from tissue banks with donation areas in the Western and Southern areas of the United States. Donors were excluded if they had a history of knee arthritis or if the cause of death was due to a high velocity impact that might cause acute knee injury. Donor joints were excluded either if osteophytes were present or if subchondral bone was exposed by cartilage erosion in the femoral compartment.

Samples used for the present study were the normal subsets of samples used, in part, in a previous study on indentation stiffness and structural indices of wear[23]. These samples were considered normal because, at harvest, they were devoid of macroscopic cartilage erosion or visible unevenness or granularity of the articular surface. Opposing tibial surfaces displayed mild degeneration or small defects (area of 0.13 ± 0.06 cm^2, mean ± SD, range of 0–0.2 cm^2). Samples were from the anterior region of the MFC ($n = 28$) and LFC ($n = 28$) approximately 1.5 cm lateral or medial to the intercondylar notch. The anterior region of each condyle was selected for study because it exhibits moderate degenerative changes in the articular surfaces during aging[20]. In addition, this region is relatively flat[27,28] and facilitates tensile mechanical testing. The samples were from donors who were distributed approximately evenly between males ($n = 4$–7) and females ($n = 3$–5) within each age group. Although donors were not selected based on body mass index (BMI), BMI was similar between age groups (ANOVA $P = 0.3$, Table I).

In the prior study, parts of these samples were examined for cartilage thickness, histopathology (Mankin–Shapiro score, surface irregularity), and surface roughening (reflectance score after India ink staining). Cartilage thickness [Fig. 1(A)] did not vary with age group and did not vary with medial or lateral locations, consistent with the selection of non-eroded cartilage specimens. The reflectance score decreased with age group, indicating more ink staining, and was lower in MFC samples compared to those of the LFC [Fig. 1(B)]. In addition, the variance of the reflectance score indicated more variation in ink staining with age in the LFC and high variation in all age groups of the MFC [Fig. 1(C)]. Consistent with this, the Mankin–Shapiro histopathology score[29] of these normal samples, while being low in general (e.g., compared to degenerate samples), increased slightly in the MFC from Young to Old age groups [Fig. 1(D)], primarily due to a higher surface irregularity score [Fig. 1(E)]. Thus, while these samples were judged to be macroscopically normal, there was evidence for very mild age- and site-associated surface roughening at the articular surface.

Samples were initially harvested in the form of 10-mm diameter osteochondral cores with a notch placed in the posterior-most edge of the core in order to maintain orientation. Samples were soaked in an excess volume (~5 ml each) of phosphate buffered saline (PBS) with proteinase inhibitors (PI; 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetic acid, 5 mM benzamidine hydrochloride, and 10 mM *N*-ethylmaleimide)[30] at 4°C for 1 h and then stored at −70°C until use. Samples were thawed in ~1 ml of PBS with PI for 15 min at room temperature prior to analysis. Control studies with cartilage slices from Young and Old

Table I
Donor description: age, BMI, and n *of human female and male donors of osteochondral cores from the LFC and MFC reported as mean* ± S.E.M.

	Lateral femoral condyle			Medial femoral condyle		
	Young	Middle	Old	Young	Middle	Old
Age (yr)						
Female	33 ± 2	47 ± 3	73 ± 5	33 ± 2	47 ± 3	70 ± 2
Male	29 ± 3	49 ± 1	69 ± 2	29 ± 3	49 ± 1	70 ± 3
Female + male	30 ± 2	48 ± 1	70 ± 3	30 ± 2	48 ± 1	70 ± 2
BMI (kg/m^2)						
Female	28 ± 4	19 ± 1	24 ± 3	28 ± 4	19 ± 1	19 ± 3
Male	25 ± 1	24 ± 2	24 ± 1	25 ± 1	24 ± 2	24 ± 1
Female + male	26 ± 2	23 ± 1	24 ± 2	26 ± 2	23 ± 1	22 ± 2
n						
Female	5	3	4	5	3	4
Male	4	6	6	4	7	5
Female + male	9	9	10	9	10	9

donors confirmed that there was negligible loss of glycosaminoglycan (GAG) or DNA into the PBS + PI bath solution (<3% of the total).

BIOMECHANICAL PROPERTIES

Portions of each core were separated into superficial, middle, and deep layers and analyzed by tensile testing. The cartilage of each core was sliced into ~0.3-mm-thick layers, at a distance from the articular surface of 0% (superficial layer, including the articular surface), 30% (middle layer), and 60% (deep layer) of the average cartilage thickness. These slices were cut into tapered specimens, with a 0.8-mm-wide and 4-mm-long gage region oriented parallel to the splitline pattern, for tensile testing using a methodology[31] that is a combination of previous equilibrium and constant strain-rate test protocols[17,32]. Each tapered specimen was elongated to 10 and 20% strains at 0.25%/s and allowed to stress relax to equilibrium at each strain. This was then followed by elongation of the specimen at a constant rate of 5 mm/min until failure. The force data were normalized to the width and thickness of the sample to obtain stress in units of megapascals (MPa), and the displacement data were normalized to the initial length to obtain strain (dimensionless). The equilibrium test results were analyzed to determine tensile equilibrium modulus, and the dynamic test results were used to assess tensile ramp modulus, tensile strength, and failure strain.

