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An Anterior Cruciate Ligament Failure Mechanism

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Abstract

Background: Nearly three-quarters of anterior cruciate ligament (ACL) injuries occur as “noncontact” failures from routine athletic maneuvers. Recent in vitro studies revealed that repetitive strenuous submaximal knee loading known to especially strain the ACL can lead to its fatigue failure, often at the ACL femoral enthesis.

Hypothesis: ACL failure can be caused by accumulated tissue fatigue damage: specifically, chemical and structural evidence of this fatigue process will be found at the femoral enthesis of ACLs from tested cadaveric knees, as well as in ACL explants removed from patients undergoing ACL reconstruction.

Study Design: Controlled laboratory study.

Methods: One knee from each of 7 pairs of adult cadaveric knees were repetitively loaded under 4 times–body weight simulated pivot landings known to strain the ACL submaximally while the contralateral, unloaded knee was used as a comparison. The chemical and structural changes associated with this repetitive loading were characterized at the ACL femoral enthesis at multiple hierarchical collagen levels by employing atomic force microscopy (AFM), AFM–infrared spectroscopy, molecular targeting with a fluorescently labeled collagen hybridizing peptide, and second harmonic imaging microscopy. Explants from ACL femoral entheses from the injured knee of 5 patients with noncontact ACL failure were also characterized via similar methods.

Results: AFM–infrared spectroscopy and collagen hybridizing peptide binding indicate that the characteristic molecular damage was an unraveling of the collagen molecular triple helix. AFM detected disruption of collagen fibrils in the forms of reduced topographical surface thickness and the induction of ~30- to 100-nm voids in the collagen fibril matrix for mechanically tested samples. Second harmonic imaging microscopy detected the induction of ~10- to 100-μm regions where the noncentrosymmetric structure of collagen had been disrupted. These mechanically

induced changes, ranging from molecular to microscale disruption of normal collagen structure, represent a previously unreported aspect of tissue fatigue damage in noncontact ACL failure. Confirmatory evidence came from the explants of 5 patients undergoing ACL reconstruction, which exhibited the same pattern of molecular, nanoscale, and microscale structural damage detected in the mechanically tested cadaveric samples.

Conclusion: The authors found evidence of accumulated damage to collagen fibrils and fibers at the ACL femoral enthesis at the time of surgery for noncontact ACL failure. This tissue damage was similar to that found in donor knees subjected *in vitro* to repetitive 4 times–body weight impulsive 3-dimensional loading known to cause a fatigue failure of the ACL.

Clinical Relevance: These findings suggest that some ACL injuries may be due to an exacerbation of preexisting hierarchical tissue damage from activities known to place larger-than-normal loads on the ACL. Too rapid an increase in these activities could cause ACL tissue damage to accumulate across length scales, thereby affecting ACL structural integrity before it has time to repair. Prevention necessitates an understanding of how ACL loading magnitude and frequency are anabolic, neutral, or catabolic to the ligament.

Keywords

anterior cruciate ligament; femoral enthesis; tissue fatigue damage; submicroscopic damage; *in vitro*; *in vivo*; atomic force microscopy; infrared spectroscopy; second harmonic imaging

INTRODUCTION

It is well recognized that nearly three-quarters of all anterior cruciate ligament (ACL) injuries occur without contact, even in a high collision sport such as National Football League football [6]. Hypotheses proposed to explain the mechanism of noncontact ACL injury include aggressive quadriceps loading [14], excessive joint compressive loading [20], awkward landing or decelerating maneuvers [7], neuromuscular control deficit [13], and the induction of macroscopic tissue damage from repetitive submaximal ligament loading after simulated strenuous jump landings [25].

The ACL consists of dense connective tissue whose major component is type I collagen [22]. Fibril-forming collagen molecules are assembled in a hierarchical order into ligaments [8]. This hierarchical assembly starts with collagen molecules aggregating to collagen fibrils at the nanometer scale, then at the micrometer scale, and eventually into bundles to form ligament at the macroscale. Disruption of the collagen helical assembly results in reduced tensile strength and abnormal development of the collagen fibrils [9].

Among all characterization methods, magnetic resonance imaging (MRI) is the most commonly used tool for the clinical diagnosis of ACL injury [21]. While MRI may also have the potential to detect molecular, nanolevel, and microlevel damage, the challenge of conducting the appropriate MRI experiment and interpreting those results remains. Widely used research techniques include micro–computed tomography and histology for characterizing ACL “microdamage” with micrometer-level spatial resolution [23]. However, these methods fail to inform on any submicrometer ACL damage that might exist.

