Interleukin-6 Reduces Spheroid Sizes of Osteophytic Cells Derived from Osteoarthritis Knee Joint via Induction of Apoptosis

Yoshifumi Negishi,* Arepati Adili,†‡ Susana de Vega,* Masahiro Momoeda,§ Haruka Kaneko,¶ Mehmet Zeynel Cilek,*‡ Chiho Yoshinaga,＊ Kazuaki Takafuji,＊ Yuta Otsuka,＊ Masayuki Shimoda,＊ Takako Negishi-Koga,＊＊ Muneaki Ishijima,＊＊＊ and Yasunori Okada＊＊＊

From the Department of Pathophysiology for Locomotive Diseases,* the Department of Medicine for Orthopaedics and Motor Organ,† and the Department of Community Medicine and Research for Bone and Joint Diseases,** Juntendo University Graduate School of Medicine, Tokyo; the Sportology Center, Kyoto; and the Department of Pathology,＊＊＊ Jikei University School of Medicine, Tokyo, Japan

Osteophytes in osteoarthritis (OA) joints contribute to restriction of joint movement, joint pain, and OA progression, but little is known about osteophyte regulators. Examination of gene expression related to cartilage extracellular matrix, endochondral ossification, and growth factor signaling in articular cartilage and osteophytes obtained from OA knee joint showed that several genes such as COL1A1, VCAN, BGLAP, BMP8B, RUNX2, and SOST are overexpressed in osteophytes compared with articular cartilage. Ratios of mesenchymal stem/progenitor cells, which were characterized by co-expression of CD105 and CD166, were significantly 2.2-fold higher in osteophytic cells than articular cells. A three-dimensional culture method for cartilage and osteophyte cells was developed by modulation of factors of self-assembled spheroid cell organoids (spheroids), and these spheroids cultured in the media for MSCs containing transforming growth factor-β showed characteristic morphologies and gene expression profiles of articular cartilage and osteophytes, respectively. The effects of IL-1β, tumor necrosis factor-α, and IL-6 on the spheroids of articular and osteophytic cells then were studied, and provided, to the best of our knowledge, the first evidence that IL-6 suppresses the spheroid size of osteophytic cells by inducing apoptosis and reducing extracellular matrix molecules. These data show that IL-6 is the suppressor of osteophyte growth and suggest that IL-6 expression and/or activity are implicated in the regulation of osteophyte formation in pathologic joints. (Am J Pathol 2023; 193: 1–15; https://doi.org/10.1016/j.ajpath.2023.10.005)
can form early in the development of OA before cartilage destruction.\textsuperscript{1,7} Concerning their clinical relevance, osteophytes may play a role in joint stabilization and prevention of OA progression, but they can be a source of pain and loss of joint function.\textsuperscript{1} Previous longitudinal studies of knee OA using radiography have suggested that the overall osteophyte score increases the risk of progression.\textsuperscript{8,9} More recently, cohort studies on knee joints in nonradiographic OA subjects have shown that osteophytes detected by conventional magnetic resonance imaging are the most common abnormality among all participants,\textsuperscript{10} and longitudinally associated with cartilage defects and knee pain.\textsuperscript{11} In addition, the studies on early stage knee OA patients or those who underwent reconstruction surgery for an anterior cruciate ligament injury have provided evidence that osteophytes at the periphery of the medial tibial cartilage detected by magnetic resonance imaging T2 mapping are implicated in medial meniscus extrusion,\textsuperscript{12,13} which is an important risk factor for knee OA progression.\textsuperscript{14} Therefore, these data indicate that osteophytes are involved in knee joint OA progression and suggest that information about how to regulate osteophyte formation is important for control and treatment of the OA knee joint.

Previous studies using experimental animal models have indicated that transforming growth factor-\(\beta\) (TGF-\(\beta\)) plays a major role in the stimulation of mesenchymal stem/progenitor cells (MSCs) in the periosteum to proliferate and differentiate to chondrocytes, and bone morphogenic protein-2 is essential to bone formation of osteophytes via endochondral ossification.\textsuperscript{1,15} A recent study of the experimental mouse OA model showed that osteophytes are formed by the Prg4-expressing and Sox9-expressing progenitors, both of which are Pdgfra-expressing, Gdf5-lineage MSCs present in the periosteum at the junction between the cartilage and bone of the OA knee joint.\textsuperscript{16} Human MSCs are characterized by the expression of certain cell surface antigens including CD105 and CD166, and cells co-expressing CD105 and CD166 represent a population of MSCs (CD105\(^+\)/CD166\(^+\) MSCs) in articular cartilage of OA and normal joints.\textsuperscript{17,18} Osteophytes also are known to contain MSCs,\textsuperscript{1,16} and MSCs isolated by culturing explant of osteophyte tissue are reported to have the ability of chondrogenic, osteogenic, and adipogenic differentiation and express markers related to cell adhesion (CD29, CD166, CD44) and stem cells (CD90, CD105, and CD73).\textsuperscript{19} However, whether Pdgfra-expressing, Gdf5-lineage MSCs are involved in osteophyte formation in human OA remains unknown, and no information is available for ratios of CD105\(^+\)/CD166\(^+\) MSCs in articular cartilage and osteophytes in the human OA joint. Differently from OA, joints in patients with rheumatoid arthritis (RA), which is characterized by prominent synovial inflammation and hyperplasia,\textsuperscript{20} show seldom or no osteophytes.\textsuperscript{21} Although the explanation of this different tissue reaction in OA and RA joints is not clear, it may be possible to speculate that proinflammatory cytokines such as IL-1\(\beta\), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\), and IL-6 may contribute to inhibition of osteophyte formation in the RA joint. However, the effects of these proinflammatory cytokines on osteophytes remain mostly unexplored. In addition, assay methods suitable for evaluation of osteophyte formation \textit{in vitro} have not been developed.

