Osteoarthritis Predominantly in Patients with Fibulin-4 Is a Target of Autoimmunity

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Fibulin-4 Is a Target of Autoimmunity Predominantly in Patients with Osteoarthritis

Yang Xiang,* Taichi Sekine,** Hiroshi Nakamura,* Shinobu Imajoh-Ohmi,† Hiroyuki Fukuda,**§ Kazuo Yudoh,* Kayo Masuko-Hongo,* Kusuki Nishioka,‖ and Tomohiro Kato*†

Autoimmunity to chondrocyte-producing proteins has been reported in patients with osteoarthritis (OA) as well as in those with rheumatoid arthritis (RA). To answer whether or not OA-specific autoimmunity exist, we performed screening of chondrocyte-producing autoantigens by two-dimensional electrophoresis and Western blotting with each of 20 OA and 20 RA serum samples. We identified an apparently OA-specific autoantigen spot with a molecular mass of 52 kDa and a Isoelectric point of 4.1 as fibulin-4 by mass fingerprinting. By preparing recombinant proteins of fibulin-4, we determined prevalence of the autoantibodies to fibulin-4 in 92 patients with OA, 67 patients with RA, 40 patients with systemic lupus erythematosus, and 43 patients with systemic scleroderma. As a result, the IgG type anti-fibulin-4 autoantibodies were detected in 23.9% of sera from patients with OA, in 8.9% of sera from patients with RA, in 2.5% of sera from patients with systemic lupus erythematosus, and in 9.3% of sera from patients with systemic scleroderma. Furthermore, we immunized DBA/1J, ICR, BALB/c, and C57BL/6 mice with the recombinant fibulin-4 proteins to investigate arthritogenicity of fibulin-4. As a result, mild synovitis was detected in all of the four strains. In addition, we demonstrated expression of fibulin-4 in chondrocytes at both mRNA and protein levels in vivo and in vitro by RT-PCR, Western blotting, and immunohistochemistry. Taken together, fibulin-4, expressed in chondrocytes and recognized as an autoantigen mainly in OA rather than in RA, may play pathogenic roles in OA.

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Fibulin-4, also called EVEC, H411, or EFEMP, is a novel extracellular matrix (ECM) protein with 443 aa residues, belonging to the fibulin family that is characterized by a unique C-terminal fibulin-type module and several repeated epidermal growth factor-like domains. Fibulin-4 is expressed in heart, placenta, skeletal muscles, and lung (11, 12). The function of fibulin-4 is not completely understood. Fibulins are demonstrated in in vitro binding to fibrinogen, fibronectin, laminins, nidogen-1, aggrecan, and several other basement membrane proteins (13–15). Thus, fibulins likely participate in the assembly and stabilization of ECM structures; they also have been implicated in regulating organogenesis, vasculogenesis, hemostasis, fibrogenesis, and tumorigenesis (16–19).

We confirmed expression of fibulin-4 in articular cartilage tissue and cultured chondrocytes by RT-PCR and immunohistochemistry (IHC). The prevalence of the Abs against fibulin-4 was found in 23.9% of patients with OA, 8.9% of patients with RA, 2.5% of patients with systemic lupus erythematosus (SLE), and 9.3% of patients with systemic scleroderma (SSc) by ELISA. Furthermore, we immunized DBA/1J, ICR, BALB/c, and C57BL/6 mice with recombinant human fibulin-4 proteins to investigate arthritogenic
potential of the autoimmunity to fibulin-4. As a result, mild synovitis was found in all four strains. These results implicate involvement of autoimmunity to fibulin-4 in the pathogenesis of OA.