We hypothesized that damage can accumulate in the ACL across the different hierarchical levels, ranging from the molecular to micrometer level, and that this damage could be responsible for ACL failures observed in vitro and in vivo [25]. To test this hypothesis, we investigated (1) molecular-level collagen unraveling using atomic force microscopy (AFM)–infrared spectroscopy (AFM-IR) and a collagen hybridizing peptide (CHP), (2) nanometer-level collagen fibril damage using AFM, and (3) micrometer-level collagen fiber damage using second harmonic imaging microscopy (SHIM) in mechanically tested cadaveric ACLs and the injured ACLs of patients.

Most orthopaedic studies focus on acute triggers of ACL injury, in accord with the view that noncontact ACL injuries are predominately caused by a single overload of a healthy ligament [12]. Fewer studies have explored the development of preinjury ACL damage caused by repetitive submaximal loading [25] that does not exceed the ligament's ultimate tensile strength [2]. In this study, we address 2 key questions: (1) What is the chemical and structural nature of any damage incurred in a cadaveric ACL during repeated simulated jump landings as compared with its untested contralateral knee? (2) Is the damage signature observed in the cadaveric specimens comparable with that observed in patient ACLs that failed in a noncontact mechanism?

We previously demonstrated in vitro that the ACL can experience fatigue failure in <100 repeated jump landings when internal femoral rotation is limited [18]. The major focus of that earlier work was the characterization of ACL microstructural change at the femoral enthesis in 1 of each pair of cadaveric knees subjected to a repetitive loading regimen. The work herein provides characterization of ACL damage from knees subjected to similar loading tests at multiple levels of the collagen hierarchical structure—namely, at the molecular, nanoscopic, and microscopic levels.

METHODS

The multiscalar ACL femoral enthesis damage present in paired adult cadaveric knees—one mechanically tested repetitively, the other untested—was evaluated at the molecular, fibril, fiber, and tissue levels. Molecular-level ACL damage was observed via AFM-IR as an amide I band shift from 1664 cm^{-1} to 1740 cm^{-1} , consistent with the unraveling or denaturation of the collagen triple helix [17]. Although the sample preparation methods employed did not generate a similar band shift in the control samples, an additional validation test was performed to ensure that the 1740-cm^{-1} band assigned to triple-helix denaturation was not the result of tissue dehydration. This spectroscopic assignment of the 1740-cm^{-1} band was confirmed with a carboxytetramethylrhodamine (TAMRA) dye conjugated to a fluorescent CHP probe that specifically binds to single-stranded collagen associated with denatured helices [26]. Fibril-level collagen nanoscopic damage was further characterized as a reduction in surface topological height ranging from 30 nm to 100 nm in diameter. Fiber- to tissue-level collagen microscopic damage was characterized as 10- to $100\text{-}\mu\text{m}$ regions of disordered collagen per SHIM. A summary of the hierarchical detection scheme is shown in Figure 1. Explants removed from the femoral enthesis of failed ACLs in 5 injured patients were also examined for multiscalar damage signatures to cross-check whether they were consistent with signatures from the tested cadaveric knees.

ACL Cadaveric Samples and Jump Landing Simulation

Seven pairs of cadaveric knees were acquired from adult donors (Table 1) at the University of Michigan Medical School and Gift of Life Michigan within 48 hours of death. Use of all human tissue was approved by the University of Michigan Institutional Review Board and given exempt status. Upon tissue harvesting, knees were stored at -20°C . One of each pair of knees was randomly selected for repetitive mechanical loading, while the contralateral knee was reserved as an internal untested control. The paired knees were set to thaw for 48 hours at room temperature before use. The knee chosen for mechanical testing was dissected down to the knee capsule, with care taken to leave intact the ligaments and tendons of the quadriceps (rectus femoris muscle), medial hamstrings (semitendinosus, semimembranosus, gracilis muscles), lateral hamstrings (biceps femoris muscle), and medial and lateral heads of the gastrocnemius muscle. After dissection, the paired knees were stored at 4°C until testing. Just before testing, the proximal femoral and distal tibial and fibular diaphyses were cut to a length of 20 cm from the joint line, and the bone extremities were potted in PMMA (polymethylmethacrylate). Once the PMMA cured, the knee was mechanically tested with a custom-built apparatus to simulate repeated single-legged pivot landings with a 4 times–body weight impulsive load that combines knee compression, knee flexion, internal tibial torque, and trans-knee muscle force loads as described previously [4]. Each tested knee was subjected to cyclic loading until ACL failure or until a minimum of 100 loading trials were completed.