In the present study, the expression levels of the genes related to cartilage extracellular matrix (ECM), endochondral ossification, and growth factor signaling in articular cartilage and osteophytes obtained from OA knee joints were examined and compared. Ratios of CD105\(^+\)/CD166\(^+\) MSCs in cells isolated from OA cartilage and osteophytes were investigated by flow cytometry. A three-dimensional culture method for articular cartilage and osteophyte cells was developed by modification of cultures of self-assembled spheroid cell organoids (ie, spheroids), and mimicry of these spheroids to articular cartilage and osteophytes was confirmed. Then, the effects of IL-1\(\beta\), TNF-\(\alpha\), and IL-6 on the spheroids of articular cartilage and osteophyte cells finally were studied. This study provides the first evidence that IL-6 suppresses the spheroid size of osteophytic cells.

**Materials and Methods**

**Clinical Samples and Histology**

The joint tissues were obtained at total knee arthroplasty from patients with knee OA (\(n = 46\); 32 women and 14 men; mean age, 74 ± 7 years; range 58 to 86 years), which was diagnosed according to the American College of Rheumatology criteria.\textsuperscript{22} The cartilage tissues were removed from the macroscopically fibrillated areas of the tibial plateaus and femoral condyles and the cartilage part of the osteophytes at the peripheries of the tibial plateaus and femoral condyles. The cartilaginous tissue was taken carefully, without including calcified tissue such as bone, and used for mRNA preparation and cell cultures as described below. Some samples of the articular cartilage and osteophytes, both of which included the subchondral bone, were fixed with 4% paraformaldehyde, decalcified with 10% EDTA (pH 7.4), and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin and Safranin O, and subjected to histologic and histochemical grading as described by Mankin et al.\textsuperscript{23} For the experimental use of the surgical samples, informed consent was obtained from the patients according to hospital ethics guidelines. The study protocols complied with the principles outlined in the Declaration of Helsinki and were approved by the Ethical Committee Review Board of Juntendo University (15-074).

**Quantitative Real-Time PCR**

Cartilaginous tissues obtained from the articular cartilage and osteophytes were minced and then freeze-milled under liquid nitrogen into a fine powder using CoolMill (Toyobo Life Science, Tokyo, Japan). Total RNA was isolated from...
the powder and reverse-transcribed to cDNA, as described previously. The relative expression levels of COL2A1, ACAN, PRG4, S0X9, COL1A1, VCAN, BGLAP, BMP5B, RUNX2, SOST, GEM1, COL10A1, ITGA5, PDGFRA, GDF5, VEGF, and MMP13 to HPRT1 were determined by a SYBR Green real-time PCR assay (Invitrogen, Carlsbad, CA) according to the ΔΔCt method. The primers used for quantitative real-time PCR (qPCR) analysis are shown in Table 1.

Cell Cultures and Flow Cytometry

Cells were isolated by enzymatic dissociation from cartilage parts of the OA articular cartilage or osteophytes according to our methods. In brief, the cartilage parts without calcified tissues were cut into pieces. Then, they were incubated with 0.4% PRONASE Protease (Merck Millipore, Darmstadt, Germany) in Dulbecco’s modified Eagle’s medium/F-12 (Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37°C, and the pellets containing tissue fragments obtained by centrifugation were digested further for approximately 16 hours at 37°C by incubation with 0.4% collagenase P (Roche Diagnostics, Tokyo, Japan) in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum. The cells were washed in phosphate-buffered saline, centrifuged, and suspended in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum, ascorbic acid (25 μg/mL; Sigma-Aldrich), 100 U/mL penicillin and streptomycin (DF medium).

For flow cytometry, single-cell suspensions were prepared from first passage of cells cultured from articular cartilage or osteophytes by enzymatic treatment with Accutase (Innovative Cell Technologies, Inc., San Diego, CA) for 5 minutes at room temperature, and washed in phosphate-buffered saline. Cells suspended in phosphate-buffered saline containing 1% fetal bovine serum were stained with the following antibodies: fluorescein isothiocyanate–conjugated anti-human CD45, phosphatidylethanolamine–conjugated anti-human CD34, phosphatidylethanolamine–conjugated anti-human CD105, APC–conjugated anti-human CD166, APC–conjugated anti-human CD90, APC–conjugated anti-human CD73, or APC–conjugated anti-human CD44 antibodies. For dead cell exclusion, 7-aminoactinomycin D was used. Multipotent MSCs in the articular cartilage and osteophytes were defined as CD29+/CD45−/CD166+/CD105+ cells. Flow cytometric analysis was performed using LSRFortessa with FlowJo software (BD Biosciences Japan, Tokyo, Japan).

Isolation and Differentiation of MSCs

CD105+/CD166− MSCPs were isolated from the first passage of cells derived from OA articular cartilage or osteophytes. Cells were reacted with BV421–conjugated anti-human CD105 and phosphatidylethanolamine–conjugated anti-human CD166 antibodies (Thermo Fisher Scientific, Waltham, MA), and subjected to fluorescence-activated cell sorting using the BD FACSAria III Cell Sorter (BD Biosciences). Purity of the sorted cells was >99.9%. The sorted cells subsequently were cultured in StemPro MSC SFM (Thermo Fisher Scientific) for two passages and subjected to analysis of multilineage differentiation potential including adipogenesis, osteogenesis, and chondrogenesis. For adipogetic and osteogenetic differentiation, cells were plated at a density of 5000 cells/cm², cultured in StemPro MFC SFM for 3 days, and stimulated for 3 weeks with the hMSC Adipogenic Differentiation Medium BulletKit (Lonza, Tokyo, Japan) and the StemPro Osteogenic Differentiation Kit (Thermo Fisher Scientific), respectively. Adipogetic and osteogenetic differentiation was assessed by staining intracellular lipid droplets with Oil red O (Muto Pure Chemicals, Tokyo, Japan) and by staining for calcium with Alizarin red S (Funakoshi, Co., Ltd., Tokyo, Japan).

