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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 Kato²*

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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3 Abbreviations used in this paper: OA, osteoarthritis; RA, rheumatoid arthritis; TPI, triosephosphate isomerase; ECM, extracellular matrix; IHC, immunohistochemistry; SLE, systemic lupus erythematosus; SSC, systemic scleroderma; 2DE, two-dimensional electrophoresis; WB, Western blotting; IFL, immobilized pH gradient; MS, mass spectrometry; CBB, Coomassie brilliant blue; DAB, diaminobenzidine; MFP, mass fingerprinting; α-CHCA, α-cyano-4-hydroxycinnamic acid; MBP, maltose binding protein.

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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 articular 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; 38 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 filtered through a sterile nylon strainer (BD Biosciences), washed in DMEM three times, and seeded into 6-well plates. The released chondrocytes were cultured in DMEM (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen Life Technologies) at 37°C in a humidified atmosphere (in ~3% CO2) in a 1% O2 atmosphere. 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 times. 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 at −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 drystrip gels for WB and up to 1000 ng of the proteins were applied onto the IPG strips for 2DE. The IPG strips were equilibrated in the first equilibration solution (1.5 M Tris-Cl (pH8.8), 6 M urea, 30% glycerol, 2% SDS, and 1% DTT) and then in the second equilibration solution (1.5 M Tris-Cl (pH8.8), 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 IPG strips were then transferred to nitrocellulose membranes (Protran; Schleicher & Schuell Microscience) after the electrophoresis. In the WB, the membranes were blocked in PBS containing 1% BSA and 0.1% Tween 20 for 1 h, washed in PBS with 0.1% Tween 20 (PBST) for 30 min, and then incubated with serum samples diluted adequately in PBST containing 1% BSA for 1 h. After washing five times in PBST, the bound Abs were reacted with HRP-conjugated goat anti-human IgG (Zymed Laboratories) for 1 h. Finally, the bound Abs were visualized by diaminobenzidine (DAB).

Protein identification

Chondrocyte proteins were newly separated with 2DE as described above. After CBB staining, protein spots corresponding to the OA-related Abs were recovered. In-gel trypsin digestion was performed as described previously with a small modification (27, 28). The recovered gel piece was digested in 50 µl of digesting solution (0.1 M ammonium hydroxide 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 centrifugal 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). e- Cyanogen hydroxyacrylamidoc 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 µM primer, 0.2 µg/µl random hexamer, RNase inhibitor, 0.2 µg/ml RNaseOUT (Invitrogen Life Technologies), and 2.5 units of reverse-transcriptase (Invitrogen Life Technologies). The reaction mixture was incubated in a DNA thermal cycler at 42°C for 2 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 expression evaluation 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.

Preparation of recombinant fusion proteins and Ab purification

The amplified cDNA fragments of fibulin-4a (447 bp), -b (420 bp), and -c (531 bp) were subcloned into a plasmid expression vector (Table I). The cDNA was inserted into pMAL-c2 (New England Biolabs). Using these constructs, recombinant fibulin-4 proteins were produced in Escherichia 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-Naffinity 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 purified autoantibodies were measured using a human IgG-ELISA quantitation kit (catalog no. E90-104; 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 multimeter plate (Cook; 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 PBST 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 preincubated with 40 µg/ml MBP in PBST 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 the
formula, sample (binding units) = (OD sample/mean OD normal sera + 3 SD of normal sera) × 100. To obtain the OD of each serum sample, the OD value for the partner MBP was subtracted from the OD value for the fusion protein. According to this formula, 100-binding unit was used as a cutoff point. Serum samples detected positively by this screening method were further tested at various dilutions of 1/125-8000, and were assayed 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 purification 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 1/50 in PBS with 0.1% Triton X-100 and were reacted to the sections for 30 min. To test the antigenicity of fibulin-4, we separated each fragment of the fusion proteins by SDS-PAGE are shown in Fig. 2. To confirm cartilage 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.