Materials and Methods

Patients and articulate cartilage tissues

Serum samples were obtained from 92 patients with OA (80 women, 12 men; ages 39–95 years; mean, 74.1 years) and 67 patients with RA (46 women, 8 men; ages 34–80 years; mean, 59.1 years) diagnosed according to their respective criteria for classification (20, 21), 40 patients with SLE (38 women, 2 men; ages 20–67 years; mean, 42.3 years), and 43 patients with SSc (41 women, 2 men; ages 29–82 years; mean, 56.1 years). Age- and sex-matched healthy serum samples were selected as a control for each group of OA and RA as follows: 43 women and 11 men (ages 42–89 years; mean, 72 years) for OA; 36 women and 10 men (ages 37–80 years; mean, 58.7 years) and 42 women and 3 men (ages 20–80 years; mean, 49.7 years) for SLE and SSc. Articular cartilage tissue samples were obtained from 8 OA patients (7 women and 1 man; ages 62–87 years; mean, 73.2 years) as well as from 4 femoral cartilage healthy people at total arthroplasty (2 women and 2 men; ages 23–87 years; mean, 69.6 years) as a control. The x-ray grading of joints in OA was evaluated by the criteria of Kellgren et al. (22). All samples were obtained with informed consent, and this study was approved by the local institutional ethics committee.

Chondrocyte culture and protein preparation

Chondrocytes were prepared as described previously (23). Briefly, cartilage of the femoral head from the 23-year-old man was minced into small pieces, and digested by rotating overnight at 37°C in DMEM (Invitrogen Life Technologies) containing 1 mg/ml bacterial collagenase (Sigma-Aldrich). The released chondrocytes were washed through a sterile nylon strainer (BD Biosciences), washed in DMEM three times, and seeded into disks coated with collagen I (BD Biosciences) with the cell number of 5 × 10^6/flask, and cultivated in DMEM containing 10% heat-inactivated FCS (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies) at 37°C in a humidified atmosphere (in ~3-wk alive) of 5% CO2. The medium was replaced every 3 days. Chondrocytes in the first three passages were used in this study.

The whole cell lysate was prepared from the cultured chondrocytes by the freeze-thaw method (24). The chondrocyte pellet, resuspended in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS), was frozen in liquid nitrogen and then thawed at 37°C for 5 min. After centrifugation at 4°C for 30 min, the supernatant was collected, and its protein concentration was determined using the Bradford method. The supernatant was stored in −80°C until use.

Two-dimensional electrophoresis (2DE) and Western blotting (WB)

The extracted protein samples were diluted in a rehydration buffer (8 M urea, 2% CHAPS, 2.8 mg/ml DTT, trace of bromophenol blue) containing 0.5% immobilized pH gradient (IPG) buffer (pH range, 3–10; Amersham Biosciences) and loaded onto 7-cm Immobiline Drystrips (Amersham Biosciences) at room temperature overnight, as described elsewhere (25). Up to 400 μg of the extracted proteins were applied onto the dry strip gels for WB and up to 1000 μg of the sample was loaded for 2DE. The IPG strips were equilibrated in the first equilibration solution (1.5 M Tris-Cl (pH9.5), 6 M urea, 30% glycerol, 2% SDS, and 2.5% iodoacetamide). The equilibrated strips were placed on top of 12.5% SDS-PAGE slab gels and sealed with 6 M urea, 30% glycerol, 2% SDS, and 2.5% iodoacetamide. The equilibrated strips were placed on top of 12.5% SDS-PAGE slab gels and sealed with 0.5% lower melt gel, and then the second-electrophoresis was performed with 40 mA constant current in separating gel at 20°C.

The extracted proteins were applied onto the drystrip gels for WB and up to 1000 μg of the sample was loaded for 2DE. The IPG strips were equilibrated in the first equilibration solution (1.5 M Tris-Cl (pH9.5), 6 M urea, 30% glycerol, 2% SDS, and 2.5% iodoacetamide). The equilibrated strips were placed on top of 12.5% SDS-PAGE slab gels and sealed with 0.5% lower melt gel, and then the second-electrophoresis was performed with 40 mA constant current in separating gel at 20°C. The equilibrated strips were placed on top of 12.5% SDS-PAGE slab gels and sealed with 0.5% lower melt gel, and then the second-electrophoresis was performed with 40 mA constant current in separating gel at 20°C. After CBB staining, protein spots corresponding to the OA-related Ags were visualized by Coomassie brilliant blue (CBB) or used for preparing the standard curve of fibulin-4 mRNA copies according to the manufacturer’s instruction.