Table 1  Primer Sequences for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>F:</th>
<th>R:</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL2A1</td>
<td>F: 5′-AGACTGGCGAGATTCGGCTCTA-3′</td>
<td>R: 5′-ATCTGGAGCTGTCAGTGTG-3′</td>
<td></td>
</tr>
<tr>
<td>ACAN</td>
<td>F: 5′-CTGAACGACACAGGGCAGCAGA-3′</td>
<td>R: 5′-CTGGCAGAGATGATCCACCA-3′</td>
<td></td>
</tr>
<tr>
<td>PRG4</td>
<td>F: 5′-GGCAGGGTTTCAAAGCCTA-3′</td>
<td>R: 5′-CGACGGAGCTTCTAACAGGTC-3′</td>
<td></td>
</tr>
<tr>
<td>SOX9</td>
<td>F: 5′-TGGAAGTAAATCTGTTGGGAGAATG-3′</td>
<td>R: 5′-TGGAAAGTTAAGCTGTTGGTTG-3′</td>
<td></td>
</tr>
<tr>
<td>COL1A1</td>
<td>F: 5′-CCCGCGTTGCAAGACACATCCT-3′</td>
<td>R: 5′-TCACTACGGTCTTATCGCGAATC-3′</td>
<td></td>
</tr>
<tr>
<td>VCAN</td>
<td>F: 5′-CTTATCTTCAGGCAAGTGGGAGA-3′</td>
<td>R: 5′-GGGTCGAGAGTGGGTACTGTC-3′</td>
<td></td>
</tr>
<tr>
<td>BGLAP</td>
<td>F: 5′-CCAGGCGGACACGGAGGACAGG-3′</td>
<td>R: 5′-GCTGACCTCTGTTGGCAGT-3′</td>
<td></td>
</tr>
<tr>
<td>BMP5B</td>
<td>F: 5′-CCAGGCTGGCAGACGACATA-3′</td>
<td>R: 5′-CCAAGGGACCTCAGCCACATC-3′</td>
<td></td>
</tr>
<tr>
<td>RUNX2</td>
<td>F: 5′-GTCACCACTCCAGGCAACAT-3′</td>
<td>R: 5′-GTCTAATCTCTGCTCTGAGTCA-3′</td>
<td></td>
</tr>
<tr>
<td>SOST</td>
<td>F: 5′-TCAGGCGCAAGTTCACCTA-3′</td>
<td>R: 5′-TCCAGAGGTTGTCAGCCTGAAATAA-3′</td>
<td></td>
</tr>
<tr>
<td>GEM1</td>
<td>F: 5′-TAATCCGCGGCAAGATGCAA-3′</td>
<td>R: 5′-GGCAAGCTGTTGTAACAGACTC-3′</td>
<td></td>
</tr>
<tr>
<td>COL10A1</td>
<td>F: 5′-GACTGATATTTGTTGGATGGCGTGA-3′</td>
<td>R: 5′-CCCTGAAAGTCTGACGATA-3′</td>
<td></td>
</tr>
<tr>
<td>ITGA5</td>
<td>F: 5′-CAGAAGCTGATGAGTCCTGCA-3′</td>
<td>R: 5′-ATCAACAGTGGGACCACATA-3′</td>
<td></td>
</tr>
<tr>
<td>PDGFRA</td>
<td>F: 5′-TTCTGCTGGCCATCGGAGA-3′</td>
<td>R: 5′-GAGGTCGGGGAAGTGGACAC-3′</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>F: 5′-GTCAAGATCAGTGGTGAGGCG-3′</td>
<td>R: 5′-ACAAGGGACGACGATGTCGAG-3′</td>
<td></td>
</tr>
<tr>
<td>MMP13</td>
<td>F: 5′-TCTGCTGACGGTATGGCTC-3′</td>
<td>R: 5′-GAGGTCGGGGAAGTGGACAC-3′</td>
<td></td>
</tr>
<tr>
<td>HPRT1</td>
<td>F: 5′-GGCAAGCTGTTGTAACAGACTC-3′</td>
<td>R: 5′-TCTGACAGTGGTGAGGCG-3′</td>
<td></td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.

Q65
respective. Chondrogenic differentiation of the cells was performed in spheroid culture (1000 cells/well) as described below. After culture, spheroid cells were subjected to histology by staining with Safranin O (as described below) and qPCR analysis as described in *Quantitative Real-Time PCR*.

**Spheroid Cultures**

Self-assembled spherical cell organoids (ie, spheroids), were prepared by culturing cells isolated from OA articular cartilage or osteophytes in a 96-well, round-bottomed, ultralow-attachment surface plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) by modification of the methods reported by Takada and Mizuno. In brief, cells isolated from cartilage parts of the OA articular cartilage or osteophytes by treatment with PRONASE and collagenase P were washed in phosphate-buffered saline, centrifuged, and suspended in DF medium. After counting the cells, 1000 cells in 150 μL DF medium were cultured in each well of the plate at 5% CO₂ in air at 37°C, and 100 μL fresh DF medium was supplemented on day 4. On day 7, the culture media in each plate were replaced with DF medium containing 10 ng/mL recombinant human TGF-β3 (DF + T medium) or MSC chondrogenic differentiation medium (hMSC differentiation BulletKit-chondrogenic; Lonza) supplemented with 10 ng/mL TGF-β3 (MSC + T medium), and cultured for an additional 1, 2, and 3 weeks by replacing with each fresh medium every week.

Pictures of spheroids in the wells were taken using an inverted microscope (Primovert; Zeiss, Oberkochen, Germany) at 1, 2, and 3 weeks after culturing in these media, and sizes of the spheroids were measured by WinROof2018 measurement software (MITANI Corp., Tokyo, Japan). At 3 weeks, spheroids were harvested and digested by incubation with collagenase P for 3 hours at 37°C, and the cell numbers of the spheroids were counted by Scepter (Merck Millipore). For some spheroids, mRNA was extracted from the spheroids by TRIzol (NIPPPON Genetics, Tokyo, Japan) and subjected to qPCR. Some spheroids also were fixed with 10% buffered formalin and subjected to histologic analysis by embedding in paraffin.

