Induced superficial chondrocyte death reduces catabolic cartilage damage in murine posttraumatic osteoarthritis

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Joints that have degenerated as a result of aging or injury contain dead chondrocytes and damaged cartilage. Some studies have suggested that chondrocyte death precedes cartilage damage, but how the loss of chondrocytes affects cartilage integrity is not clear. In this study, we examined whether chondrocyte death undermines cartilage integrity in aging and injury using a rapid 3D confocal cartilage imaging technique coupled with standard histology. We induced autonomous expression of diptheria toxin to kill articular surface chondrocytes in mice and determined that chondrocyte death did not lead to cartilage damage. Moreover, cartilage damage after surgical destabilization of the medial meniscus of the knee was increased in mice with intact chondrocytes compared with animals whose chondrocytes had been killed, suggesting that chondrocyte death does not drive cartilage damage in response to injury. These data imply that chondrocyte catabolism, not death, contributes to articular cartilage damage following injury. Therefore, therapies targeted at reducing the catabolic phenotype may protect against degenerative joint disease.

Introduction

Articular cartilage plays a crucial role in joint motion and load transmission (1, 2). The most common disease of articular cartilage, osteoarthritis, affects millions of humans and is a major cause of disability (3, 4). Joint injuries (e.g., anterior cruciate ligament and meniscal tears) are increasing in incidence, particularly among young athletes, and predispose individuals to precocious osteoarthritis (5). Inflammatory diseases, such as rheumatoid arthritis, affect approximately 1% of the population and are another significant contributor to the premature failure of articular cartilage (6). In the United States, more than 4 million persons have had knee-replacement surgery for different cartilage diseases (7). The clinical and financial burden of cartilage disease has prompted substantial research investment into its causes and treatments, including the study of animal models of these disease processes.

Chondrocyte loss and cartilage damage occur with aging and after joint injury (8–12). Although cell death and cartilage failure are correlated, the relationship between chondrocyte loss and cartilage degeneration is unknown. Some studies indicate chondrocyte loss precedes cartilage damage after injury and that chondrocyte repopulation in damaged areas can promote cartilage repair (13, 14). Other studies indicate that injured chondrocytes produce factors, such as matrix metalloproteinase 13 (MMP13), ADAM metalloproteinase with thrombospondin type 1 motif 4 (ADAMTS4), and ADAMTS5, that cause cartilage degeneration (15–18). In this paper, we report on a murine genetic modification that induces chondrocyte death and surgical destabilization of the medial meniscus (DMM) to damage the cartilage surface in order to determine whether chondrocyte death is a cause or consequence of cartilage damage and whether cells that live near the surface of cartilage have the ability to regenerate.

We also describe a confocal microscopic method for measuring cartilage volume and chondrocyte number, distribution, and proliferation in mouse articular cartilage. Samples are prepared and imaged using the confocal microscope, much more easily than when studied using standard histology. Histologic sectioning across the knee joint, the commonly used method for assessing mouse articular cartilage (19), is labor intensive, time consuming, and highly dependent on the investigator’s ability to orient, section, and stain specimens to obtain comparable images across animals. We show that confocal imaging of cartilage complements and can supersede existing histologic methods.

Results

A 3D confocal imaging method for mouse articular cartilage. We imaged 760 μm × 760 μm × 50 μm regions of 1-month-old, male C57BL/6J mouse humeral heads, femoral heads, medial femoral condyles, and lateral femoral condyles in the orientations depicted (Supplemental Figure I; supplemental material available online with this article; doi:10.1172/JCI83676DS1). It took approximately 3 minutes to image each cartilage. To assess variability in measurements associated with orienting the samples, we obtained 10
sets of images for each cartilage after having removed and reset the specimen between rounds of imaging (Supplemental Figure 2). We determined that the average of 3 measures in a single specimen provided 95% CI of falling within 1 SD of the mean from 10 measurements. Therefore, in subsequent experiments, we imaged each cartilage specimen 3 times.

