Influence of Proteoglycan on Time-Dependent Mechanical Behaviors of Articular Cartilage under Constant Total Compressive Deformation*

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Articular cartilage has biphasic property based on high water content. It is generally believed that the proteoglycan supports the compressive load, but the detailed loading mechanism has not yet been clarified. In this study, first we observed the changes in compressive stress and strain of articular cartilage under constant total compressive deflection. We evaluated the changes in modulus of elasticity, which was estimated from the stress-strain relation in equilibrium state. To examine the role of proteoglycan in compressed articular cartilage, we compared the time-dependent viscoelastic behaviors in both the intact cartilage and the cartilage treated with chondroitinase ABC under constant total compressive deformation. We could confirm that the peak stress after compression and the modulus of elasticity at equilibrium were reduced after the digestion of proteoglycan. Next, we observed the changes in local strain in both articular cartilage specimens with and without chondroitinase treatment by monitoring the position of stained chondrocyte in the confocal laser scanning microscope. These visualized images indicated that the local strain changed time-dependently and depth-dependently. The digested cartilage showed the quicker change in movement and larger thinning in surface layer than the intact cartilage. These results indicate that the proteoglycan contributes to the compressive load-carrying capacity and controls the permeability.

Key Words: Biomechanics, Articular Cartilage, Proteoglycan, Extracellular Matrix, Chondrocyte, Biphasic Material, Stress Relaxation, Depth-Dependent Deformation

1. Introduction

For various daily activities in human life, the natural synovial joints can carry high load, for example, several times of body weight in hip and knee joints during walking, stair-climbing and running. This excellent durability of natural synovial joints is considered to be supported by the optimized structure composed of compliant articular cartilage surface, subchondral bone and cancellous bone

with trabeculae corresponding to the principal stress trajectories. The natural synovial joints are high performance bearings with very low friction and low wear for longer life. This excellent tribological performance appears to be supported by the adaptive multimode lubrication mechanism^{(1)–(3)}, in which the fluid film lubrication, weeping, adsorbed film and/or gel film lubrication can become effective depending on the severity of operating conditions. In natural hip and knee joints during walking, the elastohydrodynamic lubrication (EHL) mechanism, based on the macro- and micro-scopic elastic deformation of articular cartilage surfaces and viscous effect of synovial fluid, is likely to play the main role in preserving low friction and low wear.

The elastic deformation of articular cartilage enhances the fluid film formation during various motions and spreads the contact area in the rubbing surface, which can suppress the excessive rising of contact pressure un-

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der increasing load. To suppress the contact pressure less than 5 MPa at maximum and enable to form soft-EHL film, it is required to preserve compliant property in articular cartilage. On the other hand, if the elastic modulus of articular cartilage is too low, the excessive compressive deformation of articular cartilage induces the excessive deformation of the chondrocytes resulting in damage of cells. The chondrocyte appears to correspond to mechanical stimulation of appropriate level probably less than 20%. Therefore, it is required that the elastic modulus is ranged from about 0.5 MPa to 20 MPa^{(4),(5)} in load-bearing human joints.

The articular cartilage is composed of extracellular matrix, chondrocytes and movable interstitial fluid. Chondrocyte has very low stiffness as a level of 1 kPa or so^{(6),(7)}. Thus, the extracellular matrix composed of proteoglycan and collagen fiber plays main role for load-supporting. In articular cartilage structure, the proteoglycan appears to support mainly compressive load, and the collagen fiber resist to tensile load^{(5),(8)}. In this paper, the role of proteoglycan is examined in the compressive tests.