Preparation of recombinant fusion proteins and Abs purification

The amplified cDNA fragments of fibulin-4 (447 bp), -4b (420 bp), and -4c (531 bp) were subcloned into a plasmid expression vector of pMAL-c2 (New England Biolabs). These constructs, recombinant fibulin-4 proteins were produced in E. coli (DH5α) (Toyobo) as a fusion protein with maltose binding protein (MBP) and a histidine tag (26). The recombinant proteins were purified from the bacterial cell lysate according to the histidine-N term affinity purification as described previously (26).

The autoantibodies to fibulin-4 were purified from the anti-fibulin-4-positive serum samples using the recombinant proteins transferred onto the nitrocellulose membrane after SDS-PAGE as described previously (31). The concentration of the purified autoantibodies was measured using a human IgG-ELISA quantitation kit (catalog no. E30-104; Bethyl Laboratories) according to its guidelines.

ELISA for detection of the autoantibodies to fibulin-4

ELISA was performed as described previously (26). Briefly, each well of a multiplateer (Nunc; Dynatech) was coated with 10 μg/ml of the individual purified fusion protein or MBP (as a background) in a carbonate buffer (50 mM sodium carbonate; pH 9.6), followed by blocking with PBS containing 1% BSA for 2 h at room temperature. To adsorb the reactivity of the serum samples to MBP, the serum samples were diluted and incubated with 40 μg/ml MBP in PBS containing 1% BSA for 2 h before incubation with the coated recombinants. In the screening of autoantibodies to three recombinant fibulin-4 fragments, serum samples diluted at 1/500 were used. After incubation with the recombinants for 2 h, the wells were washed 10 times in PBST. The bound Abs were reacted with the same secondary Abs as WB described above, and then developed with diaminobenzidine as a substrate, and quantified using a microplate photometer at 492 nm.

The reactivity of serum samples to the fibulin-4 fusion proteins was expressed using OD or arbitrary binding units, calculated according to

Protein identification

Chondrocyte proteins were newly separated with 2DE as described above. After CBB staining, protein spots corresponding to the OA-related Ags were recovered. In-gel trypsin digestion was performed as described previously with a small modification (27, 28). The recovered gel pieces were digested in 50 μl of digesting solution (0.1 M ammonium carbonate, 50% methanol) at 40°C for 15 min and then cut into small pieces. Proteins in the gel pieces were digested in 20 μl of trypsin (Wako Pure Chemical) solution (0.1 pmol/μl trypsin in 50 mM Tris-HCl) overnight at 37°C. The digested peptides were extracted from the gel pieces with 50 and 80% acetonitrile diluted with 0.1% trifluoroacetic acid using sonication and a centrifugation-evaporator. The peptide sample solution was dried at −20°C until MS analysis.

The mass of the digested peptides in the samples was determined using a mass spectrometer with MALDI-TOF (Voyager DE-STR; PerSeptive Biosystems). α-Cyano-4-hydroxycinnamic acid was used as an assisting matrix. A list of the determined peptide mass was conducted to the mass fingerprinting (MPF) using the Mascot software program (Matrix Science) (29), in which the National Center for Biotechnology Information (NCBI) protein databases were searched.

RT-PCR and real-time PCR

Total RNA was extracted from fresh chondrocytes separated from cartilage tissues with RNA Zol B (Friendswood) and chloroform according to the manufacturer’s instruction. The cDNA was reverse-transcribed from 20 μg of total RNA in a 20-μl reaction mixture containing 500 μM each of deoxynucleotide triphosphates, 0.4 U/μl RNase inhibitor, 0.2 μg/ml random hexamers, and 2.5 μl reverse-transcriptase (Invitrogen Life Technologies). The reaction mixture was incubated in a DNA thermal cycler at 42°C for 2.5 h then stored at −20°C before use. According to the nucleotide sequence of the human fibulin-4 cDNA (12), we prepared a pair of DNA primers for evaluating expression of fibulin-4 by RT-PCR and real-time PCR, and three pairs of primers to amplify three DNA fragments that overlappingly covered the entire protein coding regions of the fibulin-4 cDNA for preparation of recombinant proteins (Table I). PCR was performed with the sequence-specific primer pairs and Taq DNA polymerase (Takara) in a 25-μl PCR as described previously (30). Real-time PCR was performed using LightCycler and the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche) according to the manufacturer’s instruction. The Zero Blunt TOPO PCR cloning kit (Invitrogen Life Technologies) was used for preparing the standard curve of fibulin-4 mRNA copies according to the manufacturer’s instruction.
in each strain received injections with 50 μg of MBP in Freund's incomplete adjuvant with 1/1 dilution was injected s.c. into the right footpad of 10 mice in each strain. For control experiments, 10 mice with infection were further tested at various dilutions of 1/125-8000, and were tested with adsorption of 20–100 μg of fibulin-4 fusion proteins or MBP.

