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Proteoglycan Concentrations in Healthy and Diseased Articular Cartilage by Fourier Transform Infrared Imaging and Principal Component Regression

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Abstract

Fourier transform infrared imaging (FTIRI) combining with principal component regression (PCR) analysis were used to determine the reduction of proteoglycan (PG) in articular cartilage after the transection of the anterior cruciate ligament (ACL). A number of canine knee cartilage sections were harvested from the meniscus-covered and meniscus-uncovered medial tibial locations from the control joints, the ACL joints at three time points after the surgery, and their contralateral joints. The PG loss in the ACL cartilage was related positively to the durations after the surgery. The PG loss in the contralateral knees was less than that of the ACL knees. The PG loss in the meniscus-covered cartilage was less than that of the meniscus-uncovered tissue in both ACL and contralateral knees. The quantitative mapping of PG loss could monitor the disease progression and repair processes in arthritis.

Keywords

cartilage; osteoarthritis; proteoglycan; ACL-transection; FTIRI; PCR

Introduction

Articular cartilage (AC), which covers the articulating surfaces of bones in synovial joints to maintain normal joint motion and to withstand external loading, has two principal macromolecular components (type II collagen and proteoglycan (PG)) and is saturated with water and mobile ions [1–3]. The collagen fibers are woven into a 3-dimensional fibrous network, which enmeshes PG molecules in its network. Since the collagen network consists

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of a depth-dependent orientation, uncalcified cartilage can be subdivided into three structural zones from the surface to the subchondral bone, namely, a superficial zone (SZ), a transitional zone (TZ), and a radial zone (RZ) [1–5]. The zonal structure in the fibril orientation, shape and arrangement of chondrocytes [2] distinguishes AC from the more homogenous nasal cartilage and provides AC with the functional properties of depth-dependent tensile strength and resiliency [4, 6, 7]. Several characteristic changes such as the reduction in the PG content, alteration in the collagen fibril structure, and degradation of matrix macromolecules often signal the onset of cartilage degeneration as a load-bearing tissue [8], which will eventually lead to degenerative joint diseases such as osteoarthritis or other arthritis [1].

Symptomatic chondral lesions due to injury to cartilage have been reported in 23% of knees with an acute anterior cruciate ligament (ACL) injury and in 54% of patients suffering from chronic ACL insufficiency [9, 10]. It is suggested that symptomatic chondral lesions represent a significant clinical challenge for the practicing orthopedic surgeon and patients since they are slow in development. Therefore, the ability to determine the PG concentration in AC non-invasively is highly desirable for the monitoring of disease development and therapeutic progress.

ACL injury is known to result in abnormal joint kinematics and damage to other tissues around the joint that may lead to morbidity, pain, and osteoarthritis. In this study, the ACL in one knee of an animal was transected to condition the knee cartilage over different durations. Fourier transform infrared imaging (FTIRI) [11, 12] combining the principal component regression (PCR) [13–15] was used to quantify the PG concentrations depth-dependently at all zones for the cartilages from the control knees, ACL-transected knees and contralateral knees. The combination [11–20] can fully utilize all IR spectral data, identify the number of components in a mixed chemical environment and predict the component concentrations in unknown samples [21, 22]. The motivation for this study was to obtain a better understanding of osteoarthritic injury by monitoring the PG loss in different developmental conditions.

Experimental

Cartilage sample

Thirty intact knees were harvested from fifteen 1–2 year old dogs after they were sacrificed for a complex biomedical study, which was approved by the institutional review committees. The animals included 4 healthy dogs, 6 dogs scarified 8-week after the ACL transection in one knee joint, 3 dogs scarified 12-week after the ACL transection in one knee and 2 dogs scarified 2-year after the ACL transection in one knee. The tibial cartilages in both ACL-transected knees and the contralateral knees were investigated for this project. AC that was still attached to the underlining bones was cut from the joints by using a table saw and a diamond blade. The final size of the cartilage-bone specimen was approximately 2 mm × 2 mm × 2 mm. After the tissue blocks were washed in saline for one minute, they were frozen in saline by liquid nitrogen. A cryostat (Leica CM 1950, Germany) was used to cut a number of 10-μm thick sections from each frozen block. These sections were rapidly picked

up by the MirrIR slides and left to air dry for at least 2 hours before the FTIR experiments were conducted.