**Analysis of the Effects of Cytokines on Cultured Spheroids**

Spheroids of articular and osteophytic cells in DF + T or MSC + T medium were treated for 3 weeks with IL-1β (0, 0.1, 1, or 10 ng/mL) (R&D Systems, Minneapolis, MN), TNF-α (0, 0.1, 1, or 10 ng/mL) (R&D Systems), or IL-6 (0, 10, 50, or 100 ng/mL) and soluble IL-6 receptor (IL-6R) (100 ng/mL) (R&D Systems). Sizes of the spheroids were measured by WinROof2018 measurement software (MITANI Corp.). To confirm the specific effect of IL-6 on spheroids, the spheroids were treated with IL-6 (100 ng/mL)/soluble IL-6R (100 ng/mL) in the presence or absence of anti–IL-6R antibody (25 μg/mL; tocilizumab; Chugai Pharmaceutical, Co., Tokyo, Japan) or nonimmune IgG (25 μg/mL) (R&D Systems) for 3 weeks. After treatment, changes in size and cell numbers of the spheroids were determined. Time course effects of IL-6 (0, 10, 50, or 100 ng/mL)/soluble IL-6R (100 ng/mL) on the spheroids also were evaluated by examining sizes at 1, 2, and 3 weeks after treatment.

**Determination of Apoptotic Cells in Spheroids by Histology and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Histochemistry**

The spheroids were cultured in MSC + T medium in the presence or absence of IL-6 (50 ng/mL)/soluble IL-6R (100 ng/mL) for 2 or 3 weeks, fixed in 10% buffered formalin, and embedded in paraffin. The paraffin sections were subjected to hematoxylin and eosin staining. For terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) histochemistry, after blocking endogenous peroxidase with 0.3% H₂O₂, the sections of IL-6–treated or untreated spheroids for 2 weeks were incubated with reaction buffer containing terminal deoxynucleotidyl transferase enzyme using the ApopTag peroxidase in situ apoptosis detection kit (Chemicon International, Billerica, MA) for 60 minutes at 37°C according to the manufacturer’s instructions. The reaction was terminated by immersing the slides in standard saline citrate for 15 minutes. They were counterstained lightly with hematoxylin. Three fields of each section were selected randomly, and the frequency of TUNEL-positive cells was estimated at ×400 magnification.

**Proteomic Analysis**

The IL-6–treated spheroids of articular or osteophytic cells were digested with phase-transfer surfactants according to previous methods, and then subjected to liquid chromatography with tandem mass spectrometry analysis using the UltiMate 3000 Nano LC system (Thermo Fisher Scientific) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) for data-independent acquisition. Samples were injected by an autosampler and enriched on a C18 reverse-phase trap column (100 μm I.D. × 5 mm length; Thermo Fisher Scientific) at a flow rate of 8 μL/min. They then subsequently were separated on an IonOpticks 25 cm Aurora column (Bruker Daltonics) at a flow rate of 300 nL/min with a linear gradient from 3% to 35% mobile phase B. Mobile phase B consisted of 90% acetonitrile with 0.1% formic acid, whereas mobile phase A consisted of 3% acetonitrile with 0.1% formic acid. The peptides were ionized using nano-electrospray ionization in positive ion mode. A spray voltage of 2100 V was applied. MS1 spectra were collected in the range of m/z 493 to 740 at 15,000 resolution to set an AGC target of 3 × 10⁶. MS2 spectra were collected in the range of m/z 200 to 1800 at 45,000 resolution to set an AGC target of
3 × 10^6, a maximum injection time of “auto”, and stepped normalized collision energies of 22%, 26%, and 30%. DIA data were analyzed using DIA-NN version 1.8.1 against the UniProt human database (https://www.uniprot.org) (downloaded February 2022 with 20,577 sequences) in robust LC (high precision) mode with RT-dependent cross-run normalization. The enzyme was trypsin and was set to be fully specific, allowing for one missed cleavage. Mass and MS1 accuracy were set to 10 ppm. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine was set as a variable modification. A heat map was generated using MeV software and expression data are represented as normalized values (Z-scores) and displayed 62 up-regulated and 157 down-regulated proteins in IL-6-treated cartilage cell spheroids, and 256 up-regulated and 778 down-regulated proteins in IL-6-treated osteophyte cell spheroids. The Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was performed by uploading both up-regulated and down-regulated proteins (>1.5× fold-change) with significant changes (P < 0.05) together using DAVID bioinformatics resources.

Statistical Analysis

All data were analyzed using the IBM SPSS Statistics 21.0 software program (Chicago, IL) and expressed as means ± SD. Results between the two independent groups were determined by the two-sided t-test. Comparisons involving more than three groups were performed by one-way analysis of variance, followed by the Bonferroni test. P values less than 0.05 were considered significant.

Results

Histologic Characteristics of OA Articular Cartilage and Osteophytes

Articular cartilage of the samples in the present study showed mild, moderate, and severe OA changes, which correspond to Mankin’s scores of 2 to 5, 6 to 9, and 10 to 14, respectively (Supplemental Figure S1, A–F). Because the samples were obtained from superficially or deeply fibrillated areas of the cartilage, all samples showed surface irregularity, fibrillation and/or laceration (fissuring), and various degrees of depletion of Safranin O–stained proteoglycans. Osteophytes are classified into four stages based on the histologic and cell biological parameters: stage I (early chondrophyte), stage II (fibrocartilage chondrophyte), stage III (growing osteophyte), and stage IV (mature osteophyte). The osteophyte samples in the current study belonged to stages II, III, and IV (Supplemental Figure S1, G–L). Stage I osteophytes were not used for the in vitro experiments because they were too small to obtain cells sufficient for cultures. Analyses of gene expression and spheroid cultures were performed by using the cartilage part of the articular cartilage with mild or moderate OA changes and that of stage III or IV osteophytes without including calcified tissues.