**Immunohistochemical analysis**

The rabbit IgG against human fibulin-4 was prepared by immunization of rabbit with the peptide of fibulin-4 (373-YNAFQIRAGNSQGDF-387) and purified with the same peptide. The cartilage tissue samples were frozen in OCT compound (Sakura) immediately after surgery, and 5-μm thick sections were prepared on glass slides (DakoCytomation). Specimens were fixed in 4% paraformaldehyde PBS for 10 min, then treated with 0.3% H2O2 in PBS for 10 min, and blocked in 1.5% goat serum PBS for 30 min. For IHC, the rabbit anti-fibulin-4 IgG was diluted to 1/100 in PBS with 0.1% Triton X-100 and were reacted to the sections for 30 min. To test the specificity of rabbit anti-fibulin-4 IgG, the diluted Ab was adsorbed with 100 μg/ml fibulin-4 fusion protein in 1 ml PBS with 0.1% Triton X-100 at room temperature for 2 h. PBS without first Ab was used as a negative control. After washing in PBS with 0.1% Triton X-100, the glass slides were reacted for 15 min with an HRP-conjugated goat anti-rabbit IgG (Histofine Simple Stain kit; Nichirei) and washed again. The bound Abs were reacted for 15 min with an HRP-conjugated goat anti-rabbit IgG (Histofine Simple Stain kit; Nichirei). Hematoxylin was used for counterstaining.

**Induction and histological evaluation of arthritis in immunized mice**

Female DBA/1J, ICR, BALB/c, and C57BL/6 mice were purchased from Charles River. Eight- to 10-week-old mice were used for immunization. A fusion protein mixture of fibulin-4a, -4b, and -4c (each 17 kDa) was injected intracutaneously into the root of tail as a second immunization. The rabbit IgG against human fibulin-4 was prepared by immunization of rabbit with the peptide of fibulin-4 (373-YNAFQIRAGNSQGDF-387) and purified with the same peptide. The cartilage tissue samples were frozen in OCT compound (Sakura) immediately after surgery, and 5-μm thick sections were prepared on glass slides (DakoCytomation). Specimens were fixed in 4% paraformaldehyde PBS for 10 min, then treated with 0.3% H2O2 in PBS for 10 min, and blocked in 1.5% goat serum PBS for 30 min. For IHC, the rabbit anti-fibulin-4 IgG was diluted to 1/100 in PBS with 0.1% Triton X-100 and were reacted to the sections for 30 min. To test the specificity of rabbit anti-fibulin-4 IgG, the diluted Ab was adsorbed with 100 μg/ml fibulin-4 fusion protein in 1 ml PBS with 0.1% Triton X-100 at room temperature for 2 h. PBS without first Ab was used as a negative control. After washing in PBS with 0.1% Triton X-100, the glass slides were reacted for 15 min with an HRP-conjugated goat anti-rabbit IgG (Histofine Simple Stain kit; Nichirei) and washed again. The bound Abs were visualized with DAB (Histofine Simple-Staining kit; Nichirei). Hematoxylin was used for counterstaining.