We compared measures of cell number and the number of nuclei in the imaged cartilage volume (cell density) between an individual animal’s left and right limbs and between the limbs of different animals. These measurements included the number of chondrocyte nuclei within the entire volume of imaged cartilage (global cell density), the number of nuclei in the cartilage volume making up the first 10 μm beneath the surface (superficial cell density), and the number of nuclei in the center 100 μm × 100 μm area at 10 μm and 20 μm beneath the surface (Figure 1, A–F). Intraanimal variation was less than interanimal variation (Figure 1G, Supplemental Figure 3, and Supplemental Table 1).

To determine whether the length of time in fixative affected the results, we compared measures of cell density and number between sets of images for each cartilage after having removed and reset the specimen between rounds of imaging (Supplemental Figure 2). We determined that the average of 3 measures in a single specimen provided 95% CI of falling within 1 SD of the mean from 10 measurements. Therefore, in subsequent experiments, we imaged each cartilage specimen 3 times.

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We generated mice that have a \( Prg4^{CreERt2} \) allele and a \( R26^{mTmG} \) allele; the latter allele causes cells to produce membrane-bound tomato fluorescent protein (dTomato) until \( Cre \)-mediated recombination causes the cells to produce membrane-bound enhanced GFP (eGFP). We also generated mice with \( Prg4^{CreERt2} \), \( R26^{mTmG} \), and \( R26^{DTA} \) alleles. The last allele causes a cell to produce diphtheria toxin A (DTA) following \( Cre \)-mediated recombination, which results in autonomous cell death (21). We administered tamoxifen or vehicle to 3-week-old \( Prg4^{CreERt2/+R26^{mTmG/+}} \) and \( Prg4^{CreERt2/+R26^{mTmG/+DTA}} \) mice and then performed confocal microscopy on their articular cartilage 1 week, 2 months, and 8 months later.

Based on the aforementioned results, we calculated sample sizes that would be required to detect 10%, 20%, 30%, 40%, and 50% changes in global and superficial cell density and in cell number within the 100 \( \mu \)m \( \times \) 100 \( \mu \)m areas (Supplemental Table 3).

Chondrocyte death does not cause cartilage surface damage. Mice that have a cDNA encoding a chimeric GFPCreERt2 protein knocked into the first coding exon of \( Prg4 \) can have \( Cre \)-recombinase activity induced in most chondrocytes near the surface of articular cartilage (20). We generated mice that have a \( Prg4^{CreER2/2} \) allele and a \( R26^{GwmTmG/+} \) allele; the latter allele causes cells to produce membrane-bound tomato fluorescent protein (dTomato) until \( Cre \)-mediated recombination causes the cells to produce membrane-bound enhanced GFP (eGFP). We also generated mice with \( Prg4^{CreER2/2}, R26^{GwmTmG/+}, \) and \( R26^{DTA} \) alleles. The last allele causes a cell to produce diphtheria toxin A (DTA) following \( Cre \)-mediated recombination, which results in autonomous cell death (21). We administered tamoxifen or vehicle to 3-week-old \( Prg4^{CreER2/2/R26^{GwmTmG/+}} \) and \( Prg4^{CreER2/2/R26^{GwmTmG/+DTA}} \) mice and then performed confocal microscopy on their articular cartilage 1 week, 2 months, and 8 months later. The \( Prg4^{GFPCreER2/2/R26^{GwmTmG/+DTA}} \) mice...
mice that received tamoxifen (i.e., DTA ablated) had significantly fewer superficial chondrocytes compared with controls (i.e., Prg4CreERT2/−R26mTmG/DTA mice that received vehicle only and Prg4CreERT2/−R26mTmG+/+ that received tamoxifen) (Figure 2; C–F; Figure 3, B–I; and Supplemental Figure 5). Notably, DTA-induced cell killing did not cause cartilage deterioration, even 8 months later (Figure 3, B–G, and Supplemental Figure 5, A–F). Although Prg4CreERT2 is also expressed in synovium and tendon, no abnormal phenotype was observed in these tissues from DTA-ablated mice (Supplemental Figure 6).
We also used Prg4CreERT2/+R26mTmG/DTA mice to induce chondrocyte death at 3 months of age and again observed no deleterious effects on the articular cartilage surface when the mice were 6 months old (Supplemental Figure 7).