As described above, the articular cartilage has two main mechanical roles of the load-carrying surface layer of synovial joint and the protective surface layer with low friction. In the previous papers^{(3),(9)–(11)}, we discussed the protective and lubricating mechanism of cartilage surface layer. It was pointed out that the proteoglycan gel-film in the surface could contribute to the protection with low friction in slightly worn surface after removal of adsorbed film. To prevent further cartilage damage inducing the osteo-arthritis, it is important to repair this gel-film and adsorbed film. The constituents of proteoglycan gel film appear to be supplied as product of extracellular matrix by chondrocytes in repairing process, although the adsorbed constituents are supplied by synovial fluid.

The repair of damaged cartilage is controlled by not only the recovery of the surface layer but also the bulk matrix recovery controlled by chondrocyte. It is considered that articular cartilage adapts to changing mechanical environments where the chondrocyte can respond to the local stress-strain state^{(12),(13)}, but the detailed process has not yet been clarified. Chondrocytes are concerned with the mechanisms underlying remodeling, adaptation, and degeneration of articular cartilage in natural synovial joints subjected to changing loads. To study the mechanism involved, it is important to know the stress-strain state of the cartilage and extracellular matrix around chondrocytes during cartilage deformation. Its depth-dependent complicated structure causes a complex stress-strain field. Some compression studies^{(14),(15)} using confocal laser scanning microscopy (CLSM) or video microscopy have focused on the equilibrium strain state of a compressed articular cartilage specimen. However, articular cartilage has a biphasic⁽¹⁶⁾ viscoelastic property based on a high water con-

tent of up to 80% and the fluid-flow behavior concerns the time-dependent deformation of articular cartilage under compression. Therefore, it is necessary to observe the time-dependent stress and strain of compressed articular cartilage, to clarify the change of the mechanical environment around chondrocytes when the tissue is exposed to compression. To evaluate the protective roles of extracellular matrix and chondrocyte, the structural response of time-dependent strain behavior of articular cartilage under unconfined, axial and static compression should be evaluated by the simplified compression tests.

The main purpose of this paper is to point out the important role of the proteoglycan as main constituent in extracellular matrix in the load-carrying structure of articular cartilage. Repairing processes of not only bulk cartilage tissue but also damaged cartilage surfaces seem to be controlled by the supply of proteoglycan, in addition to the protective mechanism provided by synovial fluids. First, the influence of proteoglycan on the initial peak stress and elastic modulus of articular cartilage is evaluated by the treatment of chondroitinase ABC in the compressive test under constant total deformation. Second, the visualization of chondrocytes in articular cartilage cross-section is applied to evaluate the time-dependent and depth-dependent mechanical behaviors of articular cartilage as biphasic material.

2. Experimental Methods

2.1 Materials for compression tests with and without enzyme treatment

The specimens are prepared from the femoral condyle of porcine knee joints shown in Fig. 1. The pigs of 10 were 4 to 5 months old. The cylindrical specimen of 3 mm diameter was prepared by the punch. Then, the cylinder was sectioned as half size with a scalpel. The semi-cylindrical specimen includes the subchondral bone of about 0.5 mm thickness as shown in Fig. 2. These 95 specimens were divided into two groups. 75 specimens as 15 × 5 for 15

Fig. 1 Femoral articular cartilage of porcine knee joint

Fig. 2 Test specimen

specimens for each condition of the digestion were treated with the enzyme as described below, and 20 specimens in an intact group were untreated.

2.2 Enzyme treatment

In this test, the chondroitinase ABC (Sigma) was used to digest the chondroitin-sulfate of main constituents in proteoglycan as extracellular matrix in articular cartilage. The treatment was conducted at concentration of 1 unit/mL, at pH8 and 38°C. The treatment times are 1, 3, 6, 12 and 24 h. The effectiveness was confirmed by the appearance of collagen fibers with the treatment of chondroitinase ABC for intact articular cartilage surface. The changes in articular cartilage surfaces were observed in saline by atomic force microscope (AFM) as fluid tapping mode using scanning probe microscopy SPM systems (Nanoscope III, Digital Instruments, USA) reported in previous studies^{(10),(11)}.