BIOCHEMICAL PROPERTIES

Portions of tissue slices, adjacent to tensile samples, were analyzed for cell and matrix properties. These portions were weighed wet, lyophilized, weighed dry, and solubilized with proteinase K (ProK). The solubilized portions were used to determine total DNA[33], hydroxyproline[34], and GAG content[35], as well as intrinsic fluorescence at excitation (Ex) and emission (Em) wavelengths corresponding to maximum fluorescence of pyridinoline (Ex 295/Em 395 nm[36]) and pentosidine (Ex 335/Em 385 nm[37]) cross-links, consistent with peaks in fluorescence maps[33]. DNA was converted to cell number using a conversion factor of 7.3 pg DNA/human chondrocyte[3]. Hydroxyproline content was converted to collagen (COL) content using 7.1 as the mass ratio of collagen to hydroxyproline[38]. Sulfated GAG content was calculated by comparison to known concentrations of shark chondroitin sulfate. The contents of DNA, COL, and GAG were calculated as the mass normalized to wet weight. Fluorescence data are reported as a ratio of pentosidine-associated fluorescence to pyridinoline-associated fluorescence, the latter of which is stable with age[13].

The residual portions of the cartilage sections were analyzed for denatured collagen exactly as described previously[9]. Briefly, proteoglycan was extracted with guanidine hydrochloride (Gnd), degraded collagen was extracted with alpha-chymotrypsin (αCT), and the remaining tissue was digested with ProK. As a positive control for detecting denatured collagen, this analysis was also performed on portions of full-thickness cartilage from the patellae of a 56-yr-old human donor, with some portions analyzed directly and other portions heated to 80°C for 4 h in 0.1 M sodium phosphate and PI to denature the collagen in a controlled manner. The αCT and ProK solutions were analyzed for COL as described above, and the percent of COL in αCT (denatured) was calculated as that in αCT compared to the sum in αCT and ProK solutions.

STATISTICS

The effect of age group on the various mechanical and biochemical parameters was assessed using repeated measures ANOVA with anatomical location (LFC or MFC) and depth from the surface (superficial, middle, or deep) as repeated factors. When anatomical location was found to have a significant independent effect ($P < 0.05$) or a significant interactive effect with layer ($P < 0.05$), the locations were analyzed separately. When age group or depth from the articular surface had an effect ($P < 0.05$), planned comparisons were made between age groups at each depth or between depths for each age group; when age group and depth both had effects or had an interactive effect, planned comparisons were only made for age groups at each depth. Each of these planned comparisons was tested using a significance level $\alpha = 0.05$ divided by the number of comparisons made for a group (e.g., $\alpha = 0.025$ for comparisons of Young vs Middle, Young vs Old, and Middle vs Old groups, since Young, Middle, and Old groups are each used in two comparisons)[39]. For results that were expressed as a ratio or percentage, data were arcsine transformed to improve normality prior to the above statistical analyses. All data are reported as mean ± S.E.M.

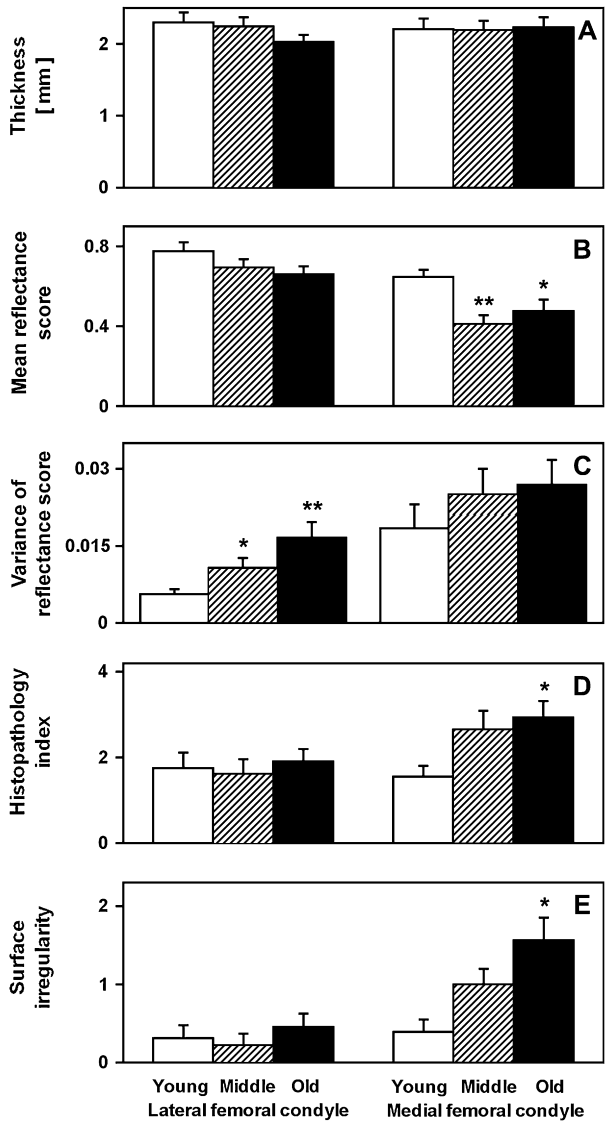

Fig. 1. Structural and surface properties of human articular cartilage from the LFC and MFC. Cartilage thickness (A), reflectance score assessed after India ink staining (B), the variance of reflectance score (C), overall histopathological index of cartilage degeneration (D), and surface irregularity assessed by histopathological grading (E) from donors of Young (21–39 yrs old), Middle (40–59 yrs old), and Old (≥60 yrs old) age groups. $n = 8-12$. $^{*}P < 0.05$, $^{**}P < 0.005$ vs Young age samples.

