MATRIX PATHOBIOLOGY

Implication of HYBID (Hyaluronan-Binding Protein Involved in Hyaluronan Depolymerization) in Hyaluronan Degradation by Synovial Fibroblasts in Patients with Knee Osteoarthritis

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Cell migration-inducing hyaluronidase 1 (CEMIP), also known as hyaluronan (HA)-binding protein involved in HA depolymerization (HYBID), plays a role in HA degradation. CEMIP2, also known as transmembrane protein 2 (TMEM2), possessing a sequence similarity with HYBID, is reported as a hyaluronidase in mice. However, the expression of these molecules in osteoarthritic synovium and their involvement in HA degradation in synovial fluid (SF) from patients with knee osteoarthritis remain elusive. This study examined their expression in synovial tissue and the relationship with molecular weight of HA in SF in knee osteoarthritis patients. Quantification of mRNA demonstrated that HYBID expression is significantly (5.5-fold) higher in osteoarthritic synovium than in normal control synovium, whereas TMEM2 expression level is similar between the two groups. By immunohistochemistry, HYBID was localized mainly to CD68-negative and fibroblast-specific protein 1—positive synovial lining cells and sublining fibroblasts in osteoarthritic synovium. The mRNA expression levels of HYBID, but not TMEM2, in osteoarthritic synovium positively correlated with distribution of lower-molecular-weight HA with below 1000 kDa in SF. HA-degrading activity in osteoarthritic synovial fibroblasts was abrogated by siRNA-mediated knockdown of HYBID. Among the 12 factors examined, IL-6 significantly up-regulated the HYBID expression and HA-degrading activity in osteoarthritic synovial fibroblasts. These data suggest that HYBID overexpressed by IL-6-stimulated synovial fibroblasts is implicated in HA degradation in osteoarthritic synovium. *(Am J Pathol 2020, 190: 1046—1058; https://doi.org/10.1016/j.ajpath.2020.01.003)*

The joint cavity is surrounded by articular cartilage and synovium and filled with synovial fluid, which is a mixture of hyaluronan (HA) and a protein-rich ultrafiltrate of blood plasma. In the normal joint, HA, which is an unbranched nonsulfated glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine and synthesized by HA synthases (HAS1, HAS2, and HAS3), is present in the range of 1000 to 10,000 kDa, that is, high-molecular-weight HA (HMW-HA). HMW-HA plays an essential role in maintaining the lubricating property and shock absorption function of synovial fluid.[1,2] These physicochemical properties of HA are lost when it is degraded to lower-molecular-weight HA (LMW-HA).[3] Other biological activities of HA are also dependent on its molecular weight[4,5]: HMW-HA is anti-inflammatory and antiangiogenic, whereas LMW-HA promotes inflammatory and angiogenic reactions.[6,7] In osteoarthritis (OA), HA degradation is enhanced and so

LMW-HA is accumulated in synovial fluid.[3] Therefore, HA in OA synovial fluid may not only enhance mechanical stress on articular cartilage due to decreased shock absorption function, but also promote synovitis, leading to the possible progression of cartilage destruction in the OA joint. Recently, the risk of knee OA progression has been associated with the preponderance of HA below 1000 kDa in synovial fluid, but not average HA molecular weight.[8]

Arthroscopic and histologic studies have shown that synovitis commonly occurs in knee OA,[9,10] and an immunohistochemical study reported that degree of synovial inflammation is more severe in early-stage knee OA than in late-stage OA.[11] However, a growing amount of evidence using noninvasive imaging techniques such as magnetic resonance imaging demonstrated that synovial inflammation is present in many patients at all stages of OA[12,13] and supports the notion that synovitis contributes to the progression of OA by accelerating cartilage breakdown.[12,14] Moreover, recent studies on the risk factors of knee OA have suggested that synovitis may be an independent cause of OA[15] and an independent driver of radiographic OA onset and progression.[13]