FTIR spectroscopy and imaging

FTIR experiments were performed using a PerkinElmer Spotlight-300 FTIR imaging system (Wellesley, MA), which consisted of an FTIR spectrometer, an infrared microscope, and a liquid N₂ cooled 16-element MCT (mercuric cadmium telluride) detector. In the imaging mode, the infrared light focused on the specimen that was mounted on a movable mechanical stage. The data were collected by the MCT detector at 6.25- μm pixel-size and 16 cm^{-1} spectral resolution over a range of 4000-960 cm^{-1} . An internal coaxial LED illumination with variable intensity was adopted to produce high quality visible images enabling tissue to be quickly located for IR sampling.

During the image analysis, the region-of-interest (ROI), uncalcified cartilage, was divided into some rectangular regions with a height of ROI and a width of 20~50 μm . Each target spectrum was extracted from the corresponding rectangular region by using the function of “co-add spectra” in the PerkinElmer Spotlight software. These target spectra were introduced into a chemometrics software, Spectrum 10 software from PerkinElmer with the built-in PCR algorithm to calculate the concentrations of PG in the specimens. Our previous work [14, 15] described in detail the building of the PCR algorithm by PerkinElmer software, Spectrum Quart+. The standard and target spectra in the range from 960 to 4000 cm^{-1} were imported into the PCR calculations, since the chondroitin 6-sulfate (CS6) as major GAG in PG was much more preponderant than the CS4 [23–25] and both CS4 and CS6 showed the identical IR spectra within the range of 4000-960 cm^{-1} [26, 27].

Statistical Analysis

Statistical analysis was performed by using the software of Origin 7.0 (OriginLab Coop., MA). PG data of the control group were compared with those of the cartilage groups of 8-week, 12-week and 2-year post ACL, as well as the contralateral cartilage group using One-Way ANOVA test. If the *p*-value is below the 0.05, the conclusion was that there was a significant difference between the two comparing groups.

Results

FTIR Images and PCR Calculation

Fig 1 shows an infrared spectrum that is extracted from the FTIR image of articular cartilage, together with the characteristic peak assignments of type II collagen and PG. The amide III (& sulfate) band is contributed jointly by collagen and PG and other amide bands are mainly originated from collagen. The sugar band (1125~960 cm^{-1}) is assigned to PG. Figure 2A–2E show the representative groups of images (each group has the visible image, the IR total absorption image, and the sugar band image from top to bottom) from the control cartilage (Fig 2A), the contralateral cartilage (Fig2B and 2C), and the 2-year ACL cartilage (Fig2D and 2E). (The same data from the 8-week and 12-week after the ACL surgery are not shown but analyzed and summarized in the later figures.) To distinguish the topographical variations of cartilage properties over any joint surface, the specimens were

separated into two regions: meniscus-uncovered (uncovered) and meniscus-covered (covered), which were consistent with a previous investigation of the ACL lesion in the tibial cartilage [28]. The intensity of the sugar images represents qualitatively the PG distributions in the uncalcified tissue (SZ, TZ and RZ). By comparing the images in Fig 2A~2E, the blue area in the images (i.e., a lower intensity) increases clearly in the cartilage sections in Fig. 2B, 2C, 2D and 2E, which implies that the PG content in the surface portion of the cartilage was reduced.

The accurate determination of the PG loss in lesioned cartilage, however, requires the quantitative calculation. The PCR program imported all target IR spectra extracted from the ROI to calculate the PG concentrations across the uncalcified tissue. Fig 2F shows the depth-dependent profiles of PG concentrations in the control section (Fig 2A) and ACL section (Fig 2D and 2E); while Fig 2G shows the similar profiles of PG concentrations from the contralateral uncovered (Fig 2B) and covered sections (Fig 2C), respectively. It was assumed that SZ is intact in the tissue depth, though it was actually abraded.

It is clear that healthy cartilage has the lowest content of PG in the SZ [1, 2, 4, 5, 7, 29], which increases to a maximum near the middle depth [1–4, 7, 29, 30]. The mean concentration (mean $\pm$ SD) for the PG in the control samples was 39.86 $\pm$ 6.43%, which agrees with the published results in the dry weight [5, 31–34]. The covered and uncovered cartilage of the ACL knee (Fig 2F) and contralateral knee (Fig 2G) all showed lower concentrations. The most severe reduction of PG occurred at both SZ and TZ for the ACL cartilage from the uncovered locations (Fig 2F).