After mechanical testing, the femoral portion of the ACL and its enthesis were removed from both the tested cadaveric knee and its contralateral control with the same tools and methods employed during ACL reconstructive surgery. With an “outside-in” approach, the femoral explant was reamed with a 10-mm trephine (Figure 2), placed in normal saline, and stored at 4°C until cryoembedding and sectioning. To determine whether the fatigue damage generated in vitro was comparable to that generated in vivo, ACL explants from 5 patients who had suffered a noncontact ACL injury within the past 3 months requiring primary ACL reconstructive surgery were analyzed (Table 1). In preparation for receiving a bone–patellar tendon–bone graft, the native femoral and tibial remnants of the ACL and its associated entheses were removed with the same surgical and storage protocols applied to the cadaveric tissue.

Preparation of ACL Explants

Before imaging, the cadaveric and patient ACL explants were longitudinally sectioned in half with a pathology diamond band saw (model 312; EXAKT Technologies, Inc). Each half was embedded in a water-soluble Super Cryo Embedding Medium (Section-Lab Co), and 20 μm –thick sections were cryosectioned (model 3050S; Leica Biosystems, Inc) with a tungsten carbide blade (TC-65; Leica Microsystems, Inc) set at an 8° angle at -25°C . The sectioned tissue was transferred to adhesive tape via the Kawamoto method [15,16] and stored in a moisturized chamber at -20°C until imaged. Immediately before imaging, sectioned sample slices were immersed in deionized water to remove the embedding media and to keep the sample hydrated. Sections were cut to a $\sim 2\text{-cm}$ square and glued to a stainless-steel AFM sample puck for AFM and AFM-IR imaging.

AFM and AFM-IR Studies

The AFM-IR data were collected with a NanoIR2 system (Anasys Instruments). AFM images were taken on a $5 \times 5\text{-}\mu\text{m}$ area at a 0.8-Hz line scan rate with 512×512 -pixel density under contact mode with nIR2 probes (parameters: gold-coated silicon cantilever; nominal radius, 25 nm; force constant, 0.07–0.4 N/m; resonance frequency, 13 ± 4 kHz). Four IR spectra were taken at a power of 2.07% and averaged for the final data. The detection range was set between 860 and 1900 cm^{-1} . Roughness and pore analysis were performed with Scanning Probe Image Processor software (v 6.7.5; Image Metrology A/S) to quantify the samples' topography thickness and fibril void density, respectively. Four AFM topography images per knee of T4, T5, T6, and T7 explants were analyzed after each raw image was processed per the following steps on the Scanning Probe Image Processor: global leveling → global bow removal → linewise bow removal → zero background → statistical difference filtering (Appendix Figure A1). Sample topography thickness was determined by the peak-peak (Sz) roughness parameter, which is defined as the height difference between the highest and lowest pixels in the image. Fibril void density was calculated by dividing the number of voids detected in 1 AFM height image by its image area ($25\text{ }\mu\text{m}^2$). A mean and SD of fibril void density were calculated for the T4 to T7 specimens after the processing of 4 images per knee. The voids were detected via pore analysis under automatic threshold settings and 3 postprocessing parameters (0.03- to $1.0\text{-}\mu\text{m}$ diameter range, 0.3–1.0 roundness, and 1.0–3.0 aspect ratio). The void diameter range was determined by transmission electron microscopy studies of cross-sectioned human ACL fibril diameters (Appendix Figure A2).

CHP-TAMRA Fluorescence Studies

After AFM and AFM-IR analyses, the samples were stained with a CHP-TAMRA reagent containing a previously published hybridization sequence (Pierce Custom Peptides) [26]. The TAMRA fluorophore was selected to avoid spectral overlap with tissue autofluorescence as well as the signal produced by SHIM when employing a laser with 910-nm incident wavelength.

Before staining, a $25\text{-}\mu\text{L}$ CHP stock solution ($150\text{ }\mu\text{m}$) was heated at 80°C for 10 minutes to disaggregate the peptide. The solution was quenched by immersion in water for 15 seconds at 4°C . The aliquot was diluted to a total volume of $500\text{ }\mu\text{L}$ and pipetted onto the ACL section. The sample was incubated in the dark at 4°C for 12 hours and then rinsed with $1\times$ phosphate-buffered saline before being immersed in Leica type F immersion oil (Leica Microsystems, Inc) for microscopic imaging.