**Results**

**Fibulin-4 is identified as a chondrocyte-produced autoantigen**

Chondrocyte proteins separated by 2DE were transferred onto membranes, which were used for surveillance of autoantibodies/autoantigens by WB using 60 serum samples from 20 patients with OA, 20 patients with RA, and 20 healthy donors. As representative results of WB show in Fig. 1, the protein spot 36, with molecular mass of ~54 kDa and a Isoelectric point value of ~4.1, was recognized as an autoantigen in 15% (3 of 20) of patients with OA and 5% (1 of 20) of patients with RA, but not in the healthy control. To characterize protein spot 36, we collected the gel spot from the 2DE gel, digested it with trypsin, and then determined the mass of the digested protein by MALDI-TOF MS. By MFP using the Mascot program and NCBI protein databases, a candidate for protein spot 36 was found to be an ECM protein, fibulin-4, as shown in Table II.

**Analysis of the antigenicity of fibulin-4 using recombinant proteins**

Next, we confirmed antigenicity of fibulin-4 by preparing recombinant fibulin-4 proteins as a fusion protein with MBP. We prepared three truncated fibulin-4 fusion proteins (Fib-4a, -4b, and -4c) that overlappingly covered the entire amino acid residues of fibulin-4 (Fig. 2, a and b). Results of the analysis of the purified fusion proteins by SDS-PAGE are shown in Fig. 2c. To confirm the antigenicity of fibulin-4, we separated each fragment of the purified fibulin-4 fusion proteins as well as MBP alone as a negative control by SDS-PAGE, and then tested their reactivity to the carilage degradation in more than one region; grade 2, localized deep cartilage degradation; grade 3, extensive deep cartilage degradation at several locations. Pannus– grade 0, no changes; grade 1, pannus formation at up to two sites; grade 2, pannus formation at up to four sites, with infiltration or flat overgrowth of joint surface; grade 3, pannus formation at more than four sites or extensive pannus formation at two sites.

**Statistical analysis**

Differences of prevalence of the anti-fibulin-4 Abs among the groups of OA, RA, SLE, SSC, and healthy controls were compared by using the χ2 test. The differences of mean binding units among the groups of OA, RA, and healthy controls and the differences of the mean x-ray grades and ages between the anti-fibulin-4-negative and -positive patients with OA, as well as the differences of mean arthritic scores between fibulin-4-immunized mice and MBP control ones were compared by the Student’s t test. The relationship of arthritic scores and titers of anti-fibulin-4 IgG in fibulin-4-immunized mice were analyzed with linear correlation analysis. p values <0.05 were considered significant.

**Table I. Primers specifications for RT-PCR and real-time PCR**

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<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>AT (°C)</th>
<th>Size (bp) (start-end)</th>
<th>Cycles</th>
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<tr>
<td>Fibulin-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sense</td>
<td>GCCCAAACTGTGTTCA</td>
<td>60</td>
<td>892–1180</td>
<td>35</td>
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<td>TGATTGGCTAAGTTAAGACCAGCCAGCAGTT</td>
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<td></td>
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<tr>
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<tr>
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<td>799–1329</td>
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<td>113–346</td>
<td>35</td>
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<tr>
<td>Antisense</td>
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* ttggatcc, etc., The sites of restriction endonucleases. AT, annealing temperature.
The matched amino acid sequences of human fibulin-4.

<table>
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<tr>
<th>Start-End</th>
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<th>Sequence of Fibulin-4</th>
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<td>157–169</td>
<td>1474.78</td>
<td>KIGPECVDIDECR</td>
<td>MLPCASCALPG SLLLWALLL LLLGSASQPDQS EEDPSYTRECT DGYEWDPDQS</td>
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<td>HCRDWNELCT IPEACKGEMK CINHYGCYL LFRSAAVINT LHERGSPPPV</td>
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<td>2195.35</td>
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<td>1199.61</td>
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<td>EQPSSIVHR</td>
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prevalence between OA patients and healthy controls, as well as between OA and RA, SLE, or SSc patients, were of statistical significance \( \chi^2 = 10.86, 5.93, 8.84, \) and \( 4.02, \) respectively; \( p < 0.05, \) but not between healthy controls and RA, SLE, or SSc patients \( (p > 0.05, \) respectively). The frequency of the anti-fibulin-4 autoantibodies in OA was found to be \( 2.5 \)-fold higher than that in RA. This result was in accordance with the result from the first screening, showing that spot 36 was recognized by 15% of 20 OA serum samples and by 5% of the 20 RA serum samples. In addition, the anti-fibulin-4 autoantibodies mainly reacted to the fragments of fibulin-4b \( (13 \text{ of } 92) \) and -4c fusion proteins \( (14 \text{ of } 92) \). Compared with the results of RA \( (1 \text{ of } 67) \) and SSc \( (0 \text{ of } 43) \), the Abs to fibulin-4b seem more specific in OA \( (\chi^2 = 4.317 \text{ and } 4.807, \) respectively; \( p < 0.05) \). Of 22 positive serum samples, six reacted to both fibulin-4b and -4c, and one reacted to all three fragments. The recognition of multiple autoepitopes would reflect the Ag-driven immune mechanism for the autoantibody production to fibulin-4.

