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Changes of human menisci in osteoarthritic knee joints

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SUMMARY

Objective: To investigate the changes of knee menisci in osteoarthritis (OA) in human.
Methods: OA and control menisci were obtained from 42 end-stage OA knees with medial involvement and 28 non-arthritic knees of age-matched donors, respectively. The change of menisci in OA was evaluated by histology, and gene expression of major matrix components and anabolic factors was analyzed in the anterior horn segments by quantitative PCR (qPCR). In those regions of menisci, the rate of collagen neo-synthesis was evaluated by [^{3}H]proline incorporation, and the change of matrix was investigated by ultrastructural observation and biomechanical measurement.
Results: In OA menisci, the change in histology was rather moderate in the anterior horn segments. However, despite the modest change in histology, the expression of type I, II, III procollagens was dramatically increased in those regions. The expression of insulin-like growth factor 1 (IGF-1) was markedly enhanced in OA menisci, which was considered to be responsible, at least partly, for the increase in procollagen gene expression. Interestingly, in spite of marked increase in procollagen gene expression, incorporation of [^{3}H]proline increased only modestly in OA menisci, and impaired collagen synthesis was suggested. This finding was consistent with the results of ultrastructural observation and biomechanical measurement, which indicated that the change of meniscal matrix was modest in the macroscopically preserved areas of OA menisci.
Conclusion: Although the expression of major matrix components was markedly enhanced, matrix synthesis was enhanced only modestly, and the changes of matrix in human OA menisci were rather modest in the non-degenerated areas.
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Introduction

With the increasing human life expectancy, osteoarthritis (OA) has now become a leading cause of disability among older adults[1]. A knee joint is one of the most susceptible joints to OA, and OA of the knees accounts for a large part of the OA-related disability. In OA knees, almost all joint structures are affected by the disease. Knee menisci are also known to undergo an obvious change in OA[2].

Menisci are C-shaped fibrocartilaginous structures that are predominantly composed of type I, with small amounts of type II, type III collagens and proteoglycans[3–11]. Located between the femoral condyles and tibial plateau, menisci play important roles in load transmission, shock absorption, and maintenance of joint stability[12]. With the progression of OA, menisci undergo degenerative changes such as wearing and laceration[2]. Subluxation of menisci also occurs[13]. Reflecting their functional importance, the changes in menisci are closely associated with the initiation and progression of OA[14,15]. Although often studied in animal models, human OA menisci have seldom been the subject of investigation, and it is still not known well how menisci change in OA.

A meniscus is not a homogeneous tissue. Regional differences in tissue composition and cell metabolism have been observed between the inner and outer areas, and structural differences have been noticed between the surface and central regions[4,8–11,16–19]. Therefore, the evaluation of meniscus pathology would be more reasonably performed if such regional differences were considered.

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In this study, human menisci from OA and control knees were investigated by histological and molecular biological analyses, paying special attention to the regional differences within menisci. The rate of collagen neosynthesis was determined, and the change of meniscal matrix was evaluated by ultrastructural analysis and biomechanical measurement. The results of these analyses revealed the unique nature of the changes in human OA menisci, which are considerably different from those reported in animal studies.

Materials and methods

Material collection

This study was performed with the approval of the Human Ethics Review Committees of the participating institutions, and informed consent was obtained in writing from each subject or family of the donor before material collection. OA menisci were obtained in pairs of lateral and medial menisci from 42 OA knee joints of 40 patients within 4 h after a prosthetic surgery. The diagnosis of OA was based on the criteria of the American College of Rheumatology for OA[20], and all knee joints were medially involved in the disease. Control menisci were obtained from 28 non-arthritic knee joints of 22 donors (obtained bilaterally in six donors; ages 73–92 with a mean of 84) within 24 h after death. The donors were chosen from those who did not have a past record of treatment for joint disease or trauma, and the normality of the joint was confirmed macroscopically at the time of sample harvest. Prior to the analyses, all OA and control menisci were photographed and their macroscopic appearance was recorded. Control menisci were inspected closely, and were not used for the study if they showed overt signs of macroscopic degeneration. In this study, evaluations were performed on the anterior horn segments of menisci unless specified otherwise. This was because that those segments were well preserved in OA menisci, which made it possible to compare the changes in OA menisci directly in a site-to-site manner with those in the control menisci. Posterior horn segments of menisci were also often preserved in OA knees, but those segments were not used for the analyses. In this work, the OA and control menisci were randomly assigned to respective analyses. Some menisci were used for two or more evaluations.

Histological evaluation

Ten pairs of OA menisci and eight pairs of control menisci were used for this evaluation. The evaluation was performed primarily on the anterior horn segments of menisci, but for OA medial menisci, the body sections were also observed in order to know the changes in degenerated regions. Meniscus tissues were fixed in 4% paraformaldehyde, and 4-μm-thick sections were prepared in a plane perpendicular to the surface. The sections were stained with hematoxylin and eosin or Masson's trichrome stain, and observed under a light microscope. For the immunodetection of insulin-like growth factor (IGF)-1, the sections were pretreated with proteinase K (DakoCytomation, Carpinteria, California) and then incubated with a monoclonal antibody for human IGF-1 (Millipore, Billerica, Massachusetts). The primary antibody was reacted with the secondary antibody conjugated with streptavidine, which was visualized with 3,3′-diaminobenzidine (DAB), tetrahydrochloride using a commercially available kit (ChemMate EnVision Detection, DakoCytomation). The nuclei were stained with hematoxylin, and the localization of staining was observed under a light microscope.

Gene expression analysis

Twelve pairs of OA and control menisci were used for the evaluation. For the analysis, meniscus tissues were obtained from the anterior horn segments with a width of approximately 5 mm (Fig. 1). Immediately after harvest, the tissue was divided into four regions under a dissection microscope (Fig. 2). The divided tissues were each embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), snap frozen in liquid nitrogen, and stored at −80 °C until use. RNA was extracted from each of these regions by a previously described method[21]. cDNA was synthesized using the Sensiscript reverse transcriptase (Qiagen, Hilden, Germany) with a routine use of DNase I (Qiagen). Gene-specific primers and probes were prepared (Table I), and quantitative PCR (qPCR) was performed using either SYBR Premix Ex Taq or Premix Ex Taq (both from Takara Bio, Shiga, Japan) on a LightCycler (Roche Diagnostics, Basel, Switzerland). The expression levels were normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). For some samples, β-actin (*ACTB*) expression was also used for normalization.