Expression of Genes Relevant to Articular or Osteophytic Cartilage Phenotype

The mRNA expression levels of the genes encoding molecules that may be involved in maintenance of cartilage ECM, endochondral ossification, and growth factor signaling were examined. As shown in Figure 1A, qPCR analysis showed that the expression levels of COL2A1, ACAN, PRG4, and SOX9 are significantly higher in articular cartilage than osteophytic cartilage, whereas COL1A1, VCAN, BGLAP, BMP8B, RUNX2, and SOST are significantly more highly expressed in the osteophytic cartilage than the articular cartilage. There were no significant changes in the expression levels of GREM1, COL10A1, ITGA5, PDGFRA, GDF5, VEGF, and MMP-13 (Figure 1A).

Expression of MSPC Markers in Articular and Osteophytic Cells

Because a previous study showed that normal and OA cartilage contains mesenchymal progenitor cells, which are characterized by co-expression of CD105 and CD166, and the potential to undergo adipogenesis, osteogenesis, and chondrogenesis, the population in isolated articular and osteophytic cells by flow cytometry was analyzed. As shown in Supplemental Figure S2, most cells were positive for CD105 (n = 4; 84.2% ± 14.7% and 89.9% ± 1.5% for articular and osteophytic cells, respectively), but 71.7% ± 10.6% of osteophytic cells were positive for CD166 (Figure 1B). The percentage of osteophytic cells that stained positive for CD90 (81.5% ± 12.8%), CD73 (84.1% ± 9.7%), or CD44 (91.4% ± 5.3%) did not differ significantly from that of articular cells (82.8% ± 4.6%, 82.2% ± 11.7%, and 88.3% ± 11.5%, respectively) (Supplemental Figure S2).

Adipogenic, Osteogenic, and Chondrogenic Potential of CD105−/CD166− Cells

Multilineage potential of CD105−/CD166− cells from articular cartilage or osteophytes were analyzed by culturing in media containing adipogenic, osteogenic, or chondrogenic supplements. As shown in Supplemental Figure S3A, both cartilage- and osteophyte-derived CD105−/CD166− cells differentiated to adipocytes, which showed many lipid vacuoles within their cytoplasm, whereas negligible lipid
was detected in control cells cultured in medium without adipogenic supplements. Osteogenic differentiation also was shown by Alizarin red S staining in both cells from cartilage and osteophytes under culture in the osteogenic differentiation medium, whereas no such staining was detected in control cells treated without osteogenic supplements (Supplemental Figure S3B). As for chondrogenesis, spheroids of CD105+/CD166+ cells from cartilage and osteophytes cultured in the chondrogenic differentiation medium showed a deposition of ECM stained with Safranin O (Supplemental Figure S3C). When the expression levels of SOX9 and COL2A1 were examined by qPCR, the levels were increased significantly in both cartilage- and osteophyte-derived CD105+/CD166+ spheroid cells treated with chondrogenic differentiation medium compared with those that were not treated with differentiation medium (Supplemental Figure S3C).

**Effects of Culture Media on Spheroid Formation of Articular and Osteophytic Cells**

The preliminary experiments showed that there were no significant differences in the ability of cell adhesion or proliferation between the isolated articular cells and osteophytic cells under the monolayer culture in DF medium and that sizes of the self-assembled spheroids of articular and osteophytic cells were almost similar at 7 days after culture in DF medium. Because the percentage of CD105+/CD166+ cells was higher in osteophytic cells than in articular cells (Figure 1B) and TGF-β is the most important factor for chondrogenesis of MSCs and maintenance of chondrocytes by preventing terminal differentiations, the effects of DF and MSC media containing TGF-β (DF + T and MSC + T) on spheroid formation of the articular and osteophytic cells were tested. Time course study showed that spheroid sizes of the osteophytic cells are increased significantly at 1, 2, and 3 weeks after culture in MSC + T medium compared with those of osteophytic cells in DF + T medium (Figure 2, A and B). Although sizes of articular cell spheroids tended to be larger under the culture in MSC + T medium than that in DF + T medium, no significant differences occurred (Figure 2, A and B). Importantly, under the culture in MSC + T medium, spheroid sizes of osteophytic cells were significantly larger than those of articular cells at 2 and 3 weeks; they were approximately 2.5-fold larger at 3 weeks (Figure 2, A and B). In accordance with the observation, the cell number of osteophytic cell spheroids cultured in MSC + T medium at 3 weeks was significantly higher (approximately 2.5-fold) than those of osteophytic cell spheroids cultured in DF + T medium or articular cell spheroids cultured in MSC + T medium (Figure 2C).

**Morphologic and Molecular Characteristics of Spheroids of Articular and Osteophytic Cells**

By histology, spheroids of articular cells cultured in MSC + T medium were composed of round cells embedded in homogenous ECM that was stained positively with Safranin O (Figure 3A). In contrast, spheroids of osteophytic cells in the MSC + T medium showed fibrocartilaginous morphology comprising spindle-shaped fibroblastic cells and fibrous ECM (Figure 3A). Spheroids of articular and osteophytic cells cultured in DF + T medium had uncharacteristic structures composed of round cells and a small amount of ECM (Figure 3A). The expression of COL2A1, ACAN, COL1A1, PRG4, SOX9, BMP8B, RUNX2, and SOST were then examined by qPCR, and found that the expression levels of COL2A1, ACAN, PRG4, and SOX9 were significantly higher in spheroids of articular cells in MSC + T medium than those of osteophytic cells, whereas COL1A1, BMP8B, RUNX2, and SOST were increased significantly in spheroids of osteophytic cells in MSC + T medium compared with those of articular cells in the medium (Figure 3B). These data show that the spheroids of articular cells and osteophytic cells cultured in MSC + T medium maintained the characteristics of articular cartilage and osteophytes, respectively, and suggest that the spheroid culture condition using MSC + T medium is suitable for analyses of effects of various arthritis modulators on articular cartilage and osteophytes.