Surviving chondrocytes divide following DTA ablation, but their distribution in cartilage remains abnormal. We monitored cell division in articular cartilage by giving 5-ethyl-2′-deoxyuridine (EdU) to control and DTA-ablated mice (Figure 2A and Figure 3A). Cell proliferation initially decreased in DTA-ablated mice compared with controls (Figure 2B, Figure 3K, and Supplemental Figure 8), but within 2 months, the DTA-ablated mice had rates of chondrocyte proliferation that were 2- to 3-fold higher than those of control mice. These data indicate that surviving cells retain the ability to divide (Figure 3K and Supplemental Figure 8). In femoral condyles, the total number of chondrocytes (not shown) and the chondrocyte density in the DTA-ablated 3-week-old mice returned to control values by 9 months of age (Figure 3H). However, the distribution of chondrocytes within the articular cartilage differed significantly between 9-month-old control and DTA-ablated mice (Figure 3J).
Chondrocyte death is a consequence of cartilage damage after joint injury. Surgically induced DMM causes osteoarthritis predominantly affecting the medial compartment of the mouse knee within 3 months of surgery (22). Therefore, we examined the medial and lateral condyles of adult mice that had undergone DMM or sham surgery 4 weeks and 12 weeks after the procedure. Four weeks after surgery, there was no cartilage degradation and no change in global cell density in either condyle of the DMM mice compared with the sham-surgery controls (Figure 4, A and B). However, superficial cell density in the medial side of the medial condyle of DMM mice was lower compared with that of controls (Figure 4, A and B). By 12 weeks after surgery, DMM mice had evidence of cartilage damage and a significant decrease in chondrocyte number and density in the medial condyle compared with the sham controls (Figure 4, C and D), but only mild cartilage surface damage and a nonsignificant...
change in chondrocyte number and density in the lateral condyle (Figure 4, C and D).

**Living chondrocytes cause cartilage degeneration after injury.** Having found that killing chondrocytes did not cause cartilage damage in an otherwise normal joint and that DMM surgery caused cartilage damage and chondrocyte loss, we next determined whether the presence of living chondrocytes reduces or enhances cartilage damage following injury. We performed sham or DMM surgery on 10-week-old mice that had or had not previously undergone DTA ablation. When we examined the joints 12 weeks later, mice whose surface chondrocytes had been DTA ablated before DMM had significantly less cartilage damage than did control mice that underwent DMM (Figure 5). Also, whereas DMM surgery led to loss of chondrocytes in the control mice, it did not further decrease chondrocyte numbers in the DTA-ablated mice (Figure 5, A–C). These data indicate that living surface chondrocytes enhance cartilage damage after DMM, even though these cells ultimately die as a consequence of DMM.

**Discussion**

To determine whether chondrocyte death and cartilage damage have a causal relationship, we examined the consequences of conditionally killing chondrocytes at the articular cartilage surface and/or physically destabilizing the medial compartment of the knee joint. We observed no deleterious effect from killing chondrocytes when knee joints were examined as much as 8 months later (Figure 3 and Supplemental Figure 5). In fact, we observed increased rates of chondrocyte cell division in DTA-ablated mice, as measured by EdU incorporation (Figure 3K and Supplemental Figure 8). Thus, DTA-induced death of articular chondrocytes did not initiate cartilage damage. This finding is consistent with work reported by de Crombrugghe and colleagues, which altered chondrocyte identity in mouse articular chondrocytes by conditionally inactivating Sox9 (23). The Sox9-inactivated cells no longer expressed chondrocyte-specific transcripts, yet a year later, the mice had no evidence of cartilage damage (23). Thus, neither survival of chondrocytes nor maintenance of chondrocyte identity is required to protect mouse cartilage over the short term. Human articular cartilage without living cells is also reported to remain free of osteoarthritic changes for several years in cancer patients who had received irradiated, cadaveric osteochondral grafts during limb-salvage surgery (24).

We observed after DTA ablation that surviving chondrocytes retained the ability to divide in vivo (Figure 3K and Supplemental Figure 8). This is compatible with earlier observations that articular chondrocytes can be expanded in culture ex vivo (25) and be induced to divide in vivo in disease states, such as osteoarthritis (26). Despite rates of chondrocyte division becoming significantly higher in DTA-ablated cartilage, the distribution of chondrocytes did not return to normal (Figure 3J). This likely reflects the inability of chondrocytes to migrate through cartilage. Thus, areas devoid of chondrocytes remained so and the progeny from chondrocyte divisions remained near one another in a manner similar to that seen in the “cloning” that is observed in osteoarthritic cartilage.