Images were collected with a Leica SP8 confocal microscopy system (Leica Microsystems, Inc). The light source was a 910-nm IR laser (10% laser power [LP], 33% gain, 38% offset, pinhole wide open). The TAMRA fluorescence signal was detected at a 570- to 590-nm window on a photomultiplier detector. A $40\times$ lens was used for imaging $300 \times 300\text{-}\mu\text{m}$ image tiles stitched together in a 5 (perpendicular to enthesis) $\times$ 3 (along with enthesis) area automatically to cover a wide area.

SHIM Studies

Microscopic images were collected with a Leica SP8 confocal microscopy system and the type F immersion oil as described earlier. The light source was a 910-nm Coherent Chameleon 2-photon IR laser (10% LP, 33% gain, 38% offset, pinhole wide open). Two photomultiplier detectors were set at 440 nm for the detection of SHIM forward/backward signal. The signal from forward channels was colored cyan, while that from the backward channel was colored yellow to increase contrast. All Z planes (8.6 μm each) were scanned to cover the entire tissue thickness across the scanning area. Similar to the CHP study, a 40 $\times$ lens was used for SHIM imaging, and image tiles with a 300 $\times$ 300- μm area were stitched together creating a 5 (perpendicular to enthesis) $\times$ 3 (along with enthesis) tile area. The mean intensity of a 100 $\times$ 100- μm area of SHIM and TAMRA images was measured for both the mechanically tested and contralateral control knees. The TAMRA fluorescence and SHIM signal of paired untested cadaveric knees were also measured to determine the extent of normal signal variation between knees (Table 1). The intensity values of SHIM and TAMRA images for mechanically tested knees were reported as a percentage of its contralateral control, set as the baseline intensity, as a way to control for age and other differences in the patient knees. For these untested control knee pairs, the SHIM and TAMRA signal variation was within 20% (Appendix Table A1). Analysis of disordered or void regions lacking SHIM signal was done on a total of 5 $\times$ 3 tiles for each sample, covering a total area of 1500 $\times$ 900 μm (1,350,000 μm^2). The diameter and density of disordered regions were quantified with ImageJ and a particle analysis macro (National Institutes of Health). For each knee, 1 tiled SHIM image (as stated earlier) with the best resolution in the forward or backward channel was selected. Then, the grayscale threshold was adjusted to include as much of the disordered regions as possible with limited noise. Finally, particle analysis was performed with a detection diameter of 100 μm and an infinity and roundness setting of 0.5 to 1.0 to avoid detecting elongated shadows.

Statistics

Based on an a priori power analysis estimated to detect a difference in fibril void density between mechanically tested (ipsilateral) and control (contralateral) specimens at a power of 0.8, given an alpha of .05 and an effect size of 1.1, a sample size of 4 paired knees was required (we included 5 pairs in our study). According to an a priori power analysis estimated to detect at least a 2-fold difference in topography thickness between mechanically tested (ipsilateral) and control (contralateral) specimens at a power of 0.8, given an alpha of .05 and an effect size of 1.6, a sample size of 4 paired knees was required (we included 5 pairs in our study). Based on an a priori power analysis estimated to detect at least a 2-fold difference in SHIM intensity between mechanically tested (ipsilateral) and control (contralateral) specimens at a power of 0.8, given an alpha of .05 and an effect size of 0.8, a sample size of 7 paired knees was required (we included 7 pairs in our study). According to an a priori power analysis estimated to detect at least a 2-fold difference in CHP-TAMRA intensity between mechanically tested (ipsilateral) and control (contralateral) specimens at a power of 0.8, given an alpha of 0.05 and an effect size of 0.8, a sample size of 7 paired knees was required (we included 7 pairs in our study). Therefore, the number of samples included in each multiscale damage assessment was sufficient for testing the null hypothesis. Paired *t* tests were performed to test for significant differences in fibril void density, topography

thickness, SHIM intensity, and CHP-TAMRA intensity between paired tested and untested cadaveric knees. Statistical analyses were not performed for patient samples, owing to the lack of control samples.