Results

BIOMECHANICAL PROPERTIES

Variation of a number of tensile properties with age group occurred in a manner that was dependent on sample anatomical location (site) and depth from the articular surface (Fig. 2). Ramp modulus [Fig. 2(B)], strength [Fig. 2(C)], and failure strain [Fig. 2(D)] were lower in the MFC than LFC ($P < 0.005$, $P < 0.01$, and $P < 0.005$, respectively), and equilibrium modulus [Fig. 2(A)] showed a similar trend ($P = 0.2$). Each of these tensile properties was depth-dependent (each, $P < 0.005$), with the equilibrium modulus being 167% higher, the ramp modulus being 176% higher, the strength being 29% higher, and the failure strain being 54% lower in the superficial layer than the deep layer. More specific age-associated differences are described below, as a function of site and depth.

In the LFC, a number of tensile properties of superficial, middle, and deep layers varied with age in a manner suggesting that the superficial layer was affected earliest (by Middle age), while the middle layer was affected later (by Old age), with the deep layer not showing any age-related variation. In the LFC, the tensile integrity of the superficial layer of the Young age group was higher than that of the Middle and Old age groups. The equilibrium modulus [Fig. 2(A)], ramp modulus [Fig. 2(B)], and strength [Fig. 2(C)] of the superficial layer were 21% ($P = 0.2$) and 30% ($P = 0.06$), 46% ($P < 0.005$) and 47% ($P < 0.005$), and 35% ($P < 0.005$) and 39% ($P < 0.005$) lower, respectively, in Middle and Old age samples than corresponding values in Young age samples. In middle layer cartilage of the LFC, the ramp modulus ($P = 0.09$), and strength ($P = 0.04$) tended to be lower in the Old age group than the Young age group. In the LFC, failure strain did not vary between age groups in superficial, middle, or deep layers [$P = 0.09-0.8$, Fig. 2(D)].

In the MFC, a number of tensile properties showed a pattern of variation distinctly different from that of the LFC; the MFC showed relatively little variation with age and depth. In the MFC, the tensile integrity of the superficial layer was relatively low in all age groups. In the superficial, middle, and deep layers, the equilibrium modulus [$P = 0.3-0.9$, Fig. 2(A)], ramp modulus [$P = 0.2-1.0$, Fig. 2(B)] and strength [$P = 0.09-0.8$, Fig. 2(C)] did not vary with age group. The failure strain of the superficial layer was higher in Middle age samples [$P < 0.025$, Fig. 2(D)], but did not vary with age in the middle or deep layer ($P = 0.05-1.0$). Taken together, these results show a pattern of age-associated tensile weakening which occurs at an early age in the LFC and evidence for a tissue that is already weak by a Young age in the MFC.

BIOCHEMICAL PROPERTIES

Variation of a number of biochemical properties with age group also occurred in a manner that was dependent on site and depth from the surface (Fig. 3). Water content [Fig. 3(A)], DNA [Fig. 3(B)], COL in αCT [Fig. 3(D)], and GAG [Fig. 3(F)] tended to be higher ($P < 0.05$, $P = 0.09$, $P < 0.005$, and $P < 0.05$, respectively), and COL [Fig. 3(C)] and fluorescence ratio [Fig. 3(E)] was lower in the MFC than the LFC ($P < 0.05$ and $P < 0.005$, respectively). There was a depth-dependence in DNA ($P < 0.005$), COL in αCT ($P < 0.005$), fluorescence ratio ($P < 0.005$), and GAG ($P < 0.005$), but not water content ($P = 0.2$) or COL ($P = 0.5$). In particular, DNA, COL in αCT, and fluorescence ratio were 180%, 29%, and 15% higher, respectively, and GAG was 66% lower in the superficial layer than the deep layer. The controlled heating of patellar cartilage increased collagen denaturation from $8 \pm 1\%$ to $76 \pm 3\%$, confirming the method of analysis of degraded collagen and the effectiveness of αCT extraction. At particular sites and depths, there were notable age-associated differences in these cartilage components, as described below. Overall, deterioration of certain biochemical properties appeared to occur at stages subsequent to the decrease of tensile integrity noted above.