Changes of the Total PG Concentration

Figure 3 summarized the averaged PG concentrations in the control cartilage, the ACL cartilages at 8 weeks, 12 weeks and 2 years post surgery, respectively, as well as their contralateral cartilage. The statistical analyses were also carried out by comparing the control group with the three ACL groups and the corresponding contralateral groups, using the one-way ANOVA test. Several conclusions can be made. First, the averaged PG concentrations for the control specimens were consistent between the covered and uncovered cartilages. Second, the control specimens had the highest PG concentrations, higher than the contralateral cartilage and the ACL cartilage (regardless of the duration post surgery). Third, the ACL cartilage had an increasingly lowered PG concentration, more reduction with a longer post-surgery duration. In comparison, although the contralateral cartilage also had a lower PG concentration, its PG reduction was not significant until reaching 12-week after the surgery. Finally, the contralateral PG concentration in the covered region 2 years after surgery was higher than that at 12 weeks, which might indicate some type of internal repair process in the contralateral joints between the initial stage and advanced stage of OA [35, 36].

A set of different analysis was also made, that used the same data as in Fig 3 but compared the two topographical regions – the covered cartilage vs. the uncovered cartilage. For both the 12-week and 2-year cartilage, the PG concentration in the uncovered region was consistently lower than the corresponding covered region, regardless of whether the tissue was from the ACL knee or the contralateral knee. For both the control and 8-week cartilage,

the differences in the PG concentrations between the covered and uncovered regions were insignificant.

PG Concentrations at Different Zones

Figure 4 summarizes the zonal averaged PG concentrations in the covered and uncovered regions from the medial tibial cartilages. Both contralateral and ACL cartilages were separately averaged and statistically compared among the control, 8-weeks and 12-weeks post ACL surgery. (We did not include the results of the 2-year ACL cartilage in this analysis, since the 2-year cartilage had visible damages to the surface tissue, which resulted in the unclear division of the sub-tissue zones in imaging experiments.) Two features can be recognized. First, the PG contents were statistically unchanged for the contralateral cartilages based on the zonal analysis (with the exception of RZ in the m-uncovered region at 12-week post surgery). Second, most reductions of the PG concentrations occurred in the ACL cartilage. For the uncovered region, the PG concentration in SZ reduced in both 8-week and 12-week ACL cartilage. For the covered region, the PG concentration in SZ reduced only in the 12-week ACL cartilage. These trends of the PG concentration changes illustrate the complex relationships among the regional differences and the disease progressions

Discussion

The biochemical composition and molecular architecture of the cartilage's extracellular matrix give rise to distinct biomechanical properties of the tissue [2]. In SZ where the fine collagen fibrils are arranged in parallel to the surface, tissue is exposed to the highest tensile and compressive stresses [2]. Principal component concentration study [15] has determined that SZ is richer in collagen and lower in PG than any other sub-tissue zones in cartilage, which makes it easy to deform to resist both compressive and shear tensions [1, 2, 5]. Any injury to the structural components in the joint (cartilage, ligament, meniscus) can trigger the development of osteoarthritis in the joint [33, 37].

The ACL plays a crucial role in controlling the motion pattern of a normal knee [38]. The ACL transection, where the ligament is damaged in surgery, will produce a mechanically unstable knee, which may have a number of consequences. For example, the joint alignment and its loading pattern can be altered [33, 39], which would change the joint reactive force and cause cartilage damage. The ACL transection may also weaken sensory function and protective mechanisms in the knee [37]. A 123% increase in valgus rotation of the knee was reported after the ACL was transected [2]. When the injury occurs only to one knee, an alteration in the gait cycle is expected, which could lead to abnormal loading and subsequent degenerative changes on the contralateral knee joint [39–41]. The results in this project certainly support the detrimental consequences of the ACL transection in the knee joint.

Comparing topographically, it has been noted that the m-uncovered ACL cartilage was, on average, significantly thicker than that from the corresponding site in the contralateral knee, as shown in Fig.2. This result agrees with the findings in the literature from cartilage that was 54-month post-ACL transection [35]. In our experiments, this trend happened even in the cartilages at 8 weeks and 12 weeks after ACL transection, in agreement with the data

taken within 10-week [42]. It suggests that the articular cartilage in the ACL joint undergoes relatively quick, progressive degenerative changes. As reported, AC damage in ACL joint can occur within 2 weeks post-transection. Detectable cartilage surface damage and PG loss were observed as early as 1-week post-transection [43]. The cartilage thickness increases in the earlier stages, which was due to the increases in both net PG synthesis and uronic acid concentration [35, 36]. However, with the time elapsing, net PG synthesis in cartilage from the OA knee tended to be lower than that in cartilage from the contralateral knee [42]; meanwhile, PG loss becomes more obvious.