Hyaluronidases (HYALs) composed of six different molecules were long thought to be key enzymes for HA degradation,[16] and among them, HYAL-1 and HYAL-2, the latter of which acts together with cell surface HA receptor CD44, were reported to play a key role in HA degradation. However, knockdown of these genes failed to change HA-degrading activity in skin fibroblasts.[17] Therefore, we searched for new molecules related to HA degradation by microarray analysis, and demonstrated that hyaluronan-binding protein involved in hyaluronan depolymerization (HYBID), which was renamed later as cell migration-inducing hyaluronidase 1 (CEMIP) by HUGO Gene Nomnclature Committee, plays a key role in HA degradation independently from HYAL1 and HYAL2/CD44 in human fibroblasts.[17,18] *HYBID* was reported as a deafness gene of unknown function (*KIAA1199*)[19] and also named as *CEMIP* (cell migration-inducing protein) by another group.[20] A previous study on skin fibroblasts has shown that HYBID is localized in clasthrin-coated vesicles and contributes to degradation of HMW-HA into LMW-HA fragments, which are released to the extracellular milieu.[17] HYBID is a secreted protein composed of one G8, two GG, and four PbH1 domains, lacking substantial homology to HYAL enzymes, HA-binding proteins, and HA-link modules. It selectively binds to HA, but does not interact with sulfated glycosaminoglycans.[17] A recent study has shown that a type II transmembrane protein with a sequence homology to HYBID, named as transmembrane protein 2 (TMEM2) [alias cell migration-inducing hyaluronidase 2 (CEMIP2)] acts as cell-surface hyaluronidase in mice and is ubiquitously expressed in adult mouse tissues.[21] However, no studies have shown the expression or function of TMEM2 in human tissues such as synovium. Although it is reported that HYBID is expressed by rheumatoid synovial lining and sublining cells,[17] little information is available for tissue or cellular localization responsible for the expression in OA synovial tissue. In addition, the relationship between the expression levels of HYBID or TMEM2 and the size distribution of HA in OA synovial fluids remains unknown. Recent studies on the regulation of the HYBID gene expression have demonstrated that transforming growth factor-β1 (TGF-β1) down-regulates the expression in skin fibroblasts,[18] and tumor necrosis factor-α (TNF-α) up-regulates the expression in OA chondrocytes.[8] However, little is known about regulators of HYBID expression in OA synovial fibroblasts.

The present study found that HYBID, but not TMEM2, is up-regulated in OA synovial tissue. This study also shows that HYBID is implicated in degradation of HMW-HA in OA synovial fluids, and that among the factors examined, IL-6 efficiently up-regulates the HYBID expression in OA synovial fibroblasts.

Materials and Methods

Clinical Samples and Histology

Synovial tissue samples were obtained at the time of arthroplasty from joints of patients with knee OA ($n = 27$; mean $\pm$ SD age, 75.7 $\pm$ 7.3 years), which was diagnosed according to the criteria of the American College of Rheumatology.[22] Normal control synovial tissue samples were obtained at reconstruction surgery for anterior cruciate ligament from knee joints of patients with anterior cruciate ligament injury ($n = 8$; mean $\pm$ SD age, 37.3 $\pm$ 11.0 years). The OA synovial tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin. Written informed consent was obtained from the patients for the experimental use of the surgical samples according to the hospital ethics guidelines. The study protocols, which complied with the principles outlined in the Declaration of Helsinki, were approved by the Ethical Committee Review Board in Juntendo University (No 15-074).

Quantification of mRNA Copy Numbers

Total RNA was isolated from OA and normal control synovial tissues using RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized from 2 μg of total RNA using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The cDNA was amplified by quantitative real-time PCR using the THUNDERBIRD SYBR qPCR Mix (Toyobo) on a QuantStudio3 (Applied Biosystems, Foster City, CA). Nucleotide sequences for the primers were as follows: for HYBID 5′-TCACA-GAGGACTCCTACCCG-3′ (forward) and 5′-ATTGGC-CATCCAGAAGGTGG-3′ (reverse); and for TMEM2 5′-TTACGGCTTTCAGGGTGGTC-3′ (forward) and 5′-TTGGGAACGTCCTGTTCCTG-3′ (reverse). To examine

