Fourier transform infrared imaging spectroscopy analysis of collagenase-induced cartilage degradation

Abstract. Collagenase treatment of cartilage serves as an in vitro model of the pathological collagen degradation that occurs in the disease osteoarthritis (OA). Fourier transform infrared spectroscopic (FT-IRIS) analysis of collagenase-treated cartilage is performed to elucidate the molecular origin of the spectral changes previously found at the articular surface of human OA cartilage. Bovine cartilage explants are treated with 0.1% collagenase for 0, 15, or 30 min. In situ collagen cleavage is assessed using immunofluorescent staining with an antibody specific for broken type II collagen. The FT-IRIS analysis of the control and treated specimens mirrors the differences previously found between normal and OA cartilage using an infrared fiber optic probe (IFOP). With collagenase treatment, the amide II/1338 cm$^{-1}$ area ratio increases while the 1238 cm$^{-1}$/1227 cm$^{-1}$ peak ratio decreases. In addition, polarized FT-IRIS demonstrates a more random orientation of the collagen fibrils that correlate spatially with the immunofluorescent-determined regions of broken type II collagen. We can therefore conclude that the spectral changes observed in the collagenase-treated cartilage, and similarly in OA cartilage, arise from changes in collagen structure. These findings support the use of mid-infrared spectral analysis, in particular the minimally invasive IFOP, as potential techniques for the diagnosis and management of degenerative joint diseases such as osteoarthritis.

Keywords: infrared imaging; collagen; collagenase; osteoarthritis; infrared fiber optic probe.

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lecular changes were related primarily to the breakdown of the collagen network. In the current study, we further investi-
gate the origin of these spectral changes using FTIR imaging spectroscopic (FT-IRIS) analysis of collagenase-treated arti-
cular cartilage combined with immunohistochemical analysis. The biochemical action of collagenase is to cleave the colla-
gen molecule within the triple helical domain at a single site 3/4 distance from the N-terminal end, resulting in 3/4-
and 1/4-length fragments that can be detected by an antibody specific for these fragments.18 Comparison of the spectral im-
aging data from these in vitro collagenase-treated samples to the spectral data previously acquired by IFOP from human OA cartilage provides support for the potential use of the IFOP to monitor collagen breakdown in an in vivo environment.

2 Materials and Methods

2.1 Tissues

Twelve full-depth cylindrical cartilage plugs (disks of 7 mm diam, 1.5 to 2 mm thick), without subchondral bone, were harvested from the femoral condyles of an adult bovine knee joint immediately after death (obtained from a local abattoir). Collagen degradation was induced by incubating six cartilage explants with 0.1% collagenase (Worthington Biochemical Company, Freehold, New Jersey) in serum-free Dulbecco’s Modified Eagle Medium (DMEM) at 37 °C in a confined chamber, such that degradation proceeded from the articular surface down. Three plugs were treated for 15 min and three for 30 min. These relatively long time points were chosen to mimic the severe degradation seen in late-stage OA (30-min time period) and a less severe degradation (15-min time pe-
period). The remaining six plugs were used as untreated controls and incubated in DMEM for either 15 or 30 min. At the end of the collagenase treatment, the explants were removed from the incubator at 37 °C, rinsed in saline, embedded in OCT media (Miles Incorporated, Elkhart, Indiana), flash frozen in liquid nitrogen, and stored at −70 °C until further processed.

2.2 Spectroscopic and Immunohistochemical Tissue Processing

Six 6-μm-thick full-depth slices were cryosectioned from each explant. Three sections were placed on BaF₂ windows for FTIR imaging spectroscopic (FT-IRIS) analysis, while the remaining three were mounted on glass slides for immunohis-
tochemical staining. In situ collagen cleavage was assessed using immunofluorescent staining for collagenase-generated neoepitopes using the Col2-3/4Cshow antibody (kindly supplied by Dr. Robin Poole, Shriners Hospital, Montreal, Quebec). Nonspecific binding in cartilage sections was blocked with 10% goat serum and 1% bovine serum albumin at room temperature. Sections were treated with 1-μg/ml testicular hyaluronidase at room temperature for 30 min to enhance ant-
gen retrieval before being incubated with primary antibodies at 37 °C for 30 min. Sections were then incubated with sec-
ondary (goat-anti-rabbit) antibody conjugated with fluorescein (Sigma-Aldrich, St. Louis, MO), and counterstained with propidium iodide. Sections were viewed using a fluorescent micro-
scope (Nikon Corporation, Japan).