RESULTS

Molecular-Level Ligament Damage Detected by AFM-IR and CHP-TAMRA

Healthy, normal-looking ACLs should exhibit an IR spectrum with the strongest signal at 1664 cm^{-1} , the amide I band from the protein backbone (Figure 3A). Indeed, the IR spectrum from the untested cadaveric ACL controls is almost identical to that reported by Spalazzi et al. [24]. However, a strong 1740-cm^{-1} feature emerges after mechanical testing (Figure 3B). The 1740-cm^{-1} band is a chemical signature of a disrupted collagen backbone structure [17] (Appendix Figure A3). Computational studies of stabilization of collagen-like peptides in water revealed that the stability is provided by interstitial water bridges anchoring amine-carbonyl (ζ bridges: $\text{N} - \text{H} \dots \text{W}_n \dots \text{O} = \text{C}$), which are further stabilized by polar $-\text{OH}$ side chain of Hyp through H-bonding with water [5,10]. Molecular dynamics simulation calculates that the average residence time of waters on external hydration sites (from Hyp and exposed carbonyl groups) of the triple helix is 50 ps, while the ζ bridge waters exhibited a residence time >100 ps [2]. Another molecular dynamics simulation exploring water gelation around collagen triple helix shows that radial distribution function of $\text{O} - \text{H}$ became distorted under $4\text{\AA}$, followed by deformation of a tetrahedral network of hydrogen bonds in the kinetically labile first hydration shell [11]. Based on these studies, the mechanical forces leading to weakened ζ bridges and destabilization of the triple helix will generate an effective local dehydration of the collagen molecules that is consistent with our localized observation of the 1740-cm^{-1} band, as illustrated in Appendix Figure A3. This effect generates change in the stoichiometric amount of water associated in a bonding fashion with collagen triple helix and has a significant effect on spectroscopic signature as well as structural and mechanical properties. We believe that it is most useful to view this as chemical change of the material. It is worth noting that the 1740-cm^{-1} band was also observed in the explants from the ruptured patient ACLs obtained before surgical reconstruction (Figure 3C). In the Discussion, we argue that this similarity in collagen backbone disruption suggests that the ACLs from the patients may also have been subjected to severe repetitive loading cycles.

To confirm the spectroscopic assignment of the 1740-cm^{-1} band, a fluorescent CHP conjugated to a TAMRA dye was deployed. The peptide sequence is $\text{GGG}(\text{GPO})_9$ with a fluorescent TAMRA dye conjugation on the N-terminus (G-glycine, P-proline, O-hydroxyproline). The TAMRA dye has an excitation wavelength at 551 nm and a maximal emission at 576 nm, which effectively avoided the interference from the possible collagen autofluorescence. The CHP sequence resembles the sequence of type I collagen, offering a specific strong binding toward dissembled collagen molecules, as demonstrated by Zitnay et al. [26]. After mechanical testing, the mean percentage intensity of the CHP-TAMRA probe was at least twice that of the untested contralateral controls ($P = .02$) (Figure 4). This indicates the disruption of the collagen triple-helical structure by unraveling of the collagen

molecule after the repeated mechanical loading. Taken with the AFM-IR data, this provides strong evidence of collagen fatigue damage at the molecular level.

To further probe the nature of the chemical and structural change associated with the collagen triple-helix disruption, we dehydrated fresh human ACL at 22°C by exposure to a stream of N₂ for 7 days. Dehydration under these conditions resulted in almost complete disruption of the collagen triple helices in the sample, as indicated by the almost complete replacement of the 1668-cm⁻¹ band by the 1740-cm⁻¹ band (Appendix Figure A3). This indicates that the 1740-cm⁻¹ band can arise from a number of chemical and structural changes, including breaking of the internal amino acid hydrogen bonding, inter- and intramolecular hydrogen bonding with water, and a change in the collagen triple helix:water stoichiometric ratio. This dehydration experiment suggests that water is an integral part of the collagen triple-helix structure and an important component of the “collagen molecule,” as it is present in ligamentous tissue.

Ligament Nanodamage Detected by AFM

Type I collagen molecules assemble into nanometer-level collagen fibrils and further stack into micrometer collagen fibers. At the nanometer scale, AFM is a commonly used tool for studying collagen order and morphology. After mechanical testing, the mean surface thickness decreased ($P < .01$) (Figure 5C) and a 49% higher mean fibril void density ($P = .06$) was observed when compared with untested contralateral controls (Figure 5F).