**Effects of Proinflammatory Cytokines on Spheroids of Articular and Osteophytic Cells**

The effects of IL-1β, TNF-α, and IL-6 were tested on spheroids of articular and osteophytic cells cultured in MSC + T medium by treating the spheroids with different concentrations of IL-1β (0, 0.1, 1, and 10 ng/mL), TNF-α (0, 0.1, 1, and 10 ng/mL), or IL-6 (0, 10, 50, and 100 ng/mL) for 3 weeks and measured the sizes of each spheroid. As shown in Figure 4, only IL-1β, but not TNF-α or IL-6, appeared to decrease the size of the spheroids of articular cells in a dose-dependent manner. Higher concentrations of

**Figure 1** Expression of the genes relevant to articular or osteophytic cartilage phenotype and flow cytometric analysis of mesenchymal stem/progenitor cells (MSCs) in the cartilaginous tissue samples from osteoarthritis (OA) articular cartilage and osteophytes. A: Quantitative real-time PCR analysis of the genes encoding molecules of cartilage extracellular matrix, endochondral ossification, and growth factor signaling in articular cartilage and osteophytes. Relative expression of mRNA for the genes was determined by normalizing to the expression level of HPRT1 transcripts using the ΔΔCT method as described in Materials and Methods. The mean target gene: HPRT1 ratio in cartilage samples was set at 1. Circles represent individual subjects B: MSCs showing CD45/CD54/CD166+/CD105+ cells isolated from OA articular cartilage and osteophytes. Single-cell suspensions prepared from the first passage of the articular or osteophytic cells were incubated with phosphatidylserine-conjugated antibody against CD105 and APC-conjugated antibody against CD166 and then subjected to flow cytometric analysis as described in Materials and Methods. Data are expressed as means ± SD. n = 5 (A); n = 4 (B). *P < 0.05, **P < 0.01, and ***P < 0.001. CA, cartilage; OP, osteophyte.
IL-1β (1 and 10 ng/mL) and TNF-α (10 ng/mL) significantly reduced and increased the sizes of articular cell spheroids, respectively (Figure 4, A and B). Only a high concentration (10 ng/mL) of IL-1β and TNF-α significantly decreased the spheroid size of osteophyte cells (Figure 4, A and B). Interestingly, IL-6 dose-dependently diminished the spheroid size of osteophytic cells without affecting that of articular cells (Figure 4C).
Interleukin-6 Effect on Osteophyte Cells

Demonstration of the IL-6–Specific Effect on Spheroids of Osteophytic Cells

To examine the specific effect of IL-6 on the spheroid size reduction of osteophytic cells, spheroid cultures of articular and osteophytic cells were performed by treating with anti–IL-6R antibody (tocilizumab) or nonimmune IgG together with IL-6. As shown in Figure 5, treatment of osteophytic cells with IL-6 significantly decreased the size and cell number of the spheroids compared with the control spheroids without IL-6 treatment, and the reduced size and cell number were recovered to control levels by treatment with anti–IL-6R antibody of the IL-6–treated spheroids of osteophytic cells. There were no apparent effects of this antibody on the sizes or cell numbers of articular cells with or without IL-6 treatment and those of IL-6–untreated osteophytic cell spheroids (Figure 5). In addition, nonimmune IgG showed no effects on the size or cell number of...
these spheroids (Figure 5). These data show that the reduced size and cell numbers of IL-6–treated osteophytic cell spheroids are the result of the IL-6–specific effect.

**Involvement of Apoptosis in IL-6–Mediated Size Reduction of Osteophytic Cell Spheroids**

Time course studies of the IL-6–treated spheroids indicated that the spheroid size of articular cartilage cells was decreased slightly by treatment with IL-6 at 1 and 2 weeks after culture, but this effect disappeared at 3 weeks, resulting in no significant size difference by treatment with various concentrations of IL-6 (0, 10, 50, and 100 ng/mL) (Figure 6A). In contrast, IL-6 dose-dependently decreased the spheroid size of osteophytic cells by IL-6 treatment at 3 weeks, although no changes in sizes were observed at 1 and 2 weeks (Figure 6A). Because the size diminished abruptly at 3 weeks after culture in the presence of IL-6, the histology of the spheroids was examined carefully and it was found that some cells in the IL-6–treated spheroids of osteophytic cells, but not articular cells, at 2 weeks showed nuclear condensation and/or fragmentation, forming nuclear rupture and fragmented bodies (Figure 6B), all of which suggest that they suffered from apoptosis. Consistent with the finding, most of the cells with condensed or fragmented nuclei as well as fragmented bodies in the IL-6–treated osteophytic cell spheroids were positive for TUNEL staining, whereas only a few cells in the IL-6–treated articular

---

**Figure 4** Effects of proinflammatory cytokines on spheroids of articular cartilage and osteophyte cells. **A:** IL-1β effects on spheroids of articular cartilage and osteophyte cells. Spheroids of articular or osteophytic cells were cultured in MSC + T medium in the absence or presence of IL-1β (0.1, 1, or 10 ng/mL) for 3 weeks, and photographs of each spheroid were taken using an inverted microscope. Areas (μm²) of spheroids were measured with WinRoof2018 measurement software. **B:** Tumor necrosis factor-α (TNF-α) effects on spheroids of articular cartilage and osteophyte cells. Spheroids were cultured in MSC + T medium in the absence or presence of TNF-α (0.1, 1, or 10 ng/mL) for 3 weeks, and areas (μm²) of spheroids on each image were measured with WinRoof2018 measurement software. **C:** IL-6 effects on spheroids of articular cartilage and osteophyte cells. Spheroids were cultured in MSC + T medium in the absence or presence of IL-6 (10, 50, or 100 ng/mL) for 3 weeks, and areas (μm²) were measured with WinRoof2018 measurement software. All assays were repeated at least three times and similar data were obtained. Data are expressed as means ± SD. n = 6. *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bars = 200 μm.
Figure 5 Demonstration of IL-6–specific reduction in size and cell numbers of osteophytic cell spheroids. A: Representative images of osteophytic cell spheroids treated without or with IL-6, nonimmune IgG (NI IgG), IL-6 and anti–IL-6–receptor antibody (tocilizumab), NI IgG or tocilizumab for 3 weeks. Osteophytic cell spheroids were treated with IL-6, nonimmune IgG (NI IgG), IL-6, and/or tocilizumab as described in Materials and Methods for 3 weeks, and photographs were taken by an inverted microscope. B: IL-6–specific reduction in size of osteophytic cell spheroids. Areas (µm²) of spheroids on each image were measured with WinRoof2018 measurement software. C: IL-6–specific reduction in cell numbers of osteophytic cell spheroids. Cell numbers of each spheroid were counted by Scepter after digestion of the spheroids with collagenase P. Note that the IL-6–induced reduction in size and cell number was recovered by treatment with tocilizumab. Data are expressed as means ± SD. n = 6. ***P < 0.001. Scale bar = 200 µm.