Evaluation of collagen neosynthesis

The rate of collagen biosynthesis was determined by quantifying the amount of [^{3}H]proline incorporation into meniscus explants. Eight pairs of OA menisci and six pairs of control menisci were used for this analysis. The explants were prepared from the anterior horn segments of those menisci. In each of these menisci, the anterior horn segment was separated from the body segment, and the entire part of that segment was cut into 1–2 mm cubes, or explants. Four to six explants were placed in each of the 24-well tissue culture plates, which were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 25 μg/ml ascorbic acid. Forty-eight hours later, the media were then replaced by those containing 0.1% FBS, 25 μg/ml ascorbic acid and 10 μCi/ml [^{3}H]proline. After 8 h, the media were removed and the

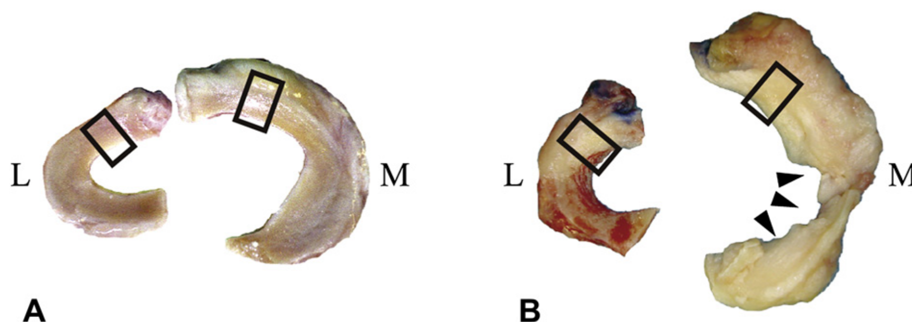

Fig. 1. Gross appearance of control and OA menisci. Representative photographs of control (A) and OA menisci (B) are shown. In each meniscus, a pair of parallel lines indicates the region in the anterior horn segment used for molecular biological analysis. In B, arrowheads indicate degenerated area in medial meniscus. M and L indicate medial and lateral menisci, respectively.

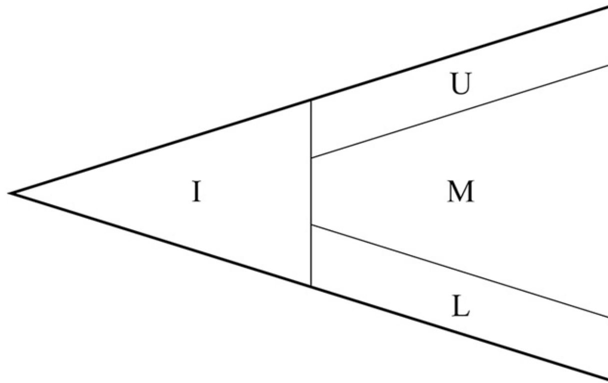

Fig. 2. Separation of meniscus regions. Anterior horn segment of meniscus was divided into inner (I), and outer halves, and the latter was further divided into upper surface (U), middle (M), and lower surface (L) regions. Gene expression was evaluated in those regions respectively. Cross-section of a meniscus is shown.

explants were rinsed three times with ice-cold phosphate-buffered saline (PBS). The samples were stored at −20 °C until use.

To determine the rate of [^{3}H]proline incorporation, the explants were minced and digested with proteinase K (2 mg/ml; Sigma, St. Louis, Missouri) at 58 °C for 48 h. After digestion, the samples were centrifuged and radioactivity of the supernatant was measured. The radioactivity was normalized by DNA content, which was determined by Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, California).

Transmission electron microscopy

Three pairs of OA menisci and three pairs of control menisci were used for the evaluation. For the analysis, the tissues were obtained as thin slices from the anterior horn segments, which were immediately immersed in 2.5% glutalaldehyde in the cacodylate buffer. The samples were then treated with 1% OsO_4 in cacodylate buffer, dehydrated in graded concentration of ethanol, and embedded in epon with careful attention to the orientation. Sections were cut at 90 nm thickness and stained sequentially with 0.2% oolong tea extract (Nisshin EM, Tokyo, Japan), aqueous uranyl acetate, and lead citrate. The sections were observed under a JOEL JEM-2000 FX-II electron microscope (Nihon Denshi, Tokyo, Japan).

Quantitative image analysis of collagen fibrils was performed following a previously described method[22] with some modifications. In brief, three to five photomicrographs were taken on each section at the magnification of ×20,000, and on each photo, the number and diameters of collagen fibrils were determined using an image analysis software (ImageJ version 1.42e; National Institute of Health, Bethesda, Maryland). For each meniscus, mean fibril diameter, number of fibrils per μm^2, and percentage of the area occupied by fibrils were determined.

Table I
Primer and probe sequences used for quantitative PCR

Gene	Primer and probe sequence	T_m
COL1A1	Forward: 5′-AGCCTGGGGCAAGACAGTGATT-3′	60
	Reverse: 5′-TTGCTTGTCTGTTTCCGGGTTG-3′	
COL1A2	Forward: 5′-ATGAGGAGACTGGCAACCTGAAAAA-3′	58
	Reverse: 5′-TCCAAAGGTGCAATATCAAGGAAGG-3′	
COL2A1	Forward: 5′-GACATAGGAGGGCCCGAGCA-3′	60
	Reverse: 5′-CGGCACCTGAAGGGAGGTCT-3′	
COL3A1	Forward: 5′-TCGAACACGCAAGGCTGTGAG-3′	60
	Reverse: 5′-TGTCGGTCACTTGCACTGGTTGA-3′	
ACAN	Forward: 5′-GCACGAGAAGGGCGAGTGGA-3′	60
	Reverse: 5′-GCTCCTGGGCTCAGCGTCCT-3′	
*IGF1**	Forward: 5′-GTACTTCAGAAGCAATGGGAAA-3′	56
	Reverse: 5′-GTTGAAATAAAAGCCCCTGTCT-3′	
	Probe Flu: 5′-GTGAAGATGCACACCATGTCCTCCTCG-3′	
	LC: 5′-ATCTCTTCTACCTGGCGCTGTGCCTGC-3′	
PDGFA	Forward: 5′-TCATTTACGAGATTCCTCGG-3′	58
	Reverse: 5′-TGCTCCTCTAACCTCACCTG-3′	
PDGFB	Forward: 5′-GGAAGAAGCCAATCTTTAAGAAG-3′	58
	Reverse: 5′-CTTCAGTGCCGTCTTGTCAT-3′	
TGFB1	Forward: 5′-ATGTTCTTCAACACATCAGAGC-3′	58
	Reverse: 5′-GGTGACATCAAAAGATAACCAC-3′	
GAPDH	Forward: 5′-AAAACCTGCCAAATATGATGAC-3′	58
	Reverse: 5′-CAGGAAATGAGCTTGACAAAGT-3′	
ACTB	Forward: 5′-ATTAAGGAGAAGCTGTGCTACGTC-3′	58
	Reverse: 5′-ATGATGGAGTTGAAGGTAGTTTCG-3′	