2.3 FT-IRIS Data Collection and Analysis

A BioRad UMA 300A FTIR microscope (Cambridge, Massa-
uchetts) with an FTS-60A step-scanning FTIR spectrometer and a 64×64 MCT focal plane array detector (Stingray Imaging Spectrometer Santa Barbara Focal Plane, Goleta, CA) was used to acquire spectra (512 scans) for each sample at 16 cm⁻¹ wavenumber resolution under N₂ purge. Data were collected from a 400×400-μm² region mapped to the individual elements of the array detector, resulting in 4096 individual spectra. Imaging data were acquired from the articular surface down through the cartilage midzone, and included all the pix-
els of the 64×64 focal plane array. FT-IR data analysis was performed using WinIR Pro software version 2.5 (BioRad). Spectra (250 pixels per image) used for IR parameter analysis were obtained from within the superficial zone, where the densely packed collagen fibrils are aligned parallel to the arti-
cular surface in normal cartilage.16 The signal-to-noise ratios (SNRs) were of the order 30/1 for all the imaging pixels in the 64/64 array for all the images. All spectra were baselined and the type II collagen absorbances were monitored in the 1690–
1600 cm⁻¹, 1590–1480 cm⁻¹, 1338 cm⁻¹, and 1300–1200 cm⁻¹ infrared region. The molecular vibrations associated with these absorbances are the amide I carbonyl stretch (C=O),19–24 the amide II combination out-of-phase C-N stretch and N-H bend,19–24 CH₂ side chain vibrations,24,25 and the amide III C-H, N-H vibrations,26–28 respectively. The follow-

ing parameters, amide II/1338 cm⁻¹ area ratio and 1238/
1227 cm⁻¹ peak ratio, have previously been shown to be di-
rectly and inversely, respectively, correlated with cartilage
degradation,17 and were calculated for each image. Polariza-
tion data were acquired by placing a wire grid polarizer be-
tween the sample and the incident IR radiation, such that the polarized radiation was directed perpendicular to the articular surface. It was assumed that the amide I peak that arises primarily from the C=O (carbonyl) stretching vibration of the type II collagen possesses a transition moment prefer-
entially oriented perpendicular to the long axis of the collagen fibril, and that the transition moment of the amide I transition moment of the amide II vibration is approximately 90 deg to that of the amide I transition moment.21,28 Therefore, the ratio of the areas of amide I/amide II absorbances in one polarization direction (perpendicular to the articular surface) was calculated and imaged as an indica-
Fig. 2 Antibodies show positive stain (green) for collagenase activity in 15- and 30-min treated specimens.

Fig. 3 (a) IR imaging spectra of untreated, 15- and 30-min collagenase-treated specimens. (b) Amide II/peak 1338 area ratio change with collagenase treatment. (* $p<0.05$ versus control as determined by one way ANOVA.) (c) 1238 cm$^{-1}$/1227 cm$^{-1}$ peak ratio change with collagenase treatment. (* $p<0.05$ versus control as determined by one way ANOVA.)

Fig. 4 Polarized FT-IR images of the amide I/II area ratio in the superficial zone of untreated and collagenase-treated cartilage.
tor of collagen orientation. For this case, a larger amide I:amide II ratio represents collagen fibrils oriented parallel to the cartilage articular surface.

A one-way analysis of variance (ANOVA) statistical test was used for comparison between the three groups (untreated, 15-min treatment, and 30-min treatment) with statistical significance determined at $p<0.05$.

3 Results

Changes in the surface morphology of the superficial zone of the collagenase-treated cartilage explants were seen after both 15 and 30 min of treatment [Figs. 1(b) and 1(c)]. Nontreated (control) explants had intact superficial zones [Fig. 1(a)]. Immunohistochemical evaluation using the Col2-3/4C_short antibody resulted in positive staining for the collagenase-treated sections only (Fig. 2). Positive staining was observed through most of the superficial zone for the 15-min treated specimens (depth of penetration 250 $\mu$m $\pm$ 20 $\mu$m), and through the superficial and into the transition zone for the 30-min specimens (depth of penetration 350 $\mu$m $\pm$ 30 $\mu$m) (Fig. 2).

A comparison of the spectral parameters (amide II/1338 cm$^{-1}$ area ratio and amide III-1238 cm$^{-1}$/1227 cm$^{-1}$ peak ratio) [Fig. 3(a)] between the treated and untreated specimens mirrored the differences previously found between normal and osteoarthritic cartilage. With collagenase treatment, the amide II/1338 cm$^{-1}$ area ratio increased for both the 15-min and 30-min treatment times ($p<0.05$) [Fig. 3(b)], while the 1238 cm$^{-1}$/1227 cm$^{-1}$ peak ratio decreased for both the 15-min and 30-min treatment times ($p<0.05$) [Fig. 3(c)]. The polarization images and spectra obtained in the superficial zone of the nontreated samples (control) showed that the collagen fibrils were highly oriented parallel to the articular surface (Fig. 4). With collagenase treatment, the amide I/amide II area ratio decreased [Figs. 5(a) and 5(b)] in this region, indicating reduced orientation in the direction parallel to the articular surface ($p<0.010$). In addition, FT-IRIS analysis below the area of collagenase activity (as measured immunohistochemically) revealed no statistical difference in the collagen orientation between the treated and untreated specimens [Fig. 5(c)]. Also note that for the parameters analyzed, the larger standard deviations for the untreated groups reflect the greater zonal variation within the untreated cartilage.