### 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 recovered 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

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

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'-3')</th>
<th>AT (°C)</th>
<th>Size (bp) (start-end)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibulin-4</td>
<td>Sense</td>
<td>GCCAAACCTGTTGCTCA</td>
<td>60</td>
<td>892–1180</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TGTATGGCCTAAGTATGAGG</td>
<td>60</td>
<td>1–447</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>tttggtaccc-ATGCTCCCCCTGCGCTTTGGCTCCTGCTAa</td>
<td>60</td>
<td>242–843</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>tttggtaccc-GCAGAGGCGTCGTGGCCAGACGCTG</td>
<td>50</td>
<td>799–1329</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>tttggtaccc-TGCGACTGCCCCAGGTTACCCCG</td>
<td>60</td>
<td>113–346</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GCCGAGCTGCTGT</td>
<td>60</td>
<td>60 113–346</td>
</tr>
</tbody>
</table>

a tttggtaccc, etc., The sites of restriction endonucleases. AT, annealing temperature.
Table II. Result of MFP: the matched peptides of fibulin-4

<table>
<thead>
<tr>
<th>Start-End</th>
<th>Mass</th>
<th>Sequence of Matched Peptide</th>
<th>Sequence of Fibulin-4*</th>
</tr>
</thead>
<tbody>
<tr>
<td>157–169</td>
<td>1474.78</td>
<td>KIGPECVDIDECLR</td>
<td>MLPCASCLPG SLLLWALLLL LLGSASQDQS EPEPSYTREC DGYEWDPSQS</td>
</tr>
<tr>
<td>177–199</td>
<td>2705.34</td>
<td>CVNLPGSFCRCQCEPG</td>
<td>HCRDVENCTL IPAECKGEMK CIHYGYLLC LPRSAAVIND LHEGGFPPPPV</td>
</tr>
<tr>
<td>228–236</td>
<td>1199.61</td>
<td>CHQGQVHNLHR</td>
<td>PFAQHNPFCPPG YEFDQDSCDVCDEQCAHLDRCPFPDQCDNLQGYSYCT</td>
</tr>
<tr>
<td>258–279</td>
<td>2478.24</td>
<td>CVNEPFCRFSCHCPQG</td>
<td>CPDQYRKIGP BCEVIIDECVC CVQGHHCVNL PGPSFRQCQCE GPQLPNNR</td>
</tr>
<tr>
<td>280–308</td>
<td>3312.58</td>
<td>LQCD1DECESGAHQGC</td>
<td>SYLQYRCVNL EPGFSCHCP QGYYLTLATR CODIDECESG AHQCSAESQTC</td>
</tr>
<tr>
<td>338–346</td>
<td>1051.58</td>
<td>EIQPSSIVHR</td>
<td>VNPHGSGYRCV DTNRCVEPYI QSNERCICL APNLPCEQRP SSVHRVMTI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSERKFPADV FQIQATSVYQ GAIYNQFQRA GNSQGDFYIR QINNVSAMLV</td>
</tr>
</tbody>
</table>


FIGURE 1. 2DE and subsequent WB of chondrocyte-derived proteins using serum samples from patients with OA or RA. Proteins extracted from human chondrocytes were separated by 2DE and then stained with CBB (a), or transferred onto nitrocellulose membranes and reacted with serum samples diluted at 1/500 from 20 patients with OA, 20 patients with RA, and 20 healthy donors. Three OA and one RA serum samples reacted to protein spot 36. Representative results from spot 36-reactive serum samples in OA (b) and RA (c) and from healthy controls (d) are shown.

Four serum samples that reacted to protein spot 36 by WB in the first screening. As a result, all four serum samples reacted positively to two or three fragments of the three fibulin-4 fusions but not to MBP alone (Fig. 3a). To confirm this in a different way, we purified the autoantibodies to fibulin-4 MBP from the positive serum samples and then stained chondrocyte proteins separated by 2DE and transferred onto membranes. As a representative result shows in Fig. 3b, only protein spot 36 was stained (right), whereas the whole serum sample before purification stained several spots including protein spot 36 (left). Furthermore, to confirm specific binding of the autoantibodies to fibulin-4, we measured anti-fibulin-4 MBP titers in series of variously diluted serum samples by ELISA. The anti-fibulin-4 MBP titers increased in a serum concentration-dependent manner, but anti-MBP titers did not (Fig. 3c-i). Moreover, the serum reactivity to fibulin-4 MBP was cancelled by addition of fibulin-4 MBP, but not by MBP alone (Fig. 3, c-II–IV). Taking these data together, autoantibodies specific for fibulin-4 were produced in these patients.