COL1A1, collagen, type I, alpha 1; *COL1A2*, collagen, type I, alpha 2; *COL2A1*, collagen, type II, alpha 1; *COL3A1*, collagen, type III, alpha 1; *ACAN*, aggrecan; *IGF1*, insulin-like growth factor 1; *PDGFA*, platelet-derived growth factor alpha polypeptide; *PDGFB*, platelet-derived growth factor beta polypeptide; *TGFB1*, transforming growth factor, beta1; *GAPDH*, glyceraldehyde-3-phsophate dehydrogenase; *ACTB*, actin, beta.

* Hybridization probes were used for quantitative PCR. Flu: probe conjugated with Fluorescein; LC: probe conjugated with LC640.

Biomechanical measurement

Six pairs of lateral and medial OA menisci and six pairs of control menisci were used for this analysis. For measurement, two to four cylindrical specimens, 4 mm in diameter, were harvested from the anterior horn segment of each meniscus. Those specimens were acquired along the periphery of the menisci, 1–2 mm inside from the outer margin, in a side-by-side manner. The number of obtained specimens varied among the menisci depending on the size and the severity of degeneration (when present). The specimens were prepared perpendicular to the tibial (lower) surface using a core reamer. After the acquisition, the upper part of the specimen was cut parallel to the tibial surface so that it would be 3 mm in height. Prior to the measurement, a wet weight of the specimen was determined.

Uniaxial confined compression test was performed according to a previously described method[23]. Each specimen was placed with the tibial surface facing down into a 4.0 mm diameter × 30 mm deep well in an acrylic chamber upon a porous filter. The chamber was then immersed in physiological saline in a reservoir, and the measurement was performed while the specimen was submerged in the saline. Compression load was applied to the specimen by a compression testing machine (Model SV-201NA, Imada Seisakusyo, Aichi, Japan). For measurement, a 4.0-mm diameter stainless indenter was gently inserted into the well in the acrylic chamber onto the specimen, and a constant load of 2 g was applied initially for 15 min as preloading. The load was then suddenly increased to 10 g, and the linear displacement rate was measured at 12 time points between 1 and 5000 s after the increase of the load. The applied load was maintained at 10 g throughout the measurement, which was monitored continuously with a load cell (Model LC-050N, Imada Seisakusyo). Two parameters, aggregate modulus (solid component stiffness) and permeability (measure of fluid flow through the tissue), which characterize the viscoelastic nature of the meniscus, were calculated using a nonlinear least squares optimization algorithm incorporating linear biphasic theory[24]. After biomechanical evaluation, the specimen was completely dried in a vacuum dryer and the dry weight was determined. Water content of the specimen was defined as the percentage of the weight lost by drying relative to that of the wet weight. Aggregate modulus, permeability, and water content were compared between OA and control menisci.

Statistics

Statistical significance of the data was evaluated using analysis of variance (ANOVA), and Fischer's Protected Least Significant Difference (PLSD) was used as a post-hoc test when necessary. Two-tailed P-values less than 0.05 were considered significant.

Results

Macroscopic observation

In control menisci, the anatomical shape was well preserved in all samples [Fig. 1(A)]. They had smooth surfaces and did not show macroscopic signs of degeneration except for the presence of slight fraying of the inner margin of the medial meniscus of four samples. For OA menisci, macroscopic appearance differed obviously between lateral and medial menisci. Since all OA menisci were obtained from medially involved knees, medial menisci underwent overt degenerative changes, while lateral menisci preserved the anatomical shape with few signs of macroscopic degeneration [Fig. 1(B)]. The changes of the medial menisci were most notable in the body and posterior horn segments, where the matrix was lost or severely fibrillated. Even in such menisci, the anterior horn segment was preserved well, showing few signs of matrix loss or degeneration.

Histological evaluation

The hematoxylin and eosin stained sections of the control lateral menisci showed short, thick wedge-shaped sections with smooth surfaces [Fig. 3(A) and (B)]. The inner area was filled with densely packed fibril bundles, and the cells were located sparsely throughout the menisci. In OA lateral menisci, fibril bundles tended to be obscure in the upper and lower surface regions, but the bundle structure was well preserved in the inner area away from the surfaces [Fig. 3(C) and (D)]. The cell density was similar to that in the controls.

Sections from control medial menisci showed a thinner, wedge-shaped cross-section with smooth surfaces [Fig. 3(E) and (F)]. Within the menisci, fibril bundles were densely packed and regularly aligned, resembling that of control lateral menisci. Meniscus cells were located sparsely, and the cell density was similar throughout the section. In medial menisci, changes with OA were more obvious than those in lateral menisci. Sections of OA medial menisci tended to be thicker than the controls, and the fibril bundles were not clearly recognized in the surface regions [Fig. 3 (G) and (H)]. In those regions, cell clusters were occasionally found. Structural change was also noticed in the inner areas, where fibril bundles were coarsely and unevenly distributed. Despite these changes, again, the cell density in OA medial menisci was similar to that of the controls.

Obvious wearing and fraying of fibril bundles was seen in the degenerated areas of OA medial menisci [Fig. 3(I) and (J)]. Even in such severely degenerated regions, cell density did not differ much from that of the controls, and cell clusters were found only occasionally.

Evaluation of matrix gene expression

Next, the expression of mRNA for matrix molecules that constitute menisci was evaluated. For this analysis, menisci were divided into inner, upper surface, middle, and lower surface regions (Fig. 2), and mRNA expression was determined in respective regions.

Considering primary components of menisci[3–11], expression of five matrix genes (*COL1A1*, *COL1A2*, *COL2A1*, *COL3A1* and *ACAN*) was evaluated in this study. First, the expression of two genes encoding type I procollagen (*COL1A1* and *COL1A2*) was evaluated. In control menisci, these genes were expressed at similar low levels in both lateral and medial menisci [Fig. 4(A) and (B)]. In OA menisci, the expression of these genes was dramatically increased in all four regions. The increase was 9- to 52-fold depending on the regions. While the expression of *COL1A1* was equally enhanced in lateral and medial OA menisci [Fig. 4(A)], *COL1A2* expression tended to be more enhanced in medial OA menisci [Fig. 4(B)]. As a consequence, in OA medial menisci, the expression ratios between *COL1A2* and *COL1A1* were significantly higher than those of the control medial menisci [Fig. 4(C)].