4 Discussion

A key factor in the development and progression of osteoarthritis is the enzyme-specific destruction of the articular cartilage extracellular macromolecules type II collagen and aggregan (PG). The matrix metalloproteinase collagenases (MMPs 1, 8, and 13) have been well documented as the primary source for the in vivo degradation of collagen.$^{3,33}$ In the current study, the effect of collagenase on type II collagen at the articular surface of mature bovine cartilage explants was successfully monitored by infrared spectroscopic analysis, and in situ collagen cleavage was confirmed by the use of immunofluorescent staining with the Col2-3/4C_short antibody. These results suggest that collagenase activity on type II collagen does result in IR spectral changes; specifically, an increase in the amide II/1338 cm$^{-1}$ area ratio ($p=0.024$), a decrease in the 1238 cm$^{-1}$/1227 cm$^{-1}$ peak height ratio ($p=0.025$), and a decreased in the polarized amide I/II area ratio ($p=0.010$). These findings corroborate our previous results obtained with the IFOP, where parallel differences were observed between normal and osteoarthritic (severely degraded) human cartilage.$^{17}$ It is important to stress at this point that the focus of this study was to relate IFOP spectral changes seen in late-stage OA to the effect of collagenase on the collagen molecule, which is why relatively strong collagenase treatment conditions were utilized. Our next step (currently underway) is to determine the threshold for IFOP detection based on lower concentrations of collagenase at shorter treatment times.

As put forward in the earlier study,$^{17}$ the decrease in the 1338 cm$^{-1}$ collagen absorbance, which arises from CH$_2$ side chain vibrations and is sensitive to the order of the triple helix, would account for the increased amide II/1338 cm$^{-1}$

![Polarized IR spectra from the superficial zone of an untreated and a 30-minute treated specimen.](Fig. 5) (a) Polarized IR spectra from the superficial zone of an untreated and a 30-minute treated specimen. (b) Amide III/area ratio change within the superficial zone with collagenase treatment. (* $p<0.01$ versus control as determined by one way ANOVA.) (c) Amide III/area ratio change below the treatment site.
area ratio. This absorbance band has also been shown to decrease in intensity as the collagen denatures in other studies.24,25 Similarly, the change in the amide III absorbance contour with collagen degradation, quantitated by a decrease in the 1238/1227 cm⁻¹ peak intensity ratio, parallels the spectral differences found between normal and OA cartilage. In this case, however, assignment of specific molecular changes to exact frequencies has proved challenging due to the highly complex nature of the amide III vibrational mode.26–28 Nonetheless, the current study supports those observed spectral changes being associated with the unraveling of the collagen triple helix structure due to enzymatic activity.

To accurately correlate our FT-IRIS findings with collagenase-treated areas, the specimens prepared in this experiment were stained for collagenase-generated neoepitopes using the Col2-3/4C_short antibody. All of the treated sections showed positive staining for collagenase activity through most of the superficial zone. FT-IRIS images generated from the polarized amide I/amide II area ratios enabled visualization of the disrupted orientation that occurs with collagen cleavage. For treated specimens, analysis of this data showed an inverse relationship between collagen organization (orientation) and collagenase treatment times. Control specimens (negative staining for collagenase activity) had the highest superficial zone amide I/amide II area ratios [Fig. 5(b)], which corresponds to the collagen fibrils having an orientation parallel to the articular surface.21 As an internal control, spectral analysis below the area of collagenase activity revealed no change in the amide I/amide II area ratio between the treated and untreated specimens, further evidence of the sensitivity of this parameter to collagen structure. These findings clearly present us with a new method for detecting collagen orientation changes in histological sections.

The use of IR spectroscopy to detect surface collagen architecture and molecular changes holds promise as an early diagnostic technique, since such changes may precede macroscopic surface alterations such as ruptures and fibrillations that occur with the progression of OA. Also encouraging is the fact that these molecular changes have previously been reported with the use of an infrared fiber optic probe.17 Currently, several IR fiber optic techniques are being investigated for clinical use.32,33 These systems have the potential to be minimally or noninvasive, nondestructive, and may ultimately allow for early detection of molecular changes in disease states. With respect to cartilage, such a system would ideally be used during arthroscopic procedures for in situ determination of articular cartilage surface integrity, and could also be used to assess the quality of tissue-engineered cartilage both before and after implantation. The current study validates the origin of the molecular changes in degenerative cartilage previously detected by IFOP, and supports its potential as a novel tool for monitoring and treating OA and other joint diseases.

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References


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