As a series of study to screen the autoantigens in OA and RA \( (10) \), we found that 38 \( (41.3\%) \) of 92 patients tested with OA were positive for Abs to fibulin-4 or TPI. Seven \( (7.6\%) \) of 92 patients were positive for both anti-fibulin-4 and anti-TPI. Statistically, we
did not recognize a relationship between these two Abs \((p = 0.38\), Fisher’s exact probability test).

**Fibulin-4 induced synovitis/arthritis in mice**

As described above, we demonstrated that fibulin-4 was expressed in chondrocytes and a target of autoantibodies predominantly in OA. In the next step, we investigated whether fibulin-4 was involved in the pathogenesis of OA. Specifically, we tested an arthritogenic potential of fibulin-4 by immunizing four strains of mice (DBA/1J, ICR, C57BL/6, and BALB/c) with a mixture of the fibulin-4a, -4b, and -4c fusion proteins. Because human and mouse fibulin-4 have 88% homology in the amino acid sequence (35), we expected that immunization of human fibulin-4 elicit autoimmunity to mouse fibulin-4. All of the mice in each of the tested strains started to develop mild joint redness and swelling with polyarthritis after the booster immunization, as shown in Fig. 7a. The arthritis scores in the fibulin-4-immunized mice were higher than that in control mice, with statistical significance \((p < 0.05\), respectively) in all four strains. To further evaluate the relationship between the anti-fibulin immunity and the severity of arthritis in fibulin-4-immunized mice, we compared the anti-fibulin-4 titers with average arthritic scores in the fibulin-4-immunized mice with linear correlation analysis. As shown in Fig. 7b, there was a moderate correlation between anti-fibulin-4 titers and arthritic scores \((r = 0.63; p < 0.01\)). Histological examination of the affected ankle joints of all mice was performed 56 days after the booster immunization. Compared with the MBP-immunized mice as a negative control, the fibulin-4-immunized mice showed infiltration of mononuclear cells into the synovial tissue and proliferation of synovial lining layer cells. This synovitis was observed in noninjected as well as injected limbs (Fig. 8). The histological arthritis scores in the fibulin-4-immunized mice were greater than in control mice \((p < 0.05\), respectively) (Fig. 7c). We did not find apparent cartilage damage in fibulin-4-immunized mice, in 56 days of observations. These findings suggest that the autoimmunity to fibulin-4 would not develop severe destructive arthritis like RA but rather develop mild synovitis as observed in OA.

**Comparison of clinical parameters between the anti-fibulin-4 IgG-positive and -negative patients**

Finally, we investigated clinical parameters between anti-fibulin-4-positive and -negative patients with OA. The age, sex, and x-ray grades of osteoarthritic joints were compared. The mean age of the anti-fibulin-4-positive OA patients was slightly older than that of the negative ones (mean age 76.7 ± 9.5 years in the positive OA patients, 73 ± 9.3 years in the negative OA patients), but the difference did not meet a statistical significance \((p > 0.05\). The differences of the ratio of female:male (10:1 in the positive and 6:1 in the negative), and the differences of the mean x-ray grades between the positive and negative patients with OA (2.1 ± 1.26 in the positive and 1.8 ± 0.85 in the negative) were of no statistical significance \((p > 0.05\), respectively).