the expression levels of HYBID and TMEM2 in synovial tissues, absolute quantification of mRNA copy numbers was performed using the standard curve method according to the methods described by Whelan et al.[23] Briefly, purified plasmids containing the coding sequences of human HYBID [pcDNA3.1(−)-HYBID][17] and human TMEM2 (RC224793; Origene, Rockville, MD) were digested with restriction enzymes, that is, EcoRI and NdeI (TAKARA, Kusatsu, Japan) for the HYBID plasmid and FauI (New England Biolabs, Ipswich, MA) for the TMEM2 plasmid, to cut out the inserts from the plasmids, both of which were purified using the QIAquick Nucleotide Removal Kit (Qiagen). Concentrations of the plasmid and insert were determined using the QuantiFluor dsDNA System (Promega, Madison, WI), and copy numbers were calculated according to the following formula: copy number (g/molecule) = (base pairs size of double-stranded plasmid containing the insert) × (330 Daltons × 2 nucleotides/base pairs) ÷ (Avogadro number 6.023×10^{23} molecules/mole). Standard curves of plasmid DNA containing the coding regions of HYBID and TMEM2 were generated with threshold cycle (C_T) values obtained from quantitative real-time PCR of various copy numbers of the plasmids. The copy numbers of HYBID and TMEM2 molecules present in the synovial tissue samples were calculated by relating the PCR signal (C_T value) of each synovial tissue sample to the standard curves.

Immunohistochemistry

Paraffin sections of OA synovial tissues were autoclaved at 121°C for 10 minutes in Target Retrieval Solution (DAKO Cytomation, Tokyo, Japan) for antigen retrieval, and nonspecific reactions were blocked with BLOCK ACE (DS Pharma Biomedical, Osaka, Japan). The sections were also treated with 3% H_2O_2 to block peroxidase reactions. They were then immunostained with rabbit anti-HYBID (KIAA1199) antibody (SAB2105467; Sigma-Aldrich, St. Louis, MO), rabbit anti−IL-6 antibody (ab6672; Abcam, Cambridge, UK) or nonimmune control IgG (X0936; DAKO Cytomation), followed by incubation with biotinylated antibody against rabbit IgG according to the ABC method (Vector Laboratories, Burlingame, CA). Color was developed with 3-amino-9-ethylcarbazole chromogen (Vector Laboratories), and sections were counterstained with hematoxylin. Specificity of anti-HYBID (KIAA1199) antibody (SAB2105467; Sigma-Aldrich) was confirmed by the absorption test by incubating the antibody with the peptide antigen (KIAA1199 Blocking Peptide, 33R-7080; Fitzgerald Industries International, Acton, MA) prior to immunohistochemistry (data not shown).

Immunofluorescence Microscopy

Paraffin sections of OA synovial tissues were subjected to antigen retrieval with Target Retrieval Solution (DAKO Cytomation), and nonspecific reactions and autofluorescence were blocked with BLOCK ACE (DS Pharma Biomedical). They were double-immunostained with mouse anti-CD68 antibody (clone PG-M1, M0876; DAKO Cytomation) and rabbit anti-HYBID (KIAA1199) antibody (SAB2105467; Sigma-Aldrich) or rabbit anti−IL-6 antibody (ab6672; Abcam, Cambridge, UK), followed by incubation with anti-mouse IgG antibody conjugated to Alexa Fluor 488 or anti-rabbit IgG antibody conjugated to Alexa Fluor 546 (Life Technologies, Carlsbad, CA). Similarly, double-immunofluorescence staining was performed with rabbit anti-HYBID (KIAA1199) antibody (SAB2105467; Sigma-Aldrich) and mouse anti−fibroblast-specific protein 1 (S100A4) antibody (ab218511; Abcam), followed by incubation with anti-rabbit IgG antibody conjugated to Alexa Fluor 546 or anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Life Technologies). Nuclei were stained with Hoechst 33342 (Thermo Fisher Scientific, Tokyo, Japan), and images were obtained with a confocal microscope TCS-SP5 (Leica, Wetzlar, Germany).

Measurement of HA and HYBID Concentrations in OA Synovial Fluids

HA in synovial fluid from OA patients was determined using QnE Hyaluronic Acid enzyme-linked immunosorbent assay (ELISA) (Biotech Trading Partners, Encinitas, CA) according to the manufacture's protocol. The assay detected HA molecular weight forms as small as 20 to 30 kDa. The molecular weight of HA in OA synovial fluid was determined by size-exclusion gel filtration using Shodex OHpak SB-807 columns (Showa Denko, Tokyo, Japan). Fractions (0.5 mL) were collected, and the HA content was determined as described previously.[17] The column was calibrated with HA species: sodium HA H2 (2500 kDa), M2 (1000 kDa), and L2 (150 kDa), all of which were purchased from PG Research (Tokyo, Japan). The HYBID protein concentrations in OA synovial fluids were evaluated by densitometric analysis of immunoblotting data according to a standard curve determined using recombinant HYBID.[17]

Cultures of OA Synovial Fibroblasts

Synovial fibroblasts were isolated from human OA synovium by enzymatic dissociation and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum according to previously published methods.[24,25] They were used for experiments at passages 1 to 4 (P1 to P4).