The semilunar menisci are situated between the femoral condyles and tibial plateau of the knee and play the vital roles in transmitting loads and providing joint lubrication with minimal wear [2]. It is clear from this project that the PG concentration reduced in the covered area in both ACL and contralateral knees at 8, 12 weeks and 2 years post ACL transection. A large PG loss was found at both SZ and TZ in the covered 12-week ACL cartilage. This result might be ascribed that the ACL transection and abnormal loading can shift the location of the tibiofemoral joint contact in the form of tearing and wearing between meniscus and cartilage and alter the stress distributions within the cartilage [44]. Possibly due to the same reason, the covered cartilage thickness of the ACL knee was thinner than that of the uncovered site from the identical animal, which was shown in Fig. 2D–E and with the literature [44]. One can therefore conclude that the change of motion pattern can cause complex degenerations in both covered cartilage as well as in the uncovered cartilage.

A final note must be made to the nature of the PG concentrations as measured in the FTIRI-PCR experiments. In this type of experiment, the PG concentration has the unit of percentage, which is based on the assumption that the sum of collagen and PG represented 100% of the dry tissue weight. This assumption was discussed in details and verified experimentally in some of the early works in the literatures [14, 15].

Conclusion

The depth-dependent profiles of the proteoglycan concentration in articular cartilage under different post-surgical conditions were quantitatively determined by FTIRI-PCR. The PG loss gradually increases with the post-surgical duration, from 8 weeks, 12 weeks to 2 years post transection. In both meniscus-covered and meniscus-uncovered sections, the PG loss in the ACL tibial cartilage is more than that in the contralateral tissue. The averaged PG concentration in the uncovered cartilage is generally lower than that in the covered cartilage, regardless of whether the tissue is from the ACL knee or the contralateral knee. The PG concentration in SZ is the lowest among the three zones under the ACL and contralateral conditions. This study by FTIRI-PCR could be helpful for predicting disease progression, and monitoring the injury and repair of osteoarthritis. It will further improve our understanding of the ACL function, which contributes to the development of novel strategies to accelerate ligament healing.

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Highlights

- FTIRI-PCR detection of proteoglycan concentration in cartilage after ACL transection
- The PG loss in cartilage was related positively to the duration after the ACL surgery
- The PG loss in the contralateral knees was less than that of the ACL knees
- The meniscus-covered cartilage lost PG less than the meniscus-uncovered tissue
- Quantitative PG mapping could monitor the disease progression

Fig. 1.

An infrared spectrum extracted from the FTIR image of articular cartilage together with the characteristic peak assignments.

Fig. 2.

The representative images in the uncovered control specimen (A), uncovered (B) and covered (C) cartilage sections in contralateral specimens, covered (D) and uncovered (E) specimens of 2-year post ACL transection. The three images from top to bottom in each group are the visible image, total absorption image and sugar band image ($1125\text{-}960\text{ cm}^{-1}$). SZ of the tissue locates at the left side in each image. (F) The depth dependent profiles of the PG concentrations by the PCR method from the images of (A), (D) and (E) across the uncalcified tissue, plotted as open and solid circles and open squares, respectively. (G) The depth dependent profiles of the PG concentrations by the PCR method from the images of (B) and (C) across the uncalcified tissue, plotted with open squares and solid circles, respectively. The x-coordinates (in microns) reflect the actual size of the cartilage section. The maximum of the color bar is 1.2 in (A)~(E) for all total absorption images, 0.2 in (A)~(D) and 0.15 in (E) for the sugar images, respectively.

Fig. 3.

The PG concentrations (averaged over the entire tissue depth) of the covered and uncovered cartilage sections in the control, the contralateral and the ACL cartilages at 8-weeks, 12-weeks and 2-years following the surgery, respectively. Each of the arrow pairs indicates the statistical analysis performed between the control and comparative group, where the corresponding p value is given above the horizontal line. The p values that are less than 0.05 are marked with double asterisks, which represent significant differences from the controls.

Fig. 4.

The zonal averaged PG concentrations of SZ, TZ and RZ of the covered and uncovered cartilage sections in the control, contralateral and ACL cartilage at 8 weeks and 12 weeks following the surgery, respectively. The pair of arrows indicates the statistical analysis performed between control and lesion group. The corresponding p value is given above the horizontal line. The p values that are less than 0.05 are marked with double asterisks, which represent significant differences from the controls.