2.3 Compression test for evaluation of compressive stress and elastic modulus

The test apparatus A constructed for the first compression test is shown in Fig. 3. The constant compressive displacement was given by manual rotation of micrometer head. The impermeable alumina ceramic plates were used as the fixed plate for the subchondral bone and the indenting plate for articular cartilage. The compression tests were conducted in immersed condition with phosphate buffered solution (PBS) at room temperature (21 to 22°C). The load cell was placed between the ceramic plate and the specimen holder to observe the changes in compressive load.

In the compression tests, 13% total strain was applied in 1.0 s and then this total displacement was kept for 10 min. This total strain approximately corresponds to the strain level of 15% or so in articular cartilage of synovial joints for lower limb at normal walking. The equilibrium modulus of elasticity was estimated as the modulus at equilibrium state after stress relaxation diminished. At this condition the equilibrium was attained after 10 min.

In the compressive tests, the elastic modulus E at equilibrium was calculated by the following equation, where σ is compressive stress and ε is nominal strain.

$$E = \sigma / \varepsilon \quad (1)$$

2.4 Visualization of movement of chondrocytes

Next, we observed the changes in local strain in articular cartilage specimens by monitoring the position of

Fig. 3 Compression apparatus A with manual operation

Fig. 4 Compression apparatus B with high precision

stained chondrocyte in the CLSM. The staining of chondrocyte is treated with calcein-AM, at 1 $\mu\text{L/mL}$ and 37°C for 30 min. In the case with enzyme treatment, the intact cartilage was stained and then treated with chondroitinase ABC. In this test, the articular cartilage with digestion for 3 h was selected as comparing specimen with the intact cartilage. The compression apparatus B (Fig. 4) with high precision within 0.2 μm for position control was newly developed. This apparatus was allocated on the stage of CLSM and the compression speed can be adjusted from 1 $\mu\text{m/s}$ to 4000 $\mu\text{m/s}$ by feed-back control of DC servomotor. In these tests, 13% total strain was applied in 1 s as the same loading condition as the above tests. The local strain was estimated as the approaching distance divided by the initial distance in perpendicular direction to the surface between corresponding chondrocytes in each layer in cartilage, as described in section 3.2. For direct

comparison between semi-cylindrical specimens with and without enzyme treatment, a cylindrical cartilage specimen was separated to paired semi-cylindrical specimens of the same thickness and structure, in which we can observe the influence of enzyme treatment on local strain. 16 pairs were tested. On the basis of these visualized images, the time-dependent and depth-dependent changes in local strain of articular cartilage were evaluated.

3. Experimental Results

3.1 The changes in peak value of compressive stress and elastic modulus

The thickness of articular cartilage specimens ranged from 1 200 to 2 300 μm , and average value is 1 700 μm . The changes in average compressive stresses after compression are shown in Fig. 5. The stress peak was observed after the total compressive deformation attained the definite target value of 0.13%, then the stress relaxation occurred, thus the compressive stress was remarkably lowered with time.

The peak value of average compressive stress (mean contact pressure) before the relaxation was clearly reduced with treatment time of digestion. The average stress at equilibrium was decreased as shown in Fig. 6. The elastic modulus at equilibrium was estimated from the average stress. As shown in Fig. 7, the modulus was gradually decreased with digesting time. The analysis of variance indicated the statistically significant results between untreated

Fig. 5 Time-dependent change in compressive stress

Fig. 6 Change in compressive stress at equilibrium with time of digestion by chondroitinase ABC. Error bars indicate standard deviations.

and 1 h digested specimens, and between 1 h digested and 3 h digested specimens, as $p < 0.005$ and $p < 0.01$, respectively. The control specimens which were immersed in PBS for 1, 3, 6, 12 and 24 h, without enzyme treatment did not exhibit the lowering of elastic modulus. Therefore, we could confirm that the digestion of chondroitin sulfate induces the lowering of elastic modulus.