Similar fibril voids were also repeatedly observed in the patient specimens (Figure 6A) and the submaximally loaded cadaveric ACLs (Figure 6B) as compared with the cadaveric control specimens (Figure 6C). These fibril voids were distributed across a size range from ~30 nm to 100 nm, often with similar shapes and consistent with the range of ACL fibril width measured with cross-sectional transmission electron microscopy (Appendix Figure A2). All AFM void distribution analyses were performed on images that covered a 5×5 - μm^2 area. The mechanically tested samples overall had 6 ± 3 voids/ μm^2 as compared with 4 ± 2 voids/ μm^2 for the untested controls, for a mean increase of about a factor of 2. Specifically, individual evaluation of samples, except T7, showed a range of 40% to 75% of fibril void density increase as compared with that of their contralateral controls (Figure 5F). Since the fibril voids were observed in the mechanically tested cadaveric knees, where biological activity had ceased, those fibril voids could not have resulted from postinjury responses such as lipid infiltration or collagen regeneration. The increase in the presence of fibril voids correlated with a concomitant decrease in surface thickness, as measured by the peak-peak (S_z) roughness parameter from a mean 750 ± 150 nm for the control samples to 430 ± 150 nm for the mechanically tested samples (Appendix Table A2).

Ligament Microdamage Detected by SHIM

Since evidence of ACL femoral enthesis damage at the molecular level and nanometer level was observed, we then extended the study to see if damage also occurred at the micrometer or fiber level. Collagen molecules are well-aligned biopolymers that can be specifically detected by SHIM. SHIM exhibited micrometer-scale regions of reduction in signal (Figure 7D) for both the mechanically tested (Figure 7B) and patient (Figure 7C) explants,

indicating the absence of organized collagen fibers. In contrast, there was less ligament disruption in the untested cadaveric control (Figure 7A). A comprehensive analysis showed that the mechanically tested ACLs contained 10- to 100- μ m regions of reduced signal (Figure 7E). The distribution and size of these regions were extracted from interest areas covering the same size (see Methods). The quantification for the disrupted region density showed that, for every square millimeter sample, the mechanically tested ligament had 73.0 ± 42.8 disrupted regions, roughly 3 times the density of untested control at 22.2 ± 20.3 (Figure 7E inset). The mean percentage intensity values of mechanically tested samples significantly differed from that of contralateral controls ($P < .01$), as well as the mean number of SHIM signal areas ($P = .01$).

DISCUSSION

These results, acquired from different analytical tools (AFM, AFM-IR, fluorescence microscopy, SHIM), provide the first independent evidence of hierarchical multiscale damage at the ACL femoral enthesis induced by strenuous repetitive impulsive knee loading known to place the ACL under significant strain in vitro [25]. These damage signatures were compared with explants acquired from patients who had an ACL injury to determine whether their tissue would exhibit the same characteristics. Indeed, the same indicators of multiscalar material fatigue damage were seen at the femoral enthesis of torn ACLs obtained from these patients. This finding suggests that the failure of the femoral ACL enthesis in these patients may be due to an accumulation of material fatigue damage of collagen fibrils and fibers resembling an injury from prior ACL multiscalar damage that has not had sufficient time to repair. It was induced by too many severe ACL loading cycles in a time frame inadequate for any physiological repair mechanisms to restore ligament homeostasis before the next severe loading cycle. The potential for ACL repair at the molecular, nanoscale, or microscale, if it exists in children and/or adults, remains unknown. However, others have shown that in the tendon that rests after the initiation of fatigue damage, there is a capacity for the extracellular matrix within the midsubstance to remodel once activity is reinitiated [1].

The present experimental study found evidence of structural and chemical degeneration at the femoral enthesis of the ACL, the same location previously shown to be particularly prone to failure under repetitive loading in vitro as well as in vivo [18]. Moreover, the finding of failure at this enthesal location is consistent with purely theoretical analyses demonstrating a strain concentration in the ACL ligament at its femoral enthesis [19]. This strain concentration increased with the acuteness of the ligament attachment angle with the femur as well as with the concavity of the femoral enthesis [3]. So, the aforementioned theoretical study shows that the ACL femoral enthesis contains a region that has a strain concentration, and this study shows that hierarchical damage can accumulate in that region under a particular ACL loading regimen. Further studies are needed to determine whether this is where ACL fatigue failure indeed initiates.

Another important observation is that our results support the hypothesis that ACL multiscalar damage can result from submaximal mechanical loading and that damage can accumulate under realistic repetitive loading to eventually become a risk factor for ACL