The water content indicated the absence of marked age-associated swelling of cartilage of these three age groups [Fig. 3(A)]. In the superficial, middle, and deep layers, the water content did not vary with age group in the LFC

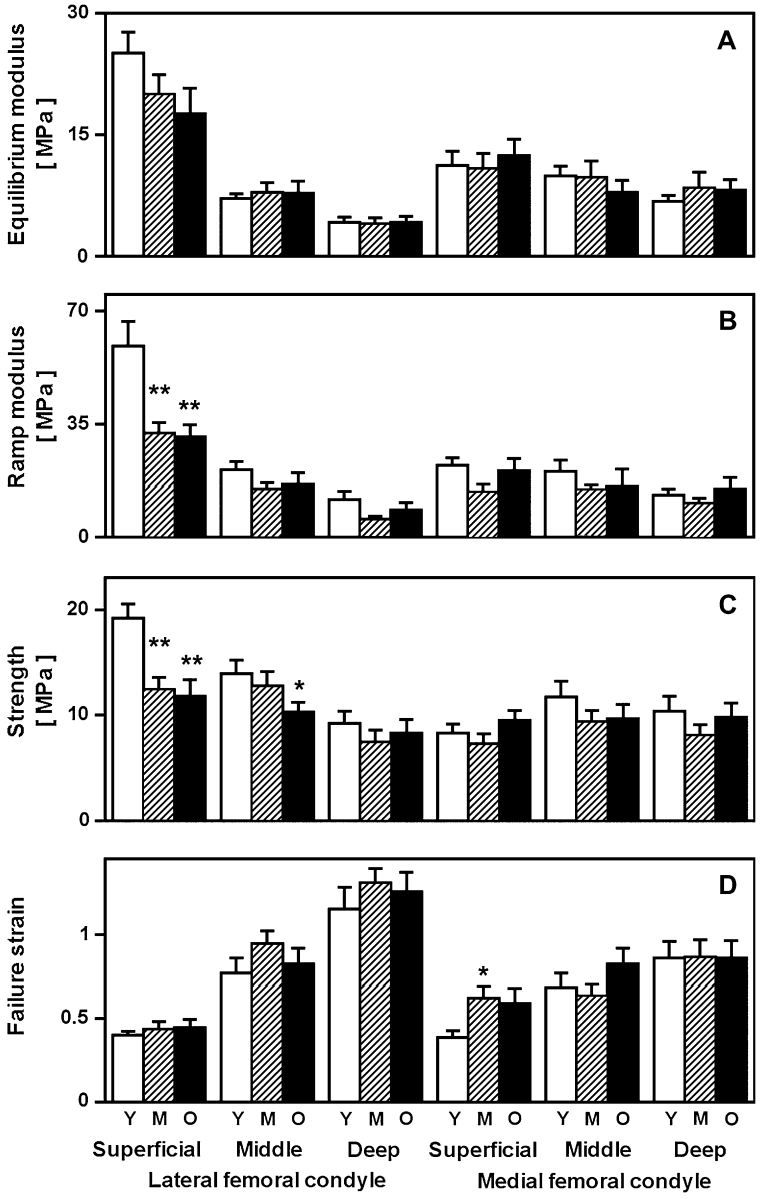

Fig. 2. Tensile biomechanical properties of samples described in Fig. 1. For specimens from the superficial, middle, and deep layers, the tensile equilibrium modulus (A), tensile ramp modulus (B), tensile strength (C), and failure strain (D) were determined from equilibrium and then non-equilibrium failure testing of articular cartilage from Young (Y), Middle (M), and Old (O) age donors. $n = 9-12$. $^{*}P < 0.05$, $^{**}P < 0.005$ vs Young age samples.

($P = 0.2-1.0$) or MFC ($P = 0.3-1.0$) and was ~73% of the wet weight.

The DNA content, a measure of cell density, of the MFC superficial layer varied with age, while that of the LFC superficial layer, and also of the middle and deep layers of the MFC and LFC did not [Fig. 3(B)]. For DNA content, there was an interactive effect of anatomical location and layer, so the sites (LFC, MFC) were analyzed separately. In the MFC, the DNA content of the superficial layer of Young age samples tended to be higher than that of both Middle and Old age samples (by 30%, $P = 0.05$ and 34%, $P = 0.05$, respectively). In the LFC, the DNA content of the superficial layer tended to decrease from Young to Old age (by 10%, $P = 0.3$). These differences in DNA content occurred at a stage after tensile properties were weak (by Middle age in the MFC whereas tensile integrity was already low at a Young age, and by Old age in the LFC whereas tensile properties diminished at the Middle age). In the LFC and MFC, the DNA content of the middle and deep layers was similar between age groups ($P = 0.1-1.0$). Thus, the pattern of age-associated decrease in cell density, indicated by decreases in DNA content, of the superficial layer was delayed relative to the age-associated decrease in tensile integrity.

Age-associated changes in the collagen network were manifest as an alteration of the fluorescence ratio [Fig. 3(E)], but not as an alteration of the total COL content [Fig. 3(C)] or COL in αCT [Fig. 3(D)]. COL content and COL in αCT in the superficial, middle, and deep layers of the LFC and MFC did not show any age-associated change