The expression of type II procollagen (*COL2A1*) was also enhanced in OA menisci in all four regions [Fig. 4(D)], although the level of increase was lower than that for type I procollagen genes (3- to 19-fold vs 9- to 52-fold).

Among the five genes evaluated, type III procollagen (*COL3A1*) was the one whose expression was most enhanced in OA menisci [Fig. 4(E)]. The increase reached as high as 400-fold. Similar to *COL1A2*, the expression of *COL3A1* tended to be more promoted in medial OA menisci than in lateral OA menisci.

In contrast to *COL3A1*, the increase of aggrecan (*ACAN*) expression in OA menisci was the least among the five genes, which never exceeded 2-fold [Fig. 4(F)].

Because the increase of gene expression in OA menisci was so dramatic, we repeated gene expression analysis of several samples using *ACTB* as an internal control. The result of this analysis confirmed the validity of the above results based on *GAPDH* expression (data not shown).

Expression of possible anabolic factors in OA menisci

In order to elucidate the mechanism(s) for enhanced matrix gene expression in OA menisci, a preliminary experiment was performed to investigate the expression of growth factor genes which could enhance anabolic activity of meniscal fibroblasts. We compared the expression of IGF-1 (*IGF1*), platelet growth factor (PDGF)-A (*PDGFA*), PDGF-B (*PDGFB*), and transforming growth factor (TGF)-β1 (*TGFB1*) between OA and control menisci, and found that the expression of *IGF1*, *PDGFA* and *PDGFB* was increased in OA menisci, while that of *TGFB1* was reduced [Fig. 5(A)–(D)]. Among these three genes, the increase in expression was most obvious with *IGF1*, which reached as high as 50-fold increase. The expression of *IGF1* tended to be higher in medial OA menisci than in lateral OA menisci, though this difference was not statistically significant. The expression of IGF-1 by OA meniscal cells was confirmed at the protein level by immunohistochemistry [Fig. 5(E) and (F)].

Evaluation of collagen neosynthesis

We next evaluated the rate of collagen neosynthesis in OA and control menisci by incorporation of [^{3}H]proline into meniscal explants. Collagens are the primary components of menisci that account for more than 75% of their dry weight[25,26]. Thus, the amount of incorporated [^{3}H]proline should represent the synthetic activity of meniscal cells. In control menisci, incorporation rate of [^{3}H]proline was similar between lateral and medial menisci (Table II). In OA menisci, the incorporation was increased above the control levels. However, the level of increase was rather low compared with the marked increase of procollagen mRNA expression in OA menisci. Because of the limited level of increase and a large variation among the samples, the increase of proline incorporation in OA menisci did not reach the level of significance in either lateral or medial OA menisci.

Ultrastructural evaluation

In order to study the change of meniscal matrix in depth, collagen fibril ultrastructure was evaluated by transmission electron microscopy. This observation was performed on the meniscal tissues obtained from the middle regions of the anterior horn segments (Fig. 2).