**Discussion**

In this study, we showed that anti-fibulin-4 IgG is predominantly detected in OA patients. It is interesting that the frequency of autoantibodies to fibulin-4 is much higher in patients with OA than in patients with RA and other rheumatic diseases. Although OA
FIGURE 6. Detection of the autoantibodies to fibulin-4 by ELISA. a, The autoantibodies to fibulin-4a, -4b, and -4c in serum samples from patients with OA or healthy control (HC)-matched sex and ages were detected by ELISA. The binding units of 100 were defined as a cutoff point.

b, The autoantibodies to fibulin-4a, -4b, and -4c in serum samples from patients with RA and healthy control-matched sex and ages were detected by ELISA. The binding units of 100 were defined as a cutoff point.

c, The autoantibodies to fibulin-4a, -4b, and -4c in serum samples from patients with SLE, SSc, as well as healthy controls were detected by ELISA. The binding units of 100 were defined as a cutoff point.

has been considered generally as a nonimmunological, degenerative disease, our result indicated that fibulin-4 was an autoantigen predominantly in OA patients like TPI investigated in our previous work (10). However, in contrast to TPI expressed ubiquitously in every cell, fibulin-4 is a component of ECM produced by chondrocytes and likely a component of articular cartilage as suggested in this study. Also, fibulin-4 was proved to be expressed in fibroblast-like synoviocytes at both mRNA and protein levels (data not shown). Thus, the autoimmunity to fiblin-4 may have direct effects on cartilage/chondrocytes or cause synovitis.

The expression and physiological roles of the fibulin family molecules (fibulin-1–6) have been reported (36–39), but those in articular cartilage tissue have not been fully understood. So far, fibulin-1 and fibulin-2 were reported to be expressed in developing articular cartilage. In particular, fibulin-2 is considered to be a specific marker for the early stages of cartilage development and bone calcification. However, the expression levels of fibulin-1 and fibulin-2 in adult cartilage were low (16, 40). In contrast to the fibulin-1 and -2, no report was found available on the expression of fibulin-4 in the articular chondrocytes. In other aspects, fibulin-4 is reported to be produced by fibroblasts and located in basement membrane zones and vessel walls in heart valve/aorta, artery/vein, skeletal muscle, lung, and kidney (12). Considering that the ligands of fibulin-1 and/or -2 include components of cartilage tissue such as fibronectin, aggrecan, and perlecan (13–15), fibulin-4 may bind to these ligands and thus play a role in maintaining normal structure of cartilage tissue..Reportedly, the bacterial LPS induces expression of fibulin-4 in fibroblasts, and cells overexpressing fibulin-4 show a higher growth rate (41), and fibulin-4 is up-regulated in vascular smooth muscle cells in two independent rodent models of vascular injury (19). Therefore, fibulin-4 may participate in the repair process in inflammatory conditions.

To our knowledge, no report was available on the expression of fibulin-4 in articular chondrocytes; therefore, we investigated expression of fibulin-4 in chondrocytes as well as its antigenicity and pathological roles. We demonstrated the expression of fibulin-4 in chondrocytes at both gene and protein levels in vivo and in vitro. Interestingly, the expression level of fibulin-4 mRNA in OA chondrocytes was higher than in apparently normal chondrocytes obtained from bone fracture patients. Speculating from the report that LPS induces expression of fibulin-4 in fibroblasts, initial inflammatory stimuli unknown so far may increase expression of fibulin-4 in OA. Alternatively, chondrocytes may produce such increased levels of fibulin-4 to repair damaged cartilage matrix in the process of OA. The increased expression of fibulin-4 may be linked to generation of autoimmunity to fibulin-4 in the OA patients. The antisera to fibulin-4 reacted to most of the chondrocytes in cartilage in the IHC; however, that did not appear to stain the ECM of cartilage, even though fibulin-4 was expected to be a component of cartilage. One reason might be that the amount of fibulin-4 is very low in the ECM of cartilage, so that it is difficult to be detected. Another reason might be that collagen II and/or proteoglycan, the dominant and huge components of cartilage, prevent the anti-fibulin-4 Abs from accessing fibulin-4 in cartilage tissue. In the latter case, fibulin-4 would not be exposed to the immune system in the intact cartilage, but would be exposed during the destruction of cartilage, which may lead to autoimmunity to fibulin-4 in the OA development. In both ways, we need to clarify whether or how much the cartilage contains fibulin-4 as a component of cartilage matrix.