Cell spheroids showed a positive reaction (Figure 6C). The TUNEL-positive cell index was significantly higher in the IL-6–treated osteophytic cells (32.5% ± 7.6%) than in the IL-6–untreated osteophytic cells (4.0% ± 2.9%). IL-6–treated articular cells (9.1% ± 8.3%), or IL-6–untreated articular cells (6.5% ± 5.6%) (Figure 6C).

To investigate the possible molecular mechanism by which IL-6 induced apoptosis of osteophytic cells, a proteomic analysis was performed by comparing the differentially expressed proteins in the spheroids of cartilage and osteophytes treated with or without IL-6 for 2 weeks (Supplemental Tables S1 and S2). As shown in Supplemental Figure S4A, a heat map of differentially expressed proteins showed that spheroids of cartilage and osteophyte cells have different expression patterns in response to IL-6 treatment. The Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis showed that several pathways with enrichment such as focal adhesion pathway and ECM-receptor interaction pathways are highlighted in IL-6–treated osteophyte cell spheroids, independently from those identified in cartilage cell spheroids (Supplemental Figure S4B). A volcano plot of differentially expressed proteins in osteophytic spheroids showed that among 31 proteins appearing in the ECM-receptor interaction pathway, 25 proteins were down-regulated significantly in response to IL-6 treatment (Supplemental Figure S4C). Some key cartilage ECM molecules including aggrecan (ACAN), versican (VCAN), biglycan (BGN), and decorin (DCN) also were down-regulated by IL-6 treatment (Supplemental Figure S4C). Although the apoptosis pathway was not up-regulated significantly by IL-6 treatment of osteophyte cells, apoptosis-related molecules annotated with GO terms including cathepsin C (CTSC), chitinase-3-like protein 1 (CHI3L1), integrin β 3 (ITGB3), CCAAT/enhancer-binding protein β (CEBPB), regulator of cell-cycle RGCC (RGCC), B-cell lymphoma 3-encoded protein (BCL3), and inhibit β A chain (INHBA) were included among the top 50 differentially up-regulated proteins (Supplemental Table S3).

Discussion

The present study showed that the cartilaginous layer of osteophyte at stage III (growing osteophyte) or stage IV (mature osteophyte) overexpresses, as compared with...
Figure 6  Involvement of apoptosis in IL-6–mediated size reduction of osteophytic cell spheroids. A: Time course study of spheroid sizes cultured in the presence of IL-6. Spheroids of cartilage (upper panel) or osteophyte cells (lower panel) were cultured in the presence of IL-6 (0, 10, 50, or 100 ng/mL) for 3 weeks and photographs were taken using an inverted microscope. Areas (μm²) of spheroids were measured with WinRoof2018 measurement software. B: Nuclear condensation and/or fragmentation of the IL-6–treated cells in osteophytic cell spheroids. Hematoxylin and eosin–stained paraffin sections of Negishi et al  

FLA 5.6.0 DTD  ■ AJPA3988_proof  ■ 7 November 2023  ■ 7:55 pm  ■ EO: AJPA-D-23-
articular cartilage, several genes including COL1A1, VCAN, BGLAP, BMP8B, RUNX2, and SOST, all of which reportedly were up-regulated by the DNA microarray analysis of osteophytes. Roelofs et al recently reported that in the experimental mouse OA model, Prg4-expressing and Sox9-expressing progenitors, both of which are Pdgfrα-expressing, Gdf5-lineage MSCs present in the periosteum at the junction of articular cartilage, bone, and synovium, are activated to form the cartilage and bone parts of osteophytes, respectively. In the present study, the expression levels of PRG4 and SOX9 were decreased in the osteophytes and there were no changes in the PDGFRα and GDF5 expression levels between osteophytes and articular cartilage. This may be because of the different time points examined for the expression. Several months or years passed before surgical removal of the human osteophytes, whereas the mouse osteophytes were examined at very early stages of 2 and 8 weeks after the OA induction. To the best of our knowledge, however, no data are available for the molecular mechanism on human osteophyte formation in human OA knee joints. Therefore, further detailed studies on human osteophytes are necessary to examine the mechanisms.

Similar to MSCs derived from bone marrow or perichondrium, CD105+/CD166+ cells isolated from human articular cartilage are known to have the potential to differentiate to adipogenic, osteogenic, and chondrogenic lineages. The present study showed their ability to differentiate by confirming the adipogenesis, osteogenesis, and chondrogenesis in vitro. The percentage of CD105+/CD166+ cells in OA cartilage reportedly is higher than that in normal cartilage (approximately 3.5% in normal cartilage and approximately 7.5% in OA cartilage), or similar in OA and normal cartilage (approximately 17% and approximately 15%, respectively). Because an osteophyte is formed by proliferation and differentiation of MSCs that originated from the periosteum covering the bone at the cartilage—bone junction, it is reasonable to think that osteophytes contain a high ratio of MSCs. Although most cells obtained from the osteophyte tissue by the explant culture reportedly expressed all markers of cell adhesion (CD29, CD166, and CD44) and stem cells (CD90, CD105, and CD73), and showed MSC-like properties such as chondrogenesis, adipogenesis, and osteogenesis, no information was available for the ratio of MSCs in osteophytes compared with that in OA articular cartilage. Therefore, the present study, to the best of our knowledge, is the first to show that osteophyte tissue contains significantly more (2.2-fold) CD105+/CD166+ MSC-like cells than OA articular cartilage.