3.2 Visualization of movement of chondrocytes

The fluorescent images of articular cartilage specimens are shown in Fig. 8, where the highlighted elliptical bodies of 10 to 20 μm indicates the chondrocytes. Some chondrocytes are enclosed with white circles. These images were observed every 2 s until 400 s at equilibrium after loading. In these tests, the articular cartilage surface was located at contact with the fixed plate, in order to keep the surface position within visual field of microscope. We estimated the local strain by calculating the changes of the distance in perpendicular direction to the cartilage surface

Fig. 7 Change in modulus of elasticity at equilibrium with time of digestion by chondroitinase ABC. Error bars indicate standard deviations.

Fig. 8 Fluorescent images of articular cartilage in compression tests (CLSM)

Fig. 9 Local strain in articular cartilage with and without enzyme treatment in compression tests

Fig. 10 Changes in local strain in articular cartilage with and without enzyme treatment in compression tests

Fig. 11 Changes in relative position of chondrocytes in articular cartilage with and without enzyme treatment in compression tests

between the definite chondrocytes as follows;

Local strain immediately after compression : $(a-b)/a$

Local strain at equilibrium : $(a-c)/a$

Change in local strain to equilibrium immediately after compression : $(b-c)/a$

Here, a , b and c means the distance between the corresponding chondrocytes before compression, immediately after compression and at equilibrium after 400 s, respectively.

In Fig. 9, the estimated values of local strain in a pair of specimens with and without enzyme treatment immediately after the total deflection of 13% was attained and at the equilibrium are plotted against the relative location in the depth direction, where 0 means surface and 1 means the tidemark as the boundary of subchondral bone. The ar-

ticular cartilage is usually discriminated to be constructed from three zones, i.e., the surface, middle and deep zone along the perpendicular direction from surface. It is noted for both cartilage specimens with and without enzyme treatment that the response of middle and deep zone is quick but the deformation of surface zone is low immediately after compression. However, the surface zone was largely compressed than average strain during stress relaxation. In contrast, the deformation of deeper zone was clearly recovered probably accompanied with flowing of fluid into the middle and deep zone. Figure 10 exhibits the remarkable changes in local strain during relaxation process, in which positive value means the increase in compressive strain and negative one indicates the reduction in compressive strain. In comparison between both cartilage

specimens with and without enzyme treatment, it is noticed that the cartilage treated with chondroitinase ABC shows remarkable compression in the surface zone during stress relaxation. Furthermore, the maximum strain at surface zone is higher in the digested cartilage than intact cartilage as shown in Fig. 9.

Figure 11 shows changes in relative position of selected chondrocytes in articular cartilage during compression tests. As shown by the moving behaviors indicated by elliptical mark, the digested articular cartilage exhibits the quicker movement than intact cartilage.

The similar phenomena in local strain as described above were observed in other 15 paired specimens. This fact designates the important role of proteoglycan in compressive load-carrying mechanism.

4. Discussions

4.1 The changes in peak value of compressive stress and elastic modulus

The physical properties of articular cartilage appears to be controlled by the properties of proteoglycan and collagen fiber, chondrocytes and the content of water. Particularly, the extracellular matrix composed of proteoglycan and collagen fiber can be dominant to control the bulk property. In the present evaluation in the first compression test by the apparatus A, the digestion of chondroitin sulfate as the main constituent in the proteoglycan showed the significant decrease in the peak value of compressive stress and elastic modulus at equilibrium. This fact reveals that the proteoglycan can play an important role in mechanical viscoelastic behaviors.