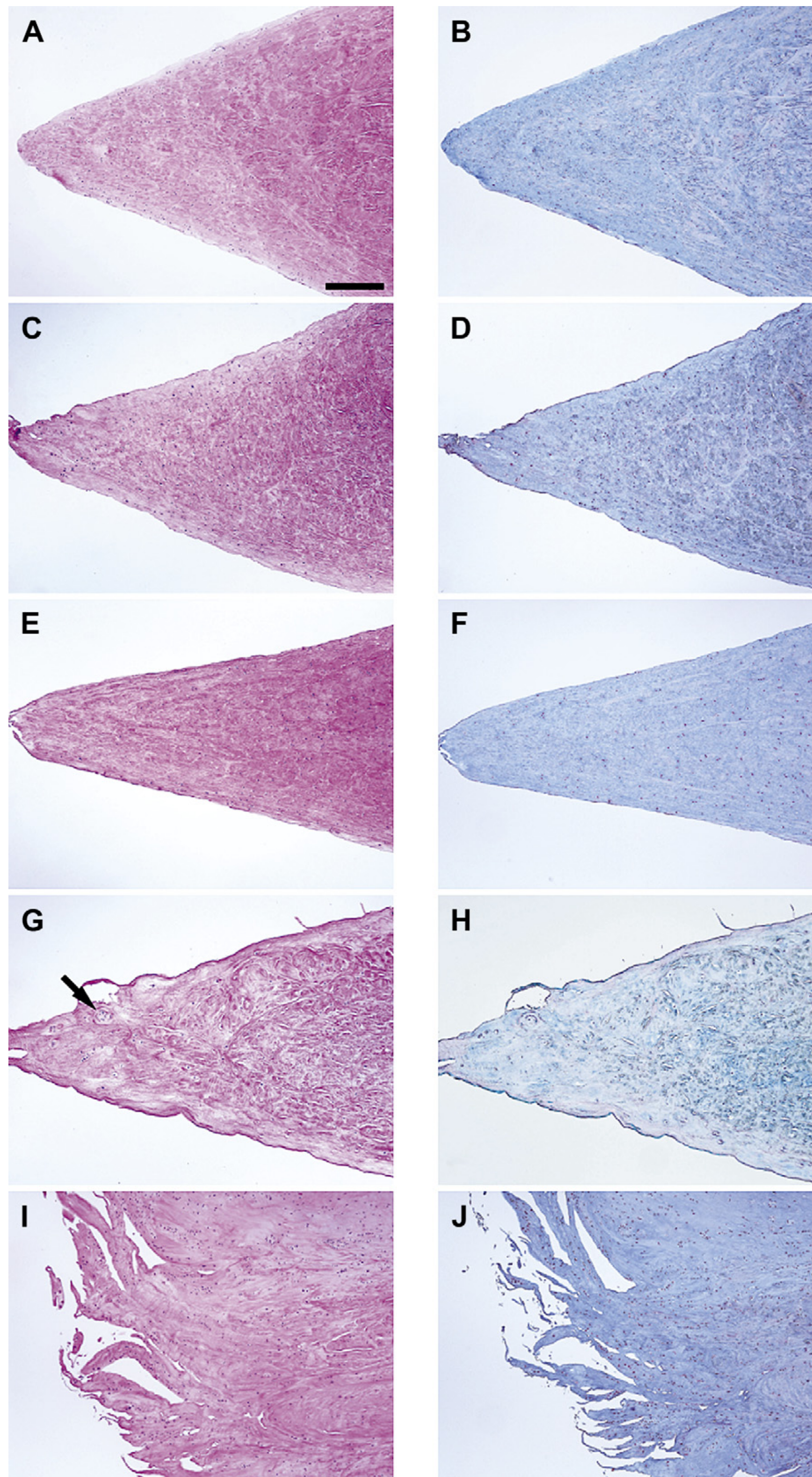

Fig. 3. Histology of OA and control menisci. Photomicrographs of sections from the anterior horn segments of control (A and B) and OA lateral menisci (C and D), and control (E and F) and OA medial menisci (G and H) are shown together with those from a degenerated area in the body segment of OA medial meniscus (I and J). In G, solid arrow indicates a cell cluster. Hematoxylin and eosin and Masson's trichrome stained sections are shown in left and right columns, respectively. Bar indicates 0.2 mm.

Consistent with previous reports[16,27], collagen fibrils in those regions were aligned in a circular orientation, and cross-sections of collagen fibrils were observed on the sections prepared perpendicular to the meniscus surfaces. Electron microscopy of control lateral menisci revealed that collagen fibrils of various diameters were densely packed in the matrix [Fig. 6(A)]. In OA lateral menisci, the change of collagen fibrils was rather modest, though fibrils of thinner diameters could have increased in

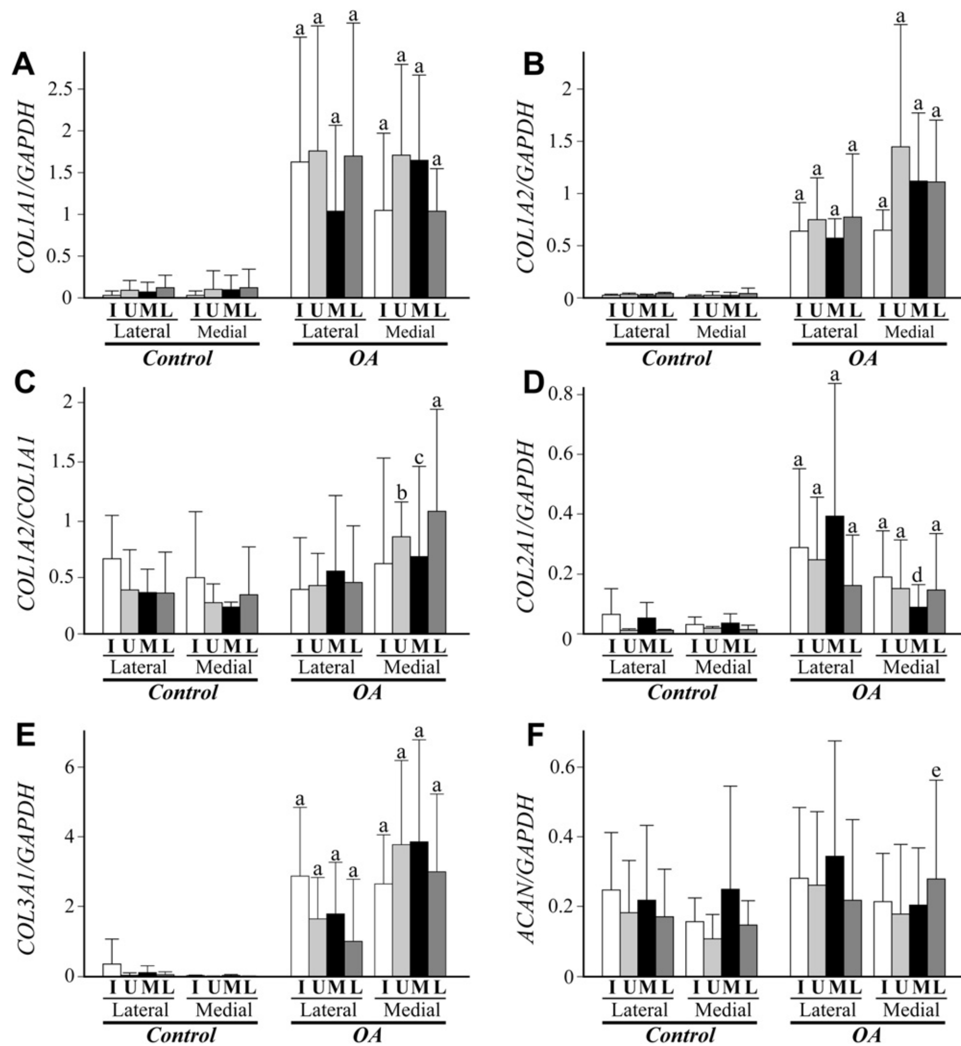

Fig. 4. Expression of matrix genes in OA and control menisci. Anterior horn segment of a meniscus was divided into four regions, and expression of matrix genes was evaluated in respective regions. Expression of type I (A and B), type II (D), type III (E) procollagen genes and aggrecan (F) is shown together with the expression ratio between two type I procollagen genes (C). In A, B, and D–F, results are shown by ratios against *GAPDH* expression. I, U, M, and L under bars indicate inner, upper surface, middle, and lower surface regions, respectively. Lateral and Medial indicate lateral and medial menisci, respectively. Results are shown by mean + 95% confidence interval. a, b, c, d and e indicate *P* values of <0.0001, 0.0092, 0.0021, 0.0086 and 0.0456, respectively, against corresponding region in control menisci.

number [Fig. 6(B)]. The change by OA was more obvious in medial menisci. Electron microscopy of the control medial menisci showed densely packed collagen fibrils with various diameters [Fig. 6(C)]. Although they resembled those of control lateral menisci, the fibrils tended to be more densely aligned. In OA medial menisci, the number of thinner fibrils increased obviously, and the fibrils were placed more sparsely [Fig. 6(D)]. The result of quantitative image analysis revealed that in OA medial menisci, the mean fibril diameter and percentage of the area occupied by fibrils were both significantly reduced, and the number of fibrils per area was significantly increased [Fig. 6(E)–(G)]. In lateral menisci, none of these parameters changed significantly by OA.