Three-dimensional cultures are superior to monolayer cultures for biological analyses of various cells, especially MSCs and chondrocytes. By using self-assembled spheroid cultures, the present study has shown that osteophytic cells cultured in MSC + T medium grow to the largest size, with a 2.5-fold increased cell number among osteophytic and articular cells cultured in DF + T medium or MSC + T medium. The finding appears to be consistent with the data that osteophytes contained 2.2-fold more CD105+/CD166+ cells compared with articular cartilage. Importantly, the spheroids of articular cells and osteophytic cells cultured in MSC + T medium showed morphologies similar to those of articular cartilage and osteophytes (ie, hyaline cartilage and fibrocartilage, respectively), and the data were supported by the expression patterns of the genes that are characteristic of articular cartilage or osteophytes. Together, these data show that spheroid cultures of articular and osteophytic cells using MSC + T medium mimic the in vivo conditions of articular cartilage and osteophytes and suggest that this method is suitable for biological analyses of articular chondrocytes and osteophyte cells in vitro.

One of the intriguing findings in the present study is that among the proinflammatory cytokines examined, only IL-6 dose-dependently reduced the spheroid size and cell number of osteophytic cells in spheroids at 3 weeks after treatment. This effect was specific to IL-6 because the anti–IL-6R antibody tocilizumab canceled the IL-6–induced size reduction in spheroids. A time course study of the culture and analyses by morphology and TUNEL indicated that apoptosis of osteophytic cells contributed to the spheroid size reduction starting 2 weeks after IL-6 treatment. Effects of IL-6 vary, depending on the cell types and/or culture conditions. Although IL-6 originally was reported to function as a factor critical for maintaining the stemness of human bone marrow–derived MSCs by suppressing their chondrocyte differentiation, a subsequent study indicated that IL-6 stimulates the chondrogenic differentiation of human bone marrow–derived MSCs. They showed that the wet weight of spheroids at 2 weeks after IL-6 treatment in MSC + T medium was approximately 1.4-fold increased. In the time course study on spheroid cultures, IL-6 showed a negligible effect on the spheroid growth of osteophytic cells at 1 and 2 weeks, but then abruptly reduced the size and cell number at 3 weeks. Therefore, it is likely that IL-6 initially contributes to the promotion of chondrogenic differentiation of MSCs and eventually to apoptosis of differentiated osteophytic chondrocytes.

The molecular mechanism by which IL-6 induces apoptosis of osteophytic cells in spheroids remains unclear in the present study. However, the proteomic analysis...
provided data that the proteins appearing in the focal adhesion pathway and the ECM-receptor interaction pathway are altered remarkably and cartilage proteoglycans are reduced significantly in osterophic spheroids treated with IL-6. Because impairment of focal adhesion and deregulation of interaction between ECM molecules and their receptors are well known to be involved in the induction of apoptosis, it may be possible to speculate that IL-6-mediated apoptosis could be triggered secondarily to activation of such pathways. However, further studies are necessary to clarify the mechanism on osteophy- cell apoptosis that is induced after long-term IL-6 treatment of more than 2 weeks.

Osteophyte formation is one of the characteristics of the OA knee joint. In sharp contrast to OA, no osteophytes are formed in joints of patients with active RA. To the best of our knowledge, little or no information was available regarding why osteophyte formation is different between OA and RA joints. The concentration of IL-6 in synovial fluid is known to be much higher in RA patients than in OA patients, and one report showed that the IL-6 level in RA synovial fluid is approximately 25 ng/mL on average, and maximally 95 ng/mL. Because IL-1β and TNF-α stimulate chondrocytes to overproduce IL-6, the levels of IL-6 in local tissue at the junction of articular cartilage, bone, and synovium, where osteophytes are developed, may be higher than 50 ng/mL, a concentration that is sufficient to suppress the growth of osteophytic cell spheroids by apoptosis induction. Taken together, it is plausible to speculate that an absence of osteophytes in RA joints may be ascribed to the continuous effects of IL-6 in the joint tissues. Further studies on the relationship between the IL-6 concentration and osteophyte size in OA and RA joints and careful evaluation of RA patients treated with anti-IL-6R antibody by focusing on the formation of osteophytes, which are composed of cartilage and bone parts and detected by magnetic resonance image T2 mapping, would provide the data to support this hypothesis.

In summary, this study showed that compared with OA articular cartilage, osteophytic cartilage is enriched with CD105+CD166-/-MSC-like cells, and, based on the data, a three-dimensional spheroid culture method was developed that reflects the in vivo condition of osteophytes and is suitable for the biological analysis of osteophyte cells in vitro. This culture method, to the best of our knowledge, provides the first evidence that IL-6 decreases the spheroid size of osteophytic cells by induction of apoptosis. Therefore, seeking for agents that are suitable for selective suppression of osteophytes by modulation of IL-6 expression and function may be a new strategy to prevent OA progression. However, because IL-6 is a pluripotent cytokine that has both destructive and protective effects on articular cartilage, deciphering the molecular pathways of IL-6 in articular cartilage and osteophytes is critical for the discovery of IL-6-related disease-modifying OA drugs in the future.

**Disclosure Statement**

Kazuaki Takafuji is an employee of Suntory Global Innovation Center, Ltd., and Yuta Otsuka is an employee of Suntory Wellness, Ltd.

**Supplemental Data**

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2023.10.005.

**References**

extrusion are strongly associated with progression of symptomatic knee osteoarthritis as assessed by quantitative magnetic resonance imaging. Ann Rheum Dis 2005, 64:556–563