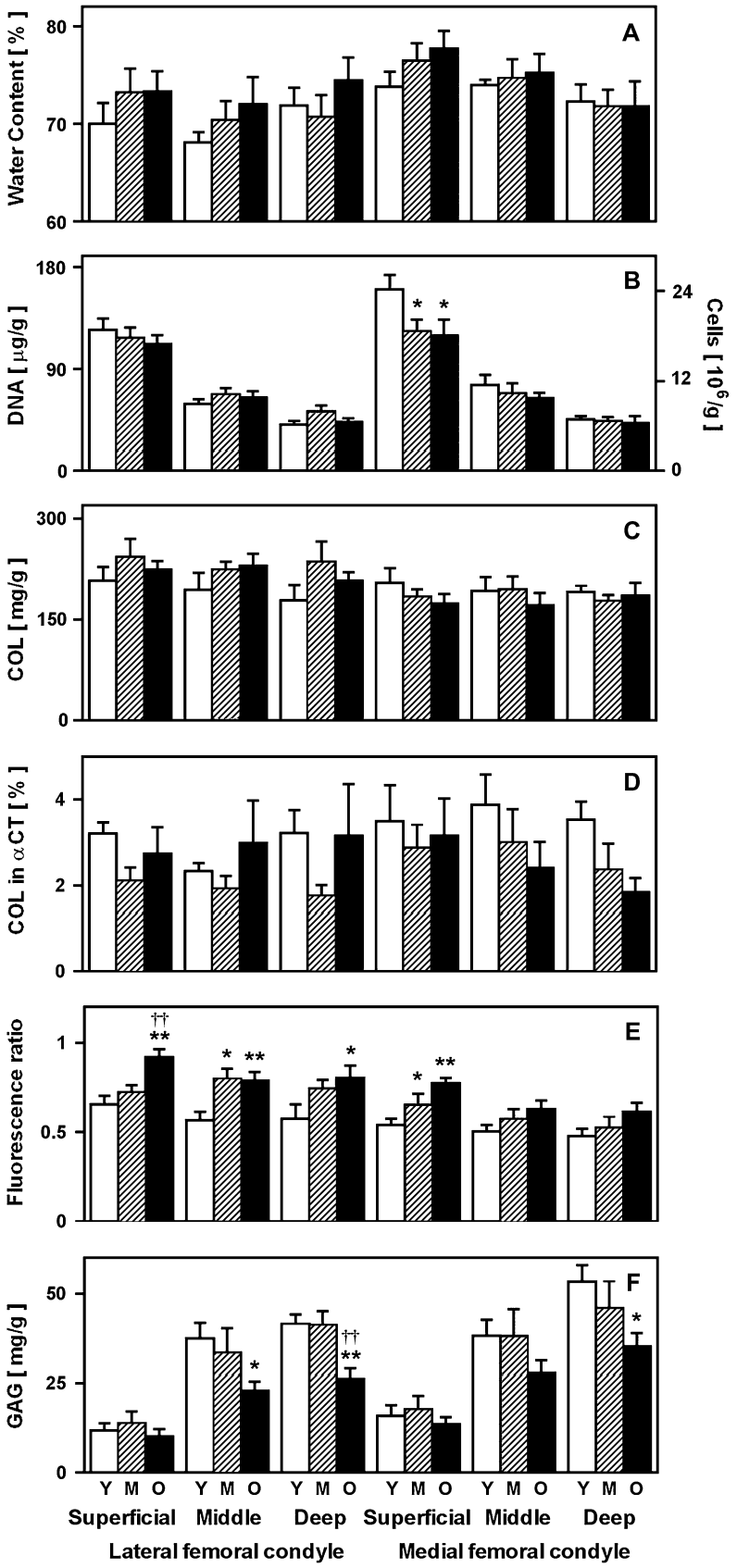

Fig. 3. Biochemical properties of human articular cartilage samples described in Fig. 1. Cartilage tissue adjacent to the mechanical test specimens was analyzed for water content (A), DNA and calculated cell number (B), COL (C), COL in αCT (D), the fluorescence ratio (E) of pentosidine-associated fluorescence (Ex 335/Em 385 nm) to pyridinoline-associated fluorescence (Ex 295/Em 395 nm) and GAG (F). DNA, COL, and GAG were each normalized to wet weight. $n = 9-12$. $^{*}P < 0.05$, $^{**}P < 0.005$ vs Young age samples. $\dagger\dagger P < 0.005$ vs Middle age samples.

($P = 0.1-0.9$). The fluorescence ratio, on the other hand, showed an increase with age at each depth, with differences depending on the site. In the LFC superficial layer, the fluorescence ratio was higher in the Old age group than in the Young age group (by 79%, $P < 0.005$). The fluorescence ratio was also higher in the LFC middle layer (by 51%, $P < 0.025$, and by 74%, $P < 0.01$, respectively), and tended to be higher in the LFC deep layer (by 26%, $P = 0.09$, and by 53%, $P < 0.025$, respectively) in Middle and Old age groups than in the Young age group. In the MFC, the fluorescence ratio in the superficial layer tended to be higher in Middle (by 30%, $P = 0.05$) and was higher in Old (by 85%, $P < 0.005$) age samples than Young age samples, but was not distinguishably different with age in middle and deep layers ($P = 0.08-0.7$). Taken together, these results indicated that although the content of collagen and degradation products in these macroscopically normal samples were indistinguishable in aging, there was an age-associated increase in pentosidine-associated fluorescence in the superficial layers, subsequent to tensile weakening by Middle age in the LFC and subsequent to the weak tensile state at Young age in the MFC.