Stimulation of OA Synovial Fibroblasts with Factors

Serum-starved OA synovial fibroblasts at P3 were treated with histamine (Wako, Osaka, Japan), TGF-β1 (R&D Systems, Minneapolis, MN), IL-6 (R&D Systems) plus soluble

IL-6 receptor (sIL-6R) (R&D Systems), IL-1α (R&D Systems), IL-8 (R&D Systems), TNF-α (R&D Systems), insulin-like growth factor-1 (IGF-1) (Sigma-Aldrich), vascular endothelial growth factor$_{165}$ (VEGF$_{165}$) (R&D Systems), basic fibroblast growth factor (bFGF) (Sigma-Aldrich), prostaglandin E2 (PGE2) (Sigma-Aldrich), tumor necrosis factor-β (TNF-β) (R&D Systems), and C-C motif chemokine ligand 5 (CCL5) (R&D Systems) or vehicle alone in Dulbecco's modified Eagle's medium containing 1% fetal bovine serum for 24 hours. The expression levels of *HYBID* and *TMEM2* were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and determined by a SYBR Green real-time PCR assay (Invitrogen, Carlsbad, CA) according to the $\Delta\Delta C_T$ method.[26] Nucleotide sequences for the primers were as follows: for *HYBID* 5′-AGGGAAGCAGGTCAGAGTGA-3′ (forward) and 5′-TCTCGGCTACAGACCCAGAG-3′ (reverse); for *TMEM2* 5′-ACTTGGTGGCTGGCATGTTC-3′ (forward) and 5′-CATGAGCTGGGCCTGAGTTG-3′ (reverse); and for *GAPDH* 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGTGAAGACGCCAGTGGA-3′ (reverse). The expression of *HYAL1*, *HYAL2*, *HAS1*, *HAS2*, and *HAS3* in IL-6−treated OA synovial fibroblasts was also examined according to the method described above using the primers as follows: for *HYAL1* 5′-CCCAAGGTTGCA-CAGCAAGA-3′ (forward) and 5′-ACTCAGTAGGAGTG-CAAGGGCTGTA-3′ (reverse); for *HYAL2* 5′-ACCAT GCACTCCCAGTCTACGTC-3′ (forward) and 5′-TCGCC AATGGTAGAGATGAGGTC-3′ (reverse); for *HAS1* 5′-ATCCTGCATCAGCGGTCCTC-3′ (forward) and 5′-CTG GTTGTACCAGGCCTCAAGAA-3′(reverse); for *HAS2* 5′-GTAGGCATCCAGCACTGGACAA-3′ (forward) and 5′-CCAGTAAATTCAGGCCACAGAACA-3′ (reverse); and for *HAS3* 5′-TTGCAATCCAGGCTGTTCTCA-3′ (forward) and 5′-GCTGTCCACCTTAGTGCTGGTTC-3′(reverse). In addition, OA synovial fibroblasts (P3) treated with IL-6 (0, 10, 50, or 100 ng/mL) in the presence of sIL-6R (100 ng/mL) for 48 hours were subjected to immunoblotting with anti-HYBID antibody (Proteintech, Rosemont, IL) and anti-GAPDH antibody (as a loading control) (ab125247; Abcam).

Effects of OA Synovial Fibroblasts with Tocilizumab

Serum-starved OA synovial fibroblasts at P3 were treated with humanized anti−IL-6R antibody (tocilizumab; 25 μg/mL) (Actemra; Chugai Pharmaceutical Co., Tokyo, Japan) or nonimmune human IgG (25 μg/mL; R&D Systems) in Dulbecco's modified Eagle's medium containing 1% fetal bovine serum for 1 hour prior to stimulation with IL-6 (0 or 100 ng/mL) and sIL-6R (100 ng/mL) for 48 hours, and then subjected to immunoblotting with anti-HYBID antibody (Proteintech). The effect of tocilizumab on HYBID mRNA and protein expression in serum-starved OA synovial fibroblasts at P1 was also tested by SYBR Green real-time PCR and immunoblotting analyses of the cells treated with

nonimmune human IgG (25 μg/mL) or tocilizumab (1, 5, or 25 μg/mL) for 24 hours and 48 hours, respectively.