We performed DMM surgery to produce medial compartment instability and ascertain the effect of this injury on chondrocyte viability (Figure 4). DMM alters joint biomechanics so that histologic changes resembling osteoarthritis first develop in the medial compartment of the knee joint and secondarily occur in the lateral compartment (22). Other investigators have shown TUNEL staining, an indicator of apoptosis, increases after DMM (27), which is consistent with the cell loss we observed by confocal imaging and histology (Figure 4). These data indicate that cartilage damage, attributable to altered biomechanics in the DMM model, is a proximate cause of chondrocyte loss. Other studies have suggested that material damage to the cartilage surface is another antecedent of chondrocyte death (28–30). For example, depletion of surface lubrication in human cartilage explants causes exaggerated mechanical strain (29) and depletion in bovine cartilage explants resulted in increased chondrocyte apoptosis following mechanical loading and movement (28). Genetic deficiency of Prg4, which increases cartilage surface friction and causes wear (30), also leads to a progressive loss of chondrocytes (31).

Protection from cartilage damage following DMM surgery has been reported in genetically engineered mice that could not express enzymes such as ADAMTS4, ADAMTS5 or MMP13 in cartilage (17, 18). These findings imply that before DMM induces cell death, it induces chondrocytes to produce factors that facilitate cartilage destruction. We directly tested this hypothesis by comparing the effect of DMM surgery in mice with and without (i.e., DTA ablated) living articular surface chondrocytes (Figure 5). Mice whose chondrocytes had been DTA ablated before DMM surgery had significantly less cartilage damage, as assessed by Osteoarthritis Research Society International (OARSI) scoring (19), than control mice. These data indicate that dead chondrocytes do less harm after DMM surgery.

We are not the first to apply confocal imaging to articular cartilage (10, 32–36). We believe that the present study is novel in its demonstration of the utility of confocal imaging for quantifying several useful measures of cartilage structure/organization, including cartilage volume, chondrocyte number, cells/volume near the surface, cell division frequency, and nearest neighbor distance. Although there are differences in the coefficients of variation (CVs) for these measurements between articular cartilage surfaces (e.g., femoral head and humeral head), most CVs were still sufficiently small (<10%) (Supplemental Table 2) so that statistically significant differences could be observed with small sample sizes (Supplemental Table 3). For example, confocal microscopy provided 90% power to detect a 30% difference in superficial chondrocyte density using 4 specimens per group for the femoral head, humeral head, and lateral femoral condyle and 6 specimens per group for the medial femoral condyle (Supplemental Table 3). At present, the confocal imaging method is limited to ex vivo analyses of cartilage structure, a limitation shared with other high-resolution imaging techniques such as μCT and μMRI (37, 38).

Compared with other methods, imaging articular cartilage using confocal microscopy is fast. Specimen processing requires little hands-on time and can be completed within 72 hours; this contrasts with standard histologic methods that require decalcification of mineralized tissue, formalin fixation, paraffin embedding, sectioning, and staining before microscopic imaging can be performed. Specimens prepared for confocal imaging can be stored for at least 3 weeks without any significant changes in measurements. Data acquisition was also quick. Obtaining 2-μm interval images

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spanning a volume of 760 \( \mu m \times 760 \mu m \times 50 \mu m \), for 4 separate fluorophores (DAPI, Alexa Fluor 647, dTomato, and eGFP) required approximately 30 minutes per specimen. The time to obtain micron level resolution using confocal microscopy was shorter than in similar micron-scale imaging with \( \mu \)CT or \( \mu \)MRI, each of which requires several hours per specimen (37, 39-41). The applications described here used confocal microscopy to measure cell number, cell density, and cell division. Other fluorophores could be used to measure other aspects of cartilage structure and chondrocyte identity, such as extracellular matrix volume and protein expression.