Biomechanical evaluation

Finally, the change in mechanical properties of meniscal matrix was evaluated by confined compression testing. Comparison of creep curves indicated that the mechanical properties could have changed by OA in medial menisci, while such changes might not be obvious in lateral menisci [Fig. 7(A)]. This was delineated by the changes in biomechanical parameters. Aggregate modulus of OA medial menisci was 40% lower than that of control medial menisci, whereas it differed little in lateral menisci [Fig. 7(B)]. In medial menisci, permeability changed more obviously. The permeability of OA medial menisci was more than 4-fold greater than that of the control medial menisci, while it changed little in lateral menisci [Fig. 7(C)]. Compared with these two parameters, the change of water content was modest. Although it tended to be higher in OA medial menisci, the increase was below the level of significance, and no significant change in this parameter was found between any two menisci [Fig. 7(D)].

Discussion

In this study, all OA menisci were obtained from OA knees with medial involvement, and the medial menisci were severely degenerated in the body segments. However, even in those menisci, the anterior horn segments were preserved well. This is why we performed most evaluations on the anterior horn segments, which made it possible to compare OA and control menisci in a site-to-site manner.

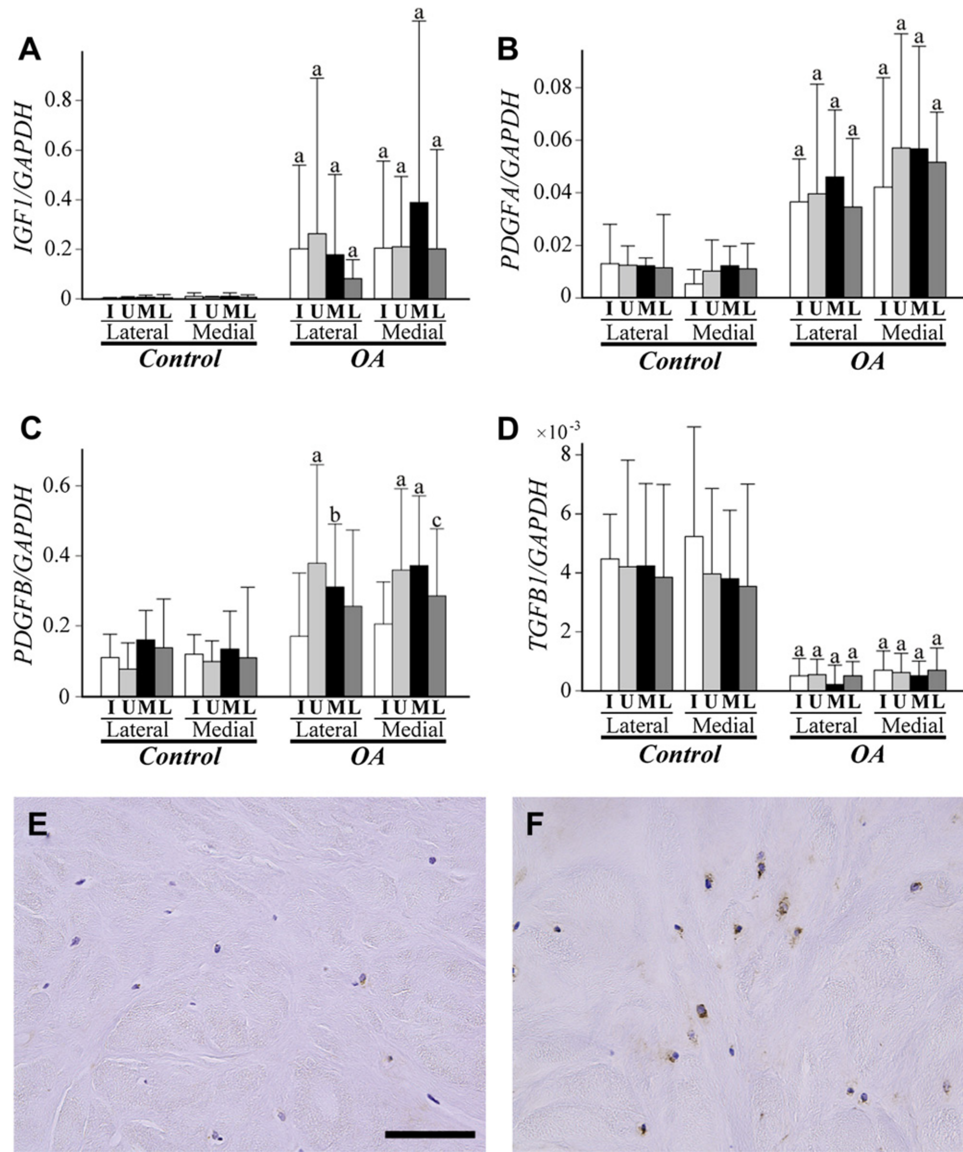

Fig. 5. Expression of possible anabolic factors in OA and control menisci. (A–D) Expression of *IGF1* (A), *PDGFA* (B), *PDGFB* (C) and *TGFB1* (D) in respective regions of OA and control menisci. Results are shown in the manner described for Fig. 4. a, b and c indicate *P* values of <0.0001, 0.0106 and 0.0028, respectively, against corresponding region in control menisci. (E and F) Immunohistochemistry for IGF-1. Photomicrographs of the sections from the middle regions of control (E) and OA menisci (F) are shown. Nuclei were stained with hematoxylin. Bar indicates 10 μm.

Consistent with the macroscopic appearance, the change in histology was moderate in the anterior horn segments of OA menisci. However, despite this limited change in histology, mRNA expression of major matrix components was markedly enhanced there. Among the five genes investigated, the increase in expression was most obvious with type I and type III procollagen, followed by type II procollagen, and least with aggrecan. This change in matrix gene expression could be the result of a reparative response that occurred within OA menisci. During the healing of fibrous tissues such as a ligament, tendon or skin, fibroblasts express type I and type III procollagens abundantly with the preponderance of type III procollagen[28,29]. We assume that this same mechanism could be involved in the enhancement of matrix gene expression in OA menisci. The result that the expression of type III procollagen tended to be more enhanced in the medial OA menisci than the lateral ones might suggest that such a reparative response could be more obvious in the medial compartment. This understanding seems reasonable since all OA knees studied here were medially involved in the disease.