RNA Interference for HYBID

A knockdown experiment was performed using two different siRNAs designed to target HYBID and non-silencing control RNAs, both of which were purchased from Thermo Fisher Scientific (Waltham, MA). OA synovial fibroblasts were transfected with these siRNAs using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific), and used for the experiment at 48 hours after transfection. Knockdown of the HYBID expression was confirmed by immunoblotting with anti-HYBID antibody (Proteintech) and anti-GAPDH antibody (Abcam).

Determination of HA-Degrading Activity, and Concentration and Molecular Size Distribution of HA

Cellular HA-degrading activity was examined by culturing siRNA-transfected or IL-6−treated OA synovial fibroblasts in medium containing fluoresceinamine-labeled HA H1 (FA-HA H1; 10 μg/mL) for 48 hours and applying the harvested medium to a Sepharose CL-2B column (1 × 60 cm; GE Healthcare, Tokyo, Japan) equilibrated with 0.5 mol/L NaCl in distilled water.[21] Calibration was performed using the FA-HA species including H1 (1800 kDa; average molecular size), M1 (1000 kDa), L1 (200 kDa), and S1 (50 kDa).[21] Concentrations of HA in the medium from OA synovial fibroblasts treated with IL-6 (0, 10, 50, and 100 ng/mL) in the presence of sIL-6R (100 ng/mL) for 48 hours were determined using a QnE HA ELISA (Biotech Trading Partners). The molecular size distribution of HA was determined by applying medium harvested from IL-6 (100 ng/mL) and sIL-6R (100 ng/mL)-treated or untreated synovial fibroblasts to the Sepharose CL-2B column and measuring HA concentrations of each fraction by HA ELISA.

Measurement of IL-6 in Culture Medium of OA Synovial Cells and Synovial Tissue Homogenates

Concentrations of IL-6 in the conditioned medium of the cells isolated from OA synovium without passage (P0) were measured using an ELISA kit (R&D Systems) according to the manufacturer's protocols. The culture medium was prepared by culturing cells (4 × 10^5 cells/6-cm dish) in 4 mL of Dulbecco's modified Eagle's medium containing 1% fetal bovine serum for 24 hours. Similarly, OA synovial tissues were homogenized in phosphate-buffered saline containing IGEPAL (CA-630; Sigma-Aldrich), and then IL-6 levels in the homogenate supernatants were determined by ELISA kit after determination of the protein concentrations using Protein Quantification Kit-Wide Range (DOJINDO, Kumamoto, Japan).

Statistical Analysis

All data were analyzed using IBM SPSS Statistics software version 21.0 (IBM, Armonk, NY) and expressed as means ± SD. For comparisons of the HYBID and TMEM2 expression levels between normal and OA synovial samples, the U-test was used. Comparisons involving more than 3 groups in the experiments using OA synovial fibroblasts were performed by the 1-way analysis of variance, followed by the Bonferroni test. Statistical significance was determined by t-test. Correlations were examined by Pearson test. $P < 0.05$ was considered significant.