In sum, employing confocal microscopy, standard histology, genetically engineered mice, and DMM surgery, we show that chondrocyte death is not a proximate cause of cartilage damage when the death has left the cartilage surface intact. We further show that, while DMM surgery causes chondrocyte death, it is the dysfunction of chondrocytes prior to their demise that enhances cartilage damage in the DMM model. Thus, postrumastric osteoarthritis, as occurs following injury (42), is the likely result of initial cartilage damage that predisposes chondrocytes to adopt a catabolic phenotype. Therapies that prevent this chondrocyte-mediated cartilage catabolism after injury should reduce the burden of degenerative joint disease.

**Methods**

**Experimental animals and procedures.** Wild-type C57BL/6J mice, R26\(^{R26}\)CreER\(^{R26}\) mice with a C57BL/6J background, R26\(^{R26}\)mT\(^{mT}\)mG\(^{mG}\) mice with a 129X1/SvJ background, and Prg4\(^{CreERt2}\) mice were obtained from the Jackson Laboratory (stock numbers 000664, 009669, 007576, and 022757, respectively).

Cre-recombinase activity in postnatal mice with the \( \text{Prg4}^{\text{CreERt2}} \) allele was induced by administering 100 mg/kg tamoxifen diluted in corn oil (both from Sigma-Aldrich) for 10 consecutive days by i.p. injection; i.p. injections of corn oil alone served as a negative control. At least 5 animals, including both sexes per genotype, treatment, and age group were studied.

To label dividing cells, EdU (Invitrogen) was given for 10 consecutive days to 40-day-old mice (50 mg/kg/dose via i.p.) or for 30 consecutive days to 2-month-old mice (0.3 mg/ml in drinking water).

DMM was performed as previously described (22, 26). Briefly, 9- to 11-week-old mice were anesthetized and their right knees were prepared for aseptic surgery. The joint capsule was immediately medial to the patellar tendon was opened. The intercondylar region was exposed to visualize the meniscotibial ligament. This ligament was cut, after which the joint capsule and skin were closed. Sham-surgery animals had the joint capsule opened without cutting the meniscotibial ligament. The knees from the animals were studied at 4 and 12 weeks after surgery. DTA ablation, prior to performing DMM or sham surgery, was accomplished by administering tamoxifen for 10 consecutive days at from 21 to 30 days of age and for another 5 consecutive days, ending 1 week before surgery was performed.

**Sample preparation.** Mice were euthanized by CO\(_2\) inhalation. Specimens consisting of whole humeri and femora were removed, cleaned of muscle and ligament, placed in approximately 1 ml of PBS containing 4% paraformaldehyde (PFA) (Affymetrix), and stored at 4°C.

Specimens consisting of knee joints with intact synovial capsules were fixed in 4% PFA for more than 72 hours, decalcified for 14 days in 14% EDTA (pH 7.5), and embedded in paraffin.

**DAPI and EdU staining.** Cell nuclei in the fixed humeri and femora were stained by adding DAPI dye (10 \( \mu g/ml \)) to the 4% PFA solution for more than 16 hours. Unincorporated dye was removed by washing specimens twice for 15 minutes in 4% PFA.

For animals that had been given EdU in vivo to label dividing cells, the EdU-containing cells in the 4% PFA-fixed humeri and femora were detected using the Alexa Fluor 647 Click-IT EdU Imaging Kit (Invitrogen). The 4% PFA fixed small intestine specimens were used as positive controls. The fixed tissues were subjected to two 15-minute washes with 3% BSA (Sigma-Aldrich) in PBS, permeabilized by being placed in 2% Triton X-100 (Sigma-Aldrich) in PBS for 2 hours at room temperature, and washed again with two 15-minute washes in 3% BSA in PBS. Click-IT reaction cocktail was prepared according to the manufacturer’s instructions and applied to the tissues for 24 hours at 4°C. Specimens were given two 30-minute washes in 3% BSA in PBS, returned to 4% PFA solution, and then stained with DAPI dye.