injury. “Realistic” here means a knee loading that has previously been shown to have the same magnitude and time course as a real jump landing. Nanoscopic detection of collagen unraveling observed by the 1740-cm^{-1} peak and CHP-TAMRA probe is consistent with the observation of a reduced SHIM signal, which identifies microscopic domains where this is occurring at a greater concentration. Similarly, the reduction in surface topological height (Sz) is also consistent with the unraveling of collagen fibrils—a value that appears largely controlled by the dimensional scale of the collagen fibril widths. Questions that remain to be answered by future studies include the following: (1) How does molecular-level collagen triple-helix unraveling relate to the observed fibril voids and decrease in surface thickness? (2) What contributes to the formation of microscopic regions of fibril disruption? (3) How are all these features associated with clinical diagnosis on MRI and, ultimately, ACL failure? The combination of AFM-IR, CHP-TAMRA, and SHIM data indicates that the collagen triple helix has unraveled into individual strands. Thus, glycine-proline intramolecular hydrogen bonding has been broken in addition to the water hydrogen bonding that is integral to the collagen structure. Our control experiments indicate that these changes are also consistent with local changes in triple-helix/water stoichiometry. Thus, we ascribe the observed changes to both their structural and their chemical nature. We believe that this wider perspective of the nature of multiscalar damage is important for the design of future studies to understand the physiological repair mechanism of this damage as well as the changes in biomechanics that lead to ACL failure.

The presence of fibril voids in the tested knees and patients as well as, to a lesser degree, in the control knees is interesting. We hypothesize that each void represents an individual collagen fibril in the ligament rupturing and pulling out during loading, leaving a fibril-sized void in the ligament. The presence of these voids in the untested control knee ACLs suggests that preexisting damage was present in the ACL that accumulated in these donors during their lifetimes. Additionally, the measured SHIM intensities were reduced in the mechanically tested knees as compared with the corresponding untested paired controls. This indicates that the noncentrosymmetric collagen crystallinity was partially broken during mechanical testing, suggesting another level of ligament damage as a result of the strenuous repetitive impulsive knee loading. These signatures of nano- and microscopic damage can be easily missed in the clinical MRI used for diagnostic purposes. The sum of these hierarchical structural changes corresponds to the multiscalar damage that was hypothesized in, but not directly demonstrated in, the original cadaveric studies of the effects of ACL repetitive loading [18].

For the purposes of this study, we clarify that “submaximal repetitive loading of the ACL” means knee loading that did not generate enough force in the ACL to fail it during the first knee loading cycle (ie, as evidenced by our data showing 3-mm anterior tibial translation during that loading cycle). Many strenuous sports activities may place large loads on the knee in stopping, turning, cutting, and landing, but only a subset of those will place larger-than-normal loads on the ACL: specifically, those that include substantial internal tibial torques while landing a jump or making a cut [18]. Hence, strenuous athletic activity in itself does not necessarily place unusual loads on the ACL and therefore would not necessarily cause a concerning increase in multiscalar fatigue damage at the femoral enthesis.

Strengths of the study were the randomization of one of each pair of cadaveric knees to mechanical testing, as well as the inclusion of data from the untested contralateral cadaveric knee. Limitations of this study include the small sample size, the nonblinded operators conducting the structural and chemical studies, and the sole assessment of fatigue damage at the femoral enthesis at the location where proximal failures are most often observed clinically [25]. In the patients included in the present study, 1 to 3 months was the usual time course to ACL reconstruction to allow for knee effusions to dissipate and knee muscle strength to be restored. It is possible that some of the collagen alterations in the patient explants may reflect partially repaired hierarchical damage. However, the same signs of multiscalar damage seen in the mechanically tested cadaveric ACLs were also observed in the patients, supporting our material fatigue hypothesis for ACL failure.

Larger randomized and blinded studies are needed to confirm these results. However, it does appear that the solution to preventing many noncontact ACL injuries may require consideration of the potential for multiscalar collagen fatigue-related damage to accumulate at the ACL femoral enthesis as identified in this article.

CONCLUSIONS

Our results suggest that the multiscalar, hierarchical structural changes observed at the femoral ACL enthesis are at least in part responsible for the reduction in structural integrity leading to noncontact ACL failure as a result of strenuous repetitive impulsive athletic maneuvers. These findings suggest that at least some ACL injuries may be attributable to an overuse injury caused by damage that accumulates in the absence of the time needed for repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Hierarchical detection of chemical and physical anterior cruciate ligament damage. (A) Mechanical force induces collagen triple-helix unraveling concomitant with the disruption of intra- and intermolecular amino acid chain and water hydrogen bonding. CHP-TAMRA binds to the unraveled collagen strands and is detected by fluorescence microscopy. AFM-infrared spectroscopy is also used to detect collagen triple-helix unraveling via the distinctive infrared band at 1740 cm^{-1} . (B) AFM is utilized to detect nanoscale topographical defects at the ~30- to 100-nm scale, which is similar to the size of collagen fibrils. Figure for AFM-IR schematic reprinted with permission from received from Bruker Corp. (C) Second harmonic imaging microscopy imaging is employed to detect regions of disordered collagen at the 10- to 100- μm scale, which is similar in size to collagen fibers. AFM, atomic force microscopy; CHP, collagen hybridizing peptide; IR, infrared; SHIM, second harmonic imaging microscopy; TAMRA, carboxytetramethylrhodamine.