The GAG content showed age-associated differences that were localized to the deep and middle layers, and not evident in the superficial layer [Fig. 3(F)]. In the LFC, the GAG contents of the middle and deep layers tended to be lower in the Old age groups than in the Young age group (by 31%, $P < 0.005$ and 34%, $P = 0.04$, respectively) and also lower in the Old age groups and Middle age groups (by 25%, $P = 0.2$ and 31%, $P < 0.005$, respectively). In the MFC, the GAG of the middle and deep layers tended to be lower in the Old age group than in the Young age group ($P = 0.2$, and $P = 0.03$, respectively). The pattern of decrease in GAG content in the middle and deep layers from Middle to Old age groups appeared subsequent to the low levels of tensile integrity in the Young (LFC) or Middle (MFC) age groups.

Discussion

This study of macroscopically normal human articular cartilage identified a pattern of age-associated changes in biomechanical integrity, cellularity, content of matrix components, and fluorescence index of non-enzymatic glycation, some of which appear related to structural indices of surface wear. These changes were prominent in cartilage at certain anatomical locations and depths. Changes of a similar sequence generally occurred at an earlier age in the MFC than in the LFC. The decrease in tensile integrity of the superficial layer (Fig. 2) and increase in surface wear (Fig. 1) occurred from Young to Middle age groups in the LFC samples while low tensile integrity of the superficial layer and surface wear were already evident in MFC samples at the Young age. This was followed by a decrease in tissue cellularity in the superficial layer [Young to Middle in the MFC, Fig. 3(B)], increased fluorescence [from Middle to Old in all layers of the LFC and a trend from Middle to Old in the superficial layer of the MFC, Fig. 3(E)], and decreased GAG content of the deep layer [from Middle to Old in the LFC and MFC, Fig. 3(F)]. These age-associated changes appear to represent stages of mild cartilage degeneration that may progress to, or predispose the joint to, the development of OA.

Several factors may limit the interpretation of the results of this study. Diet, lifestyle, and genetics of human donors were not controlled for or analyzed. However, samples were chosen to minimize effects of such factors by choosing approximately equal numbers of male and female donors for each age group, and selecting macroscopically normal, non-eroded cartilage samples; also, BMI was similar between age groups. The macroscopically normal samples of this study were obtained from donors without OA because normal-appearing cartilage in OA joints are subjected to elevated levels of matrix degrading enzymes[40]. However, such exclusion of OA donor samples may have selected for tissue properties that are present in non-OA samples and protective against OA. The sample sites, the anterior region of the MFC and LFC, provided a relatively flat region[27,28] for analysis; however, age-related changes vary with region in the knee joint, and in this region are typically less severe or delayed relative to those in the central weight-bearing regions, as well as the patella and uncovered area of the tibial plateau[20]. Finally, although 31 donors were analyzed, the sample size limits the statistical power. For certain variables of interest, such as the tensile ramp modulus and strength, significant differences were detected for the sample size of $n = 9-10$ per group. However, for other variables, such as equilibrium modulus, differences between Young, Middle, and Old age groups would have had to be ~45% for detection, and higher than the 20–30% difference seen in this study. The analysis of human cartilage samples in discrete layers and a single orientation was done to limit sources of variability in measures of biomechanical and biochemical properties. Such an analysis of cartilage layers was done at the expense of analysis of the full thickness of tissue, although indentation analysis of adjacent tissue was reported previously[23]. The standardized sampling of layers may also affect the interpretation of the results. Dimensions and orientation with respect to the splitline pattern[41] of tapered tensile test specimens were uniform among samples in this study and chosen to be similar to those of previous studies[17,42] to allow for direct comparisons.

The changes in tensile integrity, surface wear, and tissue composition were suggestive of early, intermediate, and late stages of age-associated deterioration occurring in a zonal pattern (Fig. 4) at an earlier age in the MFC than the LFC. Early-to-intermediate age-associated changes included surface wear and decreased mechanical function of the superficial zone, demonstrated here by India ink staining and histopathological indices of roughness as well as loss of, or low, tensile biomechanical integrity. This was followed by intermediate-to-late stage changes by a decrease in cellularity in the superficial zone and net depletion of GAG in the deep zone. Many of these age-associated degenerative changes are essentially identical to those regarded as features of cartilage in early OA[43].

With regard to these proposed stages of age-associated articular cartilage deterioration, some differences between the MFC and LFC locations were striking. There was a greater amount of degeneration in medial than lateral samples by Old age as indicated by the increased histopathology score. Further, the reflectance scores of medial samples were much lower than those of lateral samples, especially at Middle and Old ages, indicating an increase in surface wear. Each of these would lead to the expectation for more weakened tissue in the MFC, which was the case. Features such as higher cellularity in the superficial layer and lower failure strain in middle and deep layers of MFC samples compared to those of corresponding LFC samples may indicate intrinsic differences between articular cartilage of the MFC and LFC. Alternatively, rather than being an indicator of advanced stages of age-associated