**Fibulin-4 is expressed in chondrocytes in vivo**

Although fibulin-4 was reported to be expressed in several organs like heart and skeletal muscles, it is not known whether chondrocytes produce fibulin-4. From the above data, fibulin-4 was evidenced to be produced at least in the cultured chondrocytes. To test whether fibulin-4 was produced by chondrocytes in vivo, we first performed RT-PCR to amplify the fibulin-4 cDNA reverse-transcribed from the total RNA extracted from chondrocytes, where we used fresh chondrocytes to exclude effects of in vitro culture. As a result, the chondrocytes from either OA cartilage tissue or control cartilage tissue derived from patients with bone fracture expressed mRNA of fibulin-4 as shown in Fig. 4a. Next, we found that the expression level of fibulin-4 mRNA in OA chondrocytes was much higher than that in control chondrocytes (obtained from patients with bone fracture) by quantitative analysis of real-time PCR (p < 0.05) (Fig. 4b). These results confirmed the expression of fibulin-4 at the mRNA level. Furthermore, we tried to identify fibulin-4 at the protein level in both chondrocytes and cartilage tissue. For this purpose, we used a newly prepared rabbit IgG against human fibulin-4-specific peptide with 15 aa residues in domain III of fibulin-4. IHC revealed that fibulin-4 was produced in most of the chondrocytes (Fig. 5). The fact that these positive stainings were canceled by adding fibulin-4c indicated a specific reaction of rabbit anti-fibulin-4 IgG in cartilage chondrocytes (Fig. 5). In 2DE WB using proteins extracted from fresh chondrocytes, the rabbit anti-fibulin-4 IgG reacted to spot 36 similarly as shown in Fig. 3b (data not shown). Taking these data together, we evidenced that fibulin-4 was produced in chondrocytes at both mRNA and protein levels in vivo and in vitro.

**Prevalence of the autoantibodies to fibulin-4 in patients with OA, RA, SLE, and SSc**

We next tried to determine the frequency of the anti-fibulin-4 autoantibodies in more numbers of patients with OA and patients with RA, as well as in patients with SLE and SSc, using ELISA. Specifically, we tested 92 serum samples from patients with OA, 67 serum samples from patients with RA, 40 patients with SLE, 43 patient with SSc, as well as three groups of healthy donors as negative controls. As shown in Fig. 6, 23.9% (22 of 92) of the tested OA serum samples reacted to at least one fragment of the three fibulin-4 fusion proteins, whereas only 8.9% (6 of 67) of the tested RA serum samples, 2.5% (1 of 40) of the tested SLE serum samples, and 9.3% (4 of 43) of the tested SSc serum samples reacted to the fibulin-4 fusion proteins. Approximately 4% of the tested control samples reacted to fibulin-4. The differences of
prevalence between OA patients and healthy controls, as well as between OA and RA, SLE, or SSc patients, were of statistical significance (\( p \leq 0.05 \)), 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 of 92) and -4c fusion proteins (14 of 92). Compared with the results of RA (1 of 67) and SSc (0 of 43), the Abs to fibulin-4b seem more specific in OA (\( p < 0.05 \), respectively). 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
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 \((\text{mean age 76.7 ± 9.5 years in the positive OA patients, 73 ± 9.3 years in the negative OA patients}),\) but the difference did not meet a statistical significance \((p > 0.05)\). The differences of the ratio of female: male \((10:1 \text{ in the positive and 6:1 in the negative}),\) and the differences of the mean x-ray grades between the positive and negative patients with OA \((2.1 ± 1.26 \text{ in the positive and 1.8 ± 0.85 in the negative})\) 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...
The autoantibodies to fibulin-4a, -4b, and -4c were used. Fibulin-4a, -4b, and -4c were detected by ELISA. The binding units of 100 were defined as a cutoff point.

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 autoantibodies to fibulin-4a, -4b, and -4c were detected by ELISA. Serum samples also diluted to 1/500 were used.

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 as described in Materials and Methods. Serum samples diluted at 1/500 were used. Fib-4a = fibulin-4a, -4b = fibulin-4b, and -4c = fibulin-4c. 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. Serum samples also diluted to 1/500 were used. c. The autoantibodies to fibulin-4a, -4b, and -4c in serum samples from patients with SLE, SSc, as well in healthy controls were detected by ELISA. Serum samples also diluted to 1/500 were used.

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 fibulin-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 anti-fibulin-4 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 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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References
16. Gallagher, W. M., M. Argentini, V. Sierra, L. Bracco, L. Debsusche, and Conse-
17. Roger, P. P., P. Pujol, A. Lucas, P. Baldet, and H. Rochefort. 1998. Increased immuno-