Table II
[^{3}H]proline incorporation into meniscal explants

Meniscus		Scintillation count (cpm/μg DNA) (95% confidence interval)
Control	Lateral	3213 (0, 6963)
	Medial	3813 (1919, 5707)
OA	Lateral	7004 (0, 16,402)
	Medial	8345 (0, 20,875)

The result of qPCR analysis also revealed that the expression of IGF-1 was increased in OA menisci. Since IGF-1 has potent anabolic actions on meniscal cells[30–32], we consider that the increase in IGF-1 expression could be responsible for the enhanced matrix gene expression in OA menisci. In contrast to IGF-1, the expression of TGF-β1 was found to be reduced in OA menisci. This reduction in TGF-β1 expression could have a significant contribution to the degeneration of meniscal matrix. Besides the anabolic actions, TGF-β1

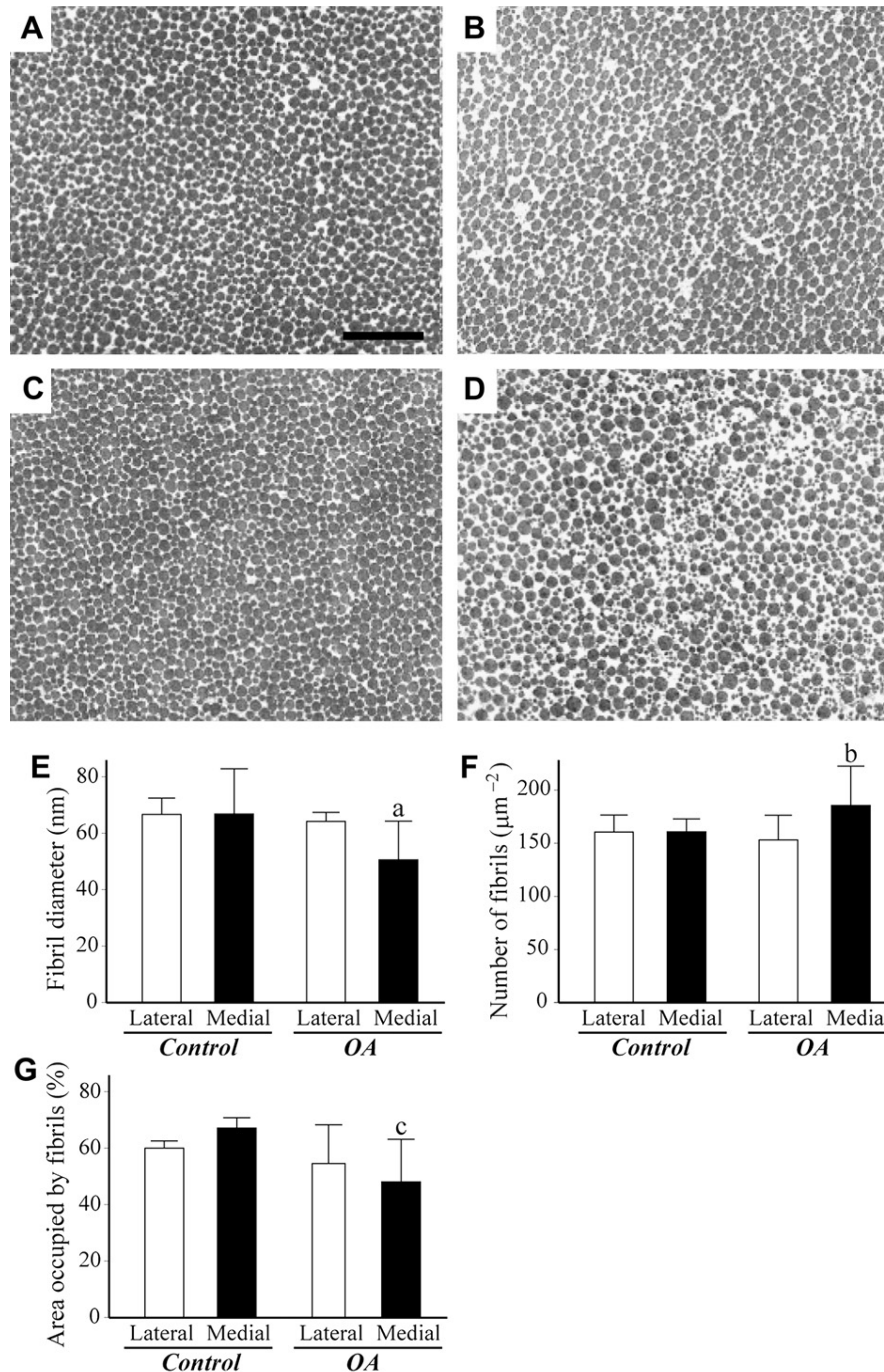

Fig. 6. Result of transmission electron microscopy. (A–D) Cross-sectional images of collagen fibrils. Electron microscopy was performed on tissues from the middle regions of control (A) and OA lateral menisci (B), and control (C) and OA medial menisci (D). Bar indicates 500 nm. (E–G) Results of quantitative image analysis. Collagen fibril diameter (E), number of fibrils per μm^2 (F), and percentage of the area occupied by fibrils (G) are shown. Lateral and Medial denote lateral and medial menisci, respectively. Results are shown by mean + 95% confidence interval. a, b and c indicate *P* values of 0.0027, 0.0423 and 0.0128, respectively, against control medial menisci.

also has a capacity to counteract the catabolic actions of proinflammatory cytokines on meniscal cells[33]. In fibroblasts and chondrocytes, TGF-β1 is known to suppress the expression of various proteinases that promote matrix degeneration, while enhancing the expression of endogenous inhibitors for those proteinases[34–37]. Therefore, the reduction of TGF-β1 expression could make the menisci more susceptible for degeneration. We assume that this might be one of the mechanisms for the loss of meniscal matrix in OA.

Interestingly, in human OA menisci, the high level of procollagen gene expression was not accompanied by a proportional increase in protein synthesis. Compared with the dramatic increase in procollagen gene expression, the increase of [^{3}H]proline incorporation was only modest. Although this seemed contradictory, the results of histological, ultrastructural, and biomechanical evaluations consistently indicated limited change of matrix in OA menisci. The reason(s) for this discrepancy is currently not known. Collagen synthesis is a complex process involving at least nine distinctive