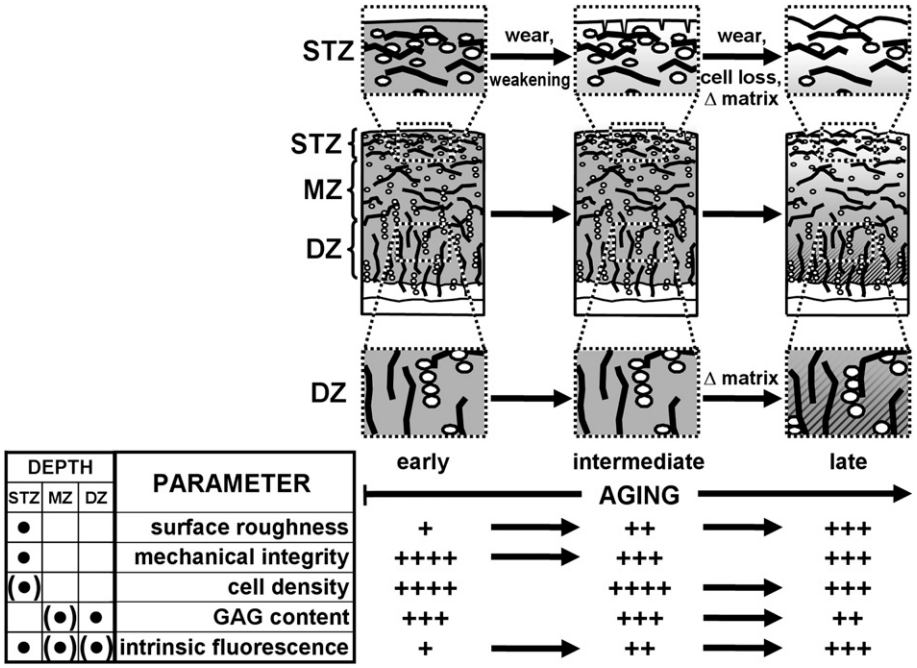

Fig. 4. Summary of cartilage changes at early, intermediate, and late stages of age-associated degeneration in particular zones. Changes in superficial tangential zone (STZ), middle zone (MZ), and deep zone (DZ) are noted by ●, with parentheses signifying variable changes. Depicted are changes in mechanical integrity (degree of gray shading), articular surface wear, alterations of chondrocyte density, increase in intrinsic fluorescence, and loss of GAG (▨).

changes, the low tensile integrity of the MFC at Young ages may be indicative of a difference in LFC and MFC maturation (age <21 yrs), due to differences in contact forces or pressures[44,45]. In addition, differential contact with the menisci between the LFC and MFC samples might cause site variations in the contact area through which load is transmitted and, therefore, alter the joint pressures experienced at those sites[46].

The observed mechanical weakening of the LFC was localized to the superficial layer and coincided with changes in articular surface structure, rather than changes in biochemical or cellular composition. Tensile weakening at that site occurred during early-to-intermediate stages [from Young to Middle age groups, Fig. 2(B, C)] and paralleled the mild age-associated decreases in reflectance score and notable age-associated increase in the variance, an indicator of the roughness of the articular surface [Fig. 1(B, C)]. Samples including the articular surface (top of specimen at 0%) were used because of its importance to the tensile properties of articular cartilage[32], to overall cartilage deformation during joint loading[47], and to its sensitivity to aging[42] and degeneration[17]. The age-associated increase in surface irregularity, detected by histology and image analysis of ink-stained surfaces performed in our prior study[23], indicated that there were mild alterations of the articular surface. How this structural alteration relates to the presence and/or loss of molecular components of the superficial zone remains to be determined. Nonetheless, the results of this study indicate that mild disruptions of the articular surface may be the structural basis for age-associated tensile weakening.

The analysis of tensile integrity expanded upon results of previous studies by assessing the effect of age at each site. This allowed separation of the confounding effects of osteoarthritic degeneration, site, and age on tensile integrity, factors which typically are present because of the prevalence of OA at advanced age[48] and because cartilage degeneration occurs earlier in the MFC than the LFC[20]. The range of values of tensile moduli and strength were generally similar to those obtained in previous studies of tensile equilibrium[17] and dynamic stiffness and failure[42]. The age-associated decrease in tensile strength of ~10% per decade of age of macroscopically normal human articular cartilage noted by Kempson[42] would appear to represent, based on the current study [Fig. 2(C)], the combination of an age-associated decrease of tensile strength of the LFC and low strength of the MFC at all ages. Furthermore, the tensile weakening with age, in addition to changes with degeneration and OA, noted by Akizuki *et al.*[17] (middle-age fibrillated and old-age OA cartilage), with tensile equilibrium moduli being ~85% and 70% lower than those of young normal cartilage, appears to be partially (~20%) attributable to age-associated tensile weakening demonstrated in this study [Fig. 2(A)]. Thus, both age and site on the femoral condyle contribute markedly to variation in cartilage tensile biomechanical properties.