The elastic modulus of intact articular cartilage at equilibrium is about 2 MPa, which are ranged in the previously reported data. By the addition of enzyme, the elastic modulus was decreased to 0.5 MPa as 1/4 of the intact condition. The peak stress immediately after the compression was decreased by the digestion, which means the enhancement of permeability. This fact corresponds that the decrease in proteoglycan reduces the elastic modulus by lowering of compressive resistance of proteoglycan and enhances the permeability as a result of reduction in water binding ability in extracellular matrix. In intact articular cartilage, the proteoglycans are polyanions due to negatively charged sulfate and carboxyl groups associated with keratan sulfate and chondroitin sulfate. These properties control the ability of proteoglycans to bind water osmotically.

The thickness of test cartilage specimens varied in the range of 2 fold but the clear difference was not found in these tests. The thickness of subchondral bone was adjusted as about 0.5 mm, and thus it behaved as the rigid body because of higher modulus of 3 order.

4.2 Visualization of movement of chondrocytes

In the observation of the stained chondrocytes, the

time-dependent and depth-dependent behaviors in the local strain could be evaluated with confocal laser scanning microscopy. The experimental results indicated that the surface zone moderately responded at the initial stage of compression and then was largely compressed during stress relaxation. In the surface zone, collagen fibrils are arranged in parallel sheets to the articular surface. On the contrary, the middle and deep zones containing larger percentage of proteoglycan than surface zone were quickly compressed at the initial stage and then recovered probably with water flowing in. Besides, it is noticed that the cartilage treated with chondroitinase ABC exhibited not only larger compression in the surface zone but also the quicker change in local strain during stress relaxation. These behaviors are considered to be caused by reduction of elastic modulus and rise of permeability due to the digestion of chondroitin sulfate as main component in proteoglycan. These facts indicate the important role of proteoglycan in supporting mechanism for compressive load.

4.3 Future subjects to be solved

The strains of articular cartilage control the deformation of chondrocytes. Therefore, the strain behaviors of articular cartilage are expected to have an important influence on the biosyntheses of proteoglycans by chondrocytes with mechanical stimuli which can control the bulk stiffness and permeability and the restoration of the lubricating gel film on the surface of articular cartilage.

In this experimental study, we indicated that the proteoglycan bears the compressive load and controls the permeability. In the future study, the treatment of collagenase which can digest the collagen is required to clarify the role of other main component in the extracellular matrix in articular cartilage.

In the visualization tests of chondrocytes, we found that the local strain shows time-dependent and depth-dependent behaviors. In our previous paper⁽¹¹⁾, the finite element analysis for biphasic articular cartilage composed of three layers with constant permeability and elastic modulus for each layer was carried out for compression tests. This analysis indicates that the local strain in the surface zone showed the larger value than average strain immediately after compression and then the local strain was recovered to average level. This time-dependent phenomenon is conflict to this experimental result where the deeper zone was compressed at the initial stage and then recovered. In this experiment, the surface layer showed the initial moderate deformation and then larger deformation during stress relaxation. The reason why the strain behavior exhibited different behavior to simplified finite element analysis is considered to have been caused by the mechanical anisotropy, non-linear property of permeability indicated by Jurvelin et al.⁽¹⁷⁾ and inhomogeneous structure with different direction of collagen fiber. In the next stage, we plan to conduct several numerical analyses

considering the non-linearity and inhomogeneous properties.

5. Conclusions

The treatment of chondroitinase ABC for intact articular cartilage exhibited the clear reduction of elastic modulus at equilibrium and peak value of compressive stress in the compression tests with definite total compression. This fact indicates that the proteoglycan can support the compressive load.

In the visualization tests of stained chondrocytes, the time-dependent and depth-dependent behaviors in the local strain could be evaluated for articular cartilage with and without enzyme treatment. The fluorescent images indicated that the surface zone moderately responded at the initial stage of compression and then was largely compressed during stress relaxation. The articular cartilage treated with chondroitinase ABC showed the quick and larger compression in the surface zone during stress relaxation process. These phenomena appear to be brought about by increase of permeability and lowering of stiffness due to digestion of proteoglycan. Thus, proteoglycan is likely to play an important role to compressive loading mechanism of articular cartilage.

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