Figure 2.

View of the anterior cruciate ligament (ACL) explant and equipment used in the outside-in extraction procedure for both patients and cadavers.

Figure 3.

Atomic force microscopy–infrared spectroscopy spectra of tissue at the anterior cruciate ligament (ACL) femoral enthesis from (A) nonmechanically tested cadaveric ACL control, (B) mechanically tested cadaveric ACL, and (C) clinically injured patient ACL. The 1740- cm^{-1} (light dashed line) disordered collagen band was prominently observed for the mechanically tested and patient sample, while only the normal 1664- cm^{-1} (dark dashed line) collagen backbone band was most prominent for the untested cadaveric ACL control.

Figure 4.

Representative fluorescent images of CHP-TAMRA binding at the femoral enthesis of the ACL in (A) an untested control knee and (B) a mechanically tested knee. Red dashed boxes represent the 5×3 tile regions of interest analyzed for pairs of knees. (C) The ACL CHP-TAMRA fluorescent intensity was compared between the tested and untested knee regions of interest and normalized as an intensity ratio. Values are presented as mean $\pm$ SD. Black dashed line represents the baseline intensity for the untested control knees. ACL, anterior cruciate ligament; AFM, atomic force microscopy; CHP, collagen hybridizing peptide; TAMRA, carboxytetramethylrhodamine.

Figure 5.

Representative 3-dimensional views of postprocessed atomic force microscopy topography images of (A) untested control knee and (B) tested knee and void density plots of (D) untested control knee and (E) tested knee of T4 donor (colors are only for visualization of the voids). (C) A comparison of the mean $\pm$ SD topography thickness for tested and untested knee images for T2 and T4 to T7. (F) A comparison of the mean $\pm$ SD void density for the same sample set as in panel C, highlighting that tested knees were denser in fibril voids as compared with nontested knees.

Figure 6.

Representative atomic force microscopy images (height) of fibril voids in the anterior cruciate ligament (ACL) associated with mechanically induced nanoscale damage. The holes with darker contrast (red arrows) are locations without the presence of collagen molecules. (A) Explant from patient ACL femoral enthesis at the time of surgical reconstruction. (B) Mechanically tested cadaveric ACL femoral enthesis. (C) Untested cadaveric contralateral (control) ACL femoral enthesis.

Figure 7.

SHIM images from the ACL femoral enthesis: (A) untested cadaveric ACL control femoral enthesis, (B) mechanically tested cadaveric ACL femoral enthesis, and (C) explant from patient ACL femoral enthesis at the time of surgical reconstruction. Red arrows indicated regions of reduced second harmonic signal induced by mechanical damage. (D) The SHIM signal intensity was also reduced after the mechanical testing as compared with the untested contralateral control ACL enthesis. Black dashed line represents the baseline intensity for the untested control knees. (E) Distribution of reduced SHIM signal areas in the femoral ACL enthesis of the mechanically tested knees (T) and the corresponding controls (U). Values are presented as mean $\pm$ SD. ACL, anterior cruciate ligament; B, bone; L, ligament; SHIM, second harmonic imaging microscopy.

Table 1.Demographic donor information.^a

Specimen	Sex	Age, y	Cause of Death
Tested cadaveric donors			
T1	F	57	COPD
T2	M	43	Strangulation
T3	M	64	Liver cancer
T4	F	36	Drug overdose
T5	M	36	Drug overdose
T6	M	34	Cardiac arrest
T7	F	31	Drug overdose
Untested cadaveric donors			
C1	F	21	Alcohol poisoning
C2	M	40	Unknown
C3	F	21	Head trauma
C4	F	26	Drug overdose
C5	M	28	Cardiac arrest
Injured patient donors			
P1	F	44	12 wk
P2	F	40	12 wk
P3	M	45	5 wk
P4	F	16	12 wk
P5	F	44	20 wk

^a Cadaveric donor knees had no external signs of surgery or trauma upon inspection and palpation. Donors were kept refrigerated upon death. Injured patient tissue was placed in saline and refrigerated immediately. COPD, chronic obstructive pulmonary disease; F, female; M, male.