The analysis of DNA content helps to clarify the controversy about age-associated changes in cartilage cellularity at different depths. Measures of DNA in macroscopically normal human articular cartilage were made possible in small portions of tissue by an assay of DNA[33] that has improved sensitivity and specificity[49] over other assays[50,51]. The measure of DNA in this study does indicate an age-associated decrease in cellularity, localized to the MFC superficial layer [Fig. 3(B)]. This result parallels the decrease of cell density as measured by cell counting in histological sections, where cell density decreased with age in macroscopically normal articular cartilage from weight-bearing zones after 40 yrs of age[1,2], especially in the superficial zone[3]. It remains unclear if the decrease in cell density

noted in the prior histological analyses is due to cell death or if the presence of empty lacunae is due to a histological artifact of cell loss[4]. The biochemical analyses support the notion that the measured DNA is within cells, rather than in the extracellular space, since tissue extracts with Gnd and αCT (that remove 90% of GAG) contained virtually no DNA (<2% of the total in the tissue, data not shown). One caveat, however, is that the biochemical measure of DNA does not describe cell activity or organization, and focal sites of hypo- and hypercellularity may exist. The decrease in cell density in the superficial zone of cartilage may contribute to the inability of remaining cells to maintain extracellular matrix in this region, and, thus, to the eventual deterioration and loss of mechanical integrity.

The constancy of total and denatured collagen content in samples from different adult age groups is consistent with and expands on the idea that collagen turnover is modest in mature, macroscopically normal cartilage. The total collagen content [Fig. 3(C)] and denatured collagen content [Fig. 3(D)] agree with previously reported values[9,10,52] and, further, varied little with age or depth from the articular surface. The low levels of collagen denaturation [2–4%, Fig. 3(D)], and absence of marked site-associated variations, are consistent with the source of tissue, i.e., joints undergoing normal aging rather than osteoarthritic degeneration, which typically exhibit extensive cartilage collagen denaturation (~10%)[9,10]. Taken together, these findings suggest that the early age-related cartilage weakening, prominent in the LFC superficial layer [Fig. 2(B, C)], is unlikely to be due to collagen denaturation, at least of the extent present in OA and inflammatory arthritis; indeed, both osteoarthritic cartilage and IL-1-treated cartilage explants exhibit tensile softening or weakening that is associated with markedly increased levels of denatured collagen[9,10,17,53].

The fluorescence ratio may reflect cumulative effects of matrix and collagen remodeling, which, in turn, affect cartilage tensile properties. The general age-associated increase in fluorescence ratio is consistent with the accumulation of end products of non-enzymatic glycation[13,14]. Such glycation and increased fluorescence have been associated, on one hand, with increased stiffness and lower collagen turnover[54] that may be protective to cartilage; evidence for a protective effect are the increases in fluorescence ratio in the LFC superficial layer from Middle to Old age and in the MFC superficial layer from Young to Middle to Old age [Fig. 3(E)], during which times tensile properties are maintained (Fig. 2). On the other hand, increased glycation has been associated with increased brittleness (lower failure strain) and/or lack of collagen remodeling that may precipitate weakening (lowered strength and ramp modulus) of cartilage[15]; the absence of such changes in tensile properties in this study suggests that glycation does not have such detrimental effects. It should be noted, however, that this measure of intrinsic fluorescence was an index of crosslink fluorescence and that may be affected by other matrix components.

The pattern of variation in GAG content with depth is generally similar to that found in previous studies[32,55], but also showed an age-associated decrease of GAG content in the deep layer of articular cartilage [Fig. 3(F)]. The measure of sulfated GAG, by reaction with DMMB, was standardized to a chondroitin sulfate standard, and is indicative of charge content since keratan sulfate (one anionic charge group per disaccharide) gives half as much signal per mass as does chondroitin sulfate (two anionic charge groups per disaccharide)[35]. No attempt was made to assess specific alterations of GAG or aggrecan structure that change with skeletal development and aging[56–59] and OA[25]. The decrease with age of GAG in the deep layer from Middle to Old age [Fig. 2(F)] occurs without additional elevation in fluorescence [Fig. 2(G)], suggesting that substantial turnover of both GAG and collagen matrix is occurring. The localization in the deep layer is intriguing, particularly given recent evidence that changes in subchondral bone turnover can markedly affect the overlying articular cartilage[60–62].

More work is needed to fully elucidate the causes and consequences of age-associated tensile weakening. The results of this study point to a role for surface wear and fatigue as major changes in early cartilage deterioration with cell loss being a downstream event. Tensile weakening may be directly related to wear at the articular surface[5,6], with disruption of the collagen network in areas within or adjacent to striations. Consequences of this initial surface wear may include further disruption of the collagen network, loss of extracellular matrix molecules, and loss of cells. Initial age-associated tensile weakening appears not to be due to enzyme-mediated degradation of the collagen network and proteoglycan, or to an age-associated increase in collagen crosslinking, because tensile weakening occurs early at the articular surface, and occurs prior to the alteration of these matrix components. Further study is needed of joints with more advanced stages of degeneration to establish the sequence of events leading to the development of OA. The extent and localization of changes in particular tissue properties sets the stage for further studies with specific and graded manipulations to test the mechanistic relationship for the sequence of events described in Fig. 4. Diminished tissue integrity, associated with aging in the LFC, and with the MFC in general, appears to be an early and sensitive marker of cartilage degeneration and may causally contribute to degeneration and the development of OA.

Acknowledgments

This work was supported by the Arthritis Foundation, HHMI, NIH, and NSF. We thank Karen Bowden for preparation of the histological slides and Drs. Kenji Yamada, Kenji Kobayashi, Tsuyoshi Watanabe, and Hirohito Mitsuyama, UCSD, La Jolla, CA for preparing tissue samples for this study.

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