**Confocal laser scanning.** Each individual specimen was placed on a glass-bottom plate and immersed in PBS in the orientation indicated (Supplemental Figure 1A). The cartilage surface in contact with the glass was imaged at 1- or 2-\( \mu m \) intervals to a depth of 50 \( \mu m \) using an inverted Zeiss LSM780 confocal laser-scanning microscopy system (Carl Zeiss) (Supplemental Figure 1B). The objective used was Zeiss ×20 Plan Apochromat NA 0.8 WD 550 mm dry. The confocal imaging was controlled through the software ZEN 2009 from Zeiss. The frame size of the image was 760 \( \mu m \) × 760 \( \mu m \). A Z-stack 50-\( \mu m \) thick for femoral heads and femoral condyles or 60-\( \mu m \) thick for humeral heads (1 or 2 \( \mu m \)/slice) was projected from the surface with a speed of 6. Samples were labeled with up to 4 fluorophores including DAPI, eGFP, dTomato, and Alexa Fluor 647. Excitation wavelengths used were 358 nm for DAPI, 488 nm for eGFP, 561 nm for dTomato, and 647 nm for Alexa Fluor 647. Emission wavelengths used were 385 to 500 nm for DAPI, 500 to 555 nm for eGFP, 585 to 650 nm for dTomato, and 647 nm for Alexa Fluor 647. Gains were 700 for DAPI, 800 for eGFP, 750 for dTomato, and 800 for Alexa Fluor 647.

**3D reconstruction of the articular cartilage and quantitative analysis.** The imaging software IMARIS was used to perform 3D image reconstruction and calculate chondrocyte number and density (43). The spots function within IMARIS was used to obtain the center of mass in each cell nucleus and count the number of nuclei with a specified radius (Figure 1A). The radii of the DAPI- and EdU-labeled cell nuclei were set at 5 \( \mu m \), and the radii of membrane-bound eGFP and dTomato fluorescence-labeled cells were set at 10 \( \mu m \). Cell nuclei were counted in a specified region within the image (Figure 1, A, C, E, and F). The surface function was used to compute the volume of cartilage surface (Figure 1, B and D).

**Histologic analysis.** The 4% PFA-fixed, 14% EDTA-decalcified, and paraffin-embedded intact knee joints were sectioned in the sagittal plane and stained with H&E or Safranin O and Fast Green (Thermo Scientific). Sections from equivalent regions of the joint were obtained in order to compare findings between animals. OARSI scoring was performed as previously described (19) on coronal sections of paired
femoral condyles and tibial plateaus. Six independent observers, who were blind to whether the joint had had DTA ablation or DDM surgery, scored the sections. Medial and lateral femoral condyle and medial and lateral tibial plateau scores were summed for each animal, and the averages of the 6 independent observers’ summed scores were used in the statistical analyses.

Statistics. Bland-Altman paired analyses with 95% CIs were performed to compare confocal microscopy measurements obtained for each mouse’s left and right femoral head, humeral head, lateral condyle, and medial condyle in order to assess agreement and correlation. A 1-sample t test was used to supplement the analysis on agreement using the difference in the measurements between the left and right sides. Repeated measures ANOVA using generalized estimating equations (GEE) followed by a 1-sided t test was used to assess the impact of time in 4% PFA on each of the 4 primary measures for femoral head, humeral head, lateral femoral condyle, and medial femoral condyle. CV was determined to assess differences between variability in relation to the calculated mean for each scan or time point. Sample size estimates were calculated based on an independent Student’s t test with equal sample sizes for each percentage change, and t tests were used to assess statistical significance for confocal microscopy measurements between DTA-ablated and control joints. Comparison of chondrocyte nearest neighbor distances in control and DTA-ablated mice was performed using Student’s t test, and variability of histogram shape was assessed according to Levene’s test. The nonparametric Wilcoxon rank-sum test was used to compare OARSI scores between mice that did or did not experience DTA ablation and DMM surgery.

Two-tailed P values of less than 0.05 were considered significant. Two articular cartilage surfaces were damaged when they were being dissected from mice and were not included in the confocal microscopy and histologic assessments.

Study approval. The Institutional Animal Care and Use Committee at the Boston Children’s Hospital and the Harvard School of Dental Medicine approved these studies.

Author contributions
MZ, GDJ, and MLW conceived the research. MZ, SBM, and YH performed confocal imaging and analysis, EDU incorporation, and DTA-induction experiments. MZ, LX, and YL performed DMM experiments. AMH and DZ performed statistical analyses.

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