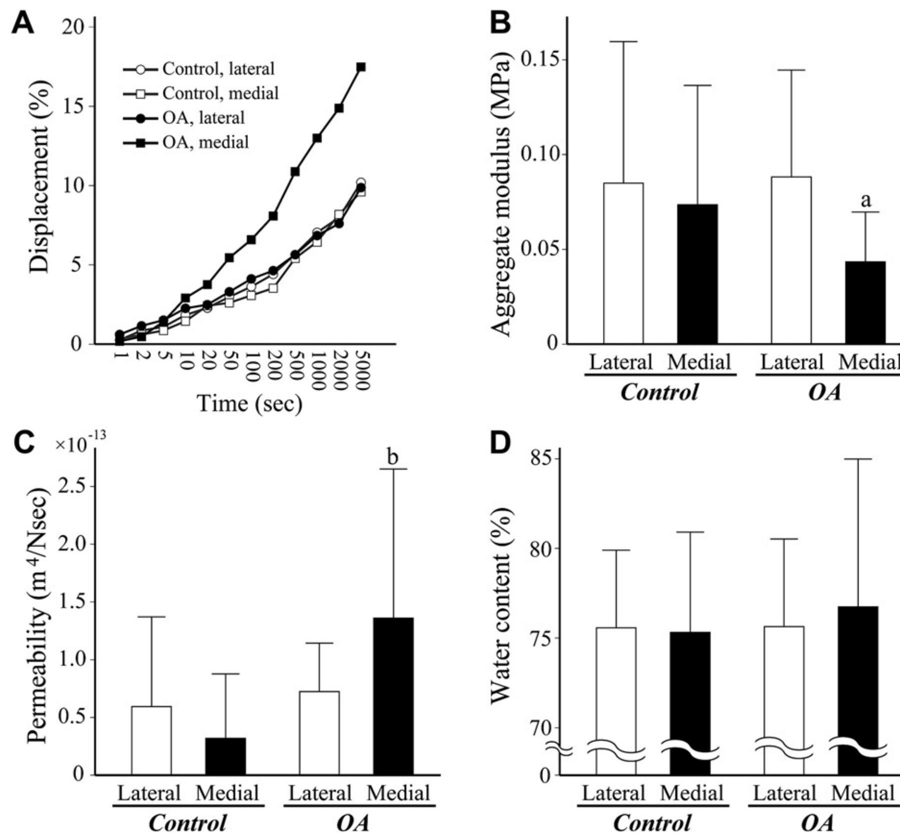

Fig. 7. Result of biomechanical measurement. Creep curve (A), aggregate modulus (B) and permeability (C) of OA and control menisci are shown together with the water content (D). In A, gray and black lines indicate the results of control and OA menisci, respectively, where circles and squares, either open or closed, denote those of lateral and medial menisci, respectively. Results are shown by mean (A) or mean + 95% confidence interval (B–D). a and b indicate P values of 0.0096 and <0.0001, respectively, against control medial menisci.

steps, each requiring a specific set of enzymes and chaperons[38,39]. Even after secretion, collagen molecules need to be properly processed and cross-linked outside the cells, which require another set of enzymes[40–42]. Considering these complexities, the synthetic process may well be significantly impaired at a certain step(s) in OA menisci. Future studies will clarify at which step(s), in fact, the process is impaired.

Although the change of menisci in OA has not been known well in human, several researchers have investigated it in animals. In an OA model in rabbits, cell density in the menisci was either increased or decreased depending on the regions, and cell clusters were frequently found in the degenerated areas[5,27]. However, these changes were not apparent in human OA menisci. Meanwhile, enhanced matrix gene expression was observed for both human and animal OA menisci[6,43], though the enhancement might be more intense and more comprehensive in humans.

Results of histological and molecular biological analyses revealed another difference between human and animal menisci. In animals, structural and functional heterogeneity within menisci has been reported repeatedly by many investigators: the inner areas are more like hyaline cartilage, while the outer areas have characteristics of fibrocartilage[8–11,17–19]. Differences between surface and central regions have been reported[4,17], and these were our rationale to evaluate gene expression in respective regions of menisci. However, such regional differences were not obvious in human menisci. Also, although reported with rabbit menisci[44], the difference in cell metabolism between medial and lateral menisci was not observed in human. These discrepancies between human and animal menisci should be kept in mind when meniscus pathology is studied in animal experiments.

The results of ultrastructural and biomechanical evaluations delineated the difference of matrix between lateral and medial OA menisci. This difference could be partly ascribed to the difference in type III procollagen expression. During fibril formation, type III procollagen is known to bind to type I collagen molecules and inhibit their lateral assembly[45]. The expression of type III procollagen was more promoted in OA medial menisci, and we assume that it could account for the appearance of thin collagen fibrils in those menisci. Of course, the change of meniscal matrix is not so simple, and other factors are likely involved in the process. For example, given that all OA medial menisci were severely degenerated in the body segment, the loss of structural integrity could be related to the change. Again, since catabolism is more promoted in the involved side of the knee, it might explain the change of matrix in the medial menisci. During the long course of disease progression, these factors could have worked together to alter the matrix in medial menisci.

Although we have identified several unique features of the changes in menisci with OA, this study has several limitations as well. First, since all menisci were obtained from aged donors, the results could be biased by age-related changes. We assume that this might explain, at least partly, the observed difference between human and animal menisci. If menisci from younger donors have been analyzed, that difference might not be so obvious as we observed in this study. Second, in this work, the analyses were performed mostly on the anterior horn segments of menisci, and the changes in the other parts of menisci were not investigated.

Thus, the difference among the three segments of menisci is not known, nor are the changes in the degenerated regions of OA menisci. Third, the analyses on OA menisci were done only on those from end-stage OA knees, and the menisci from early stage OA knees were not investigated. Fourth, although the involvement of IGF-1 has been suggested, the entire mechanism(s) for enhanced matrix gene expression in OA menisci has not been determined. Lastly, as stated earlier, the mechanism(s) for dissociation between enhanced matrix gene expression and modest increase in collagen synthesis has not been identified. Those points should be clarified by future studies.

As mentioned before, menisci are essential components of a knee joint. Clinically, it is well recognized that meniscus pathology is closely linked to the development and progression of knee OA[14,15,46]. Therefore, to understand the pathology of knee OA, it is essential to dissect the change of menisci in OA. Although many questions are left unanswered, we believe that the results of this study would provide some novel information on this issue.

Author contributions

Katsuragawa and Fukui had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analyses.

Conception and design. Katsuragawa and Fukui.

Analysis and interpretation of data. Katsuragawa, Furukawa, Yagishita, Mitomi and Fukui.

Drafting of the article. Katsuragawa and Fukui.

Critical revision of the article. Suzuki and Tohma.

Provision of study materials. Katsuragawa and Sawabe.

Technical support. Saitoh, Tanaka, Wake, Ikeda and Ishiyama.

Conflict of interest

The authors have no conflict of interest to disclose with regard to the subject matter of this present manuscript.

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