Human fibulin-4 is a highly conservative protein that shares 88% homology with mouse fibulin-4 (35). We immunized four strains of mice with human fibulin-4 to investigate arthritogenicity of fibulin-4. All of the four mouse strains developed mild chronic polyarthritis after the second immunization of fibulin-4. Histological examination of the affected ankle joints of fibulin-4-immunized mice showed inflammatory changes in synovial tissue. Elevated anti-fibulin-4 IgG titers were observed, and a moderate correlation between anti-fibulin-4 titers and arthritic scores was observed in fibulin-4-immunized mice. However, no obvious destruction of bone or cartilage was detected as far as we observed for up to 2 mo. Thus, the autoimmunity to fibulin-4 would be linked to mild chronic synovitis as commonly observed in OA but not linked to destructive arthritis as observed in RA or collagen-induced arthritis in mice or rats. Fibulin-4 may be one of the autoantigens that drive chronic synovitis in OA. It remains to be solved whether long-lasting autoimmunity to fibulin-4 leads to damage of cartilage tissue.

In addition, in this study we detected IgG types of the autoantibodies using three fragments of fibulin-4 fusion proteins. Over 30% of positive serum samples recognized more than one epitope of fibulin-4. This recognition of the multiple...
epitopes would not be produced by cross-reaction, but rather likely produced by the Ag-driven mechanism, that is, B cells specific for each of the epitopes were activated by Ag-specific T cell help. However, in this study we only prepared three overlappingly fragments of fibulin-4, the precise epitope mapping on fibulin-4 should be further performed.

Clinically, the differences of age, sex, and x-ray grades of osteoarthritic joints between the anti-fibulin-4-positive and -negative patient groups in OA did not meet statistical significance. Further studies using more numbers of patients would be needed to elucidate correlation of this autoantibody with clinical parameters.

In our study, we detected anti-TPI IgG in 24.7% of the same group of OA patients (10). Combined with anti-fibulin-4 IgG, the total positive rate reached 41.3%, 7.6% were both anti-TPI and anti-fibulin-4 positive. No statistical relationship was found between the anti-fibulin-4 and anti-TPI IgG in OA patients. A combined detection of these two autoantibodies in patients with OA may be helpful in the diagnosis and choice of therapy of OA.

FIGURE 7. Induction of arthritis in fibulin-4-immunized mice. a, Average arthritis scores of fibulin-4/MBP induced arthritis in ICR, DAB/1J, C57BL/6, and BALB/c mice. Fib-4, Mice (n = 10 in each group) were immunized with 50 μg of a mixture of fibulin-4a, -4b, and -4c fusion proteins. MBP: mice (n = 10 in each group) were immunized with 50 μg of MBP only. *, p < 0.05. b, Correlation analysis of mouse arthritis scores and the anti-fibulin-4 titers. The arthritis score and the titer of anti-fibulin-4 in each of the fibulin-4-immunized mice were analyzed. OD: the titer of anti-fibulin-4 IgG in fibulin-4-immunized mice. r, Correlation coefficient. *, p < 0.05. c, Average histological scores of fibulin-4/MBP induced arthritis in ICR, DAB/1J, C57BL/6, and BALB/c mice. The differences of histological arthritis scores between fibulin-4-immunized mice (Fib-4) and MBP-controls (MBP) are compared. *, p < 0.05.

FIGURE 8. Histological findings in the fibulin-4/MBP-immunized mice. Both of the injected (right) and noninjected (left) hind ankles were used in histological evaluation by H&E staining. AC, Articular cartilage; ST, synovial tissue; Jcav, joint cavity.
In summary, we demonstrated that fibulin-4 was expressed in articular chondrocytes in vivo and in vitro and was a target of autoimmunity mainly in OA rather than in RA. These results, together with the fact that immunization of fibulin-4 induced arthritis in mice, indicate that autoimmunity to fibulin-4 may play pathogenic roles in the development of OA.

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Disclosures
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