Results

Expression and Tissue Localization of HYBID in OA Synovium

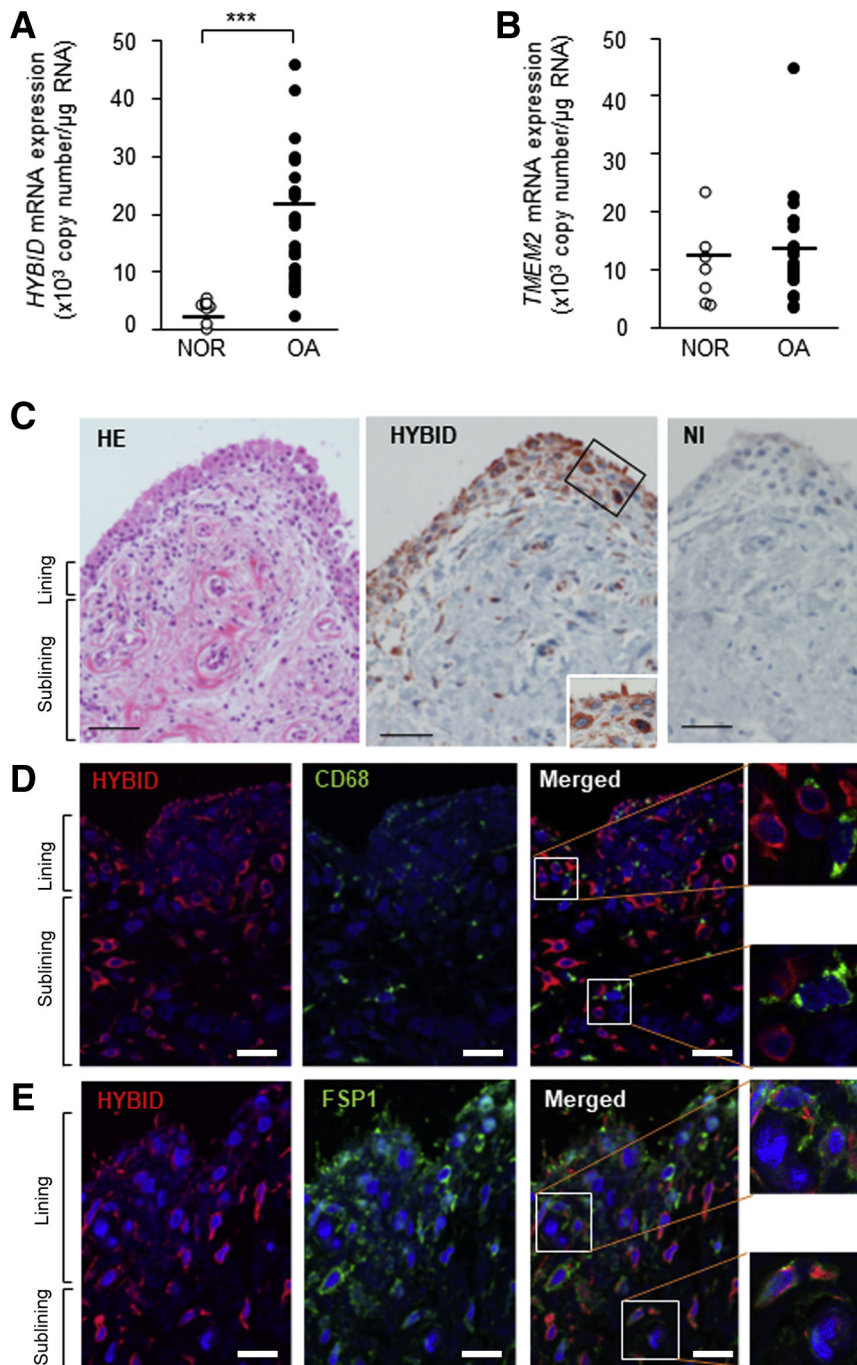This study measured the mRNA expression levels of HYBID and TMEM2, and found that HYBID is

Figure 1 Expression of hyaluronan (HA)-binding protein involved in HA depolymerization (HYBID) and transmembrane protein 2 (TMEM2), and tissue localization of HYBID in osteoarthritic (OA) synovium. **A** and **B:** Copy numbers of *HYBID* and *TMEM2* transcripts in control normal (NOR) and OA synovial samples. The copy numbers were determined by quantitative real-time PCR assay with standard curves generated from reference plasmids. Symbols represent individual subjects, and **horizontal lines** indicate means. **C:** Immunohistochemistry for HYBID in OA synovial tissue. Paraffin sections of OA synovium were stained with hematoxylin and eosin (HE) or immunostained with anti-HYBID antibody (HYBID) or nonimmune IgG (NI). **Boxed area** is shown at higher magnification in the **inset**. **D:** Double immunofluorescence staining of HYBID and CD68. Paraffin sections of OA synovial tissue were subjected to double-immunofluorescence staining for HYBID (red) and CD68 (green). **E:** Double-immunofluorescence staining of HYBID and fibroblast-specific protein 1 (FSP1). Paraffin sections of OA synovial tissue were subjected to double-immunofluorescence staining for HYBID (red) and FSP1 (green). $n = 8$ NOR samples (**A** and **B**); $n = 27$ OA synovial samples (**A** and **B**). ***$P < 0.001$ (U-test). Scale bars: 50 μm (**C**); 25 μm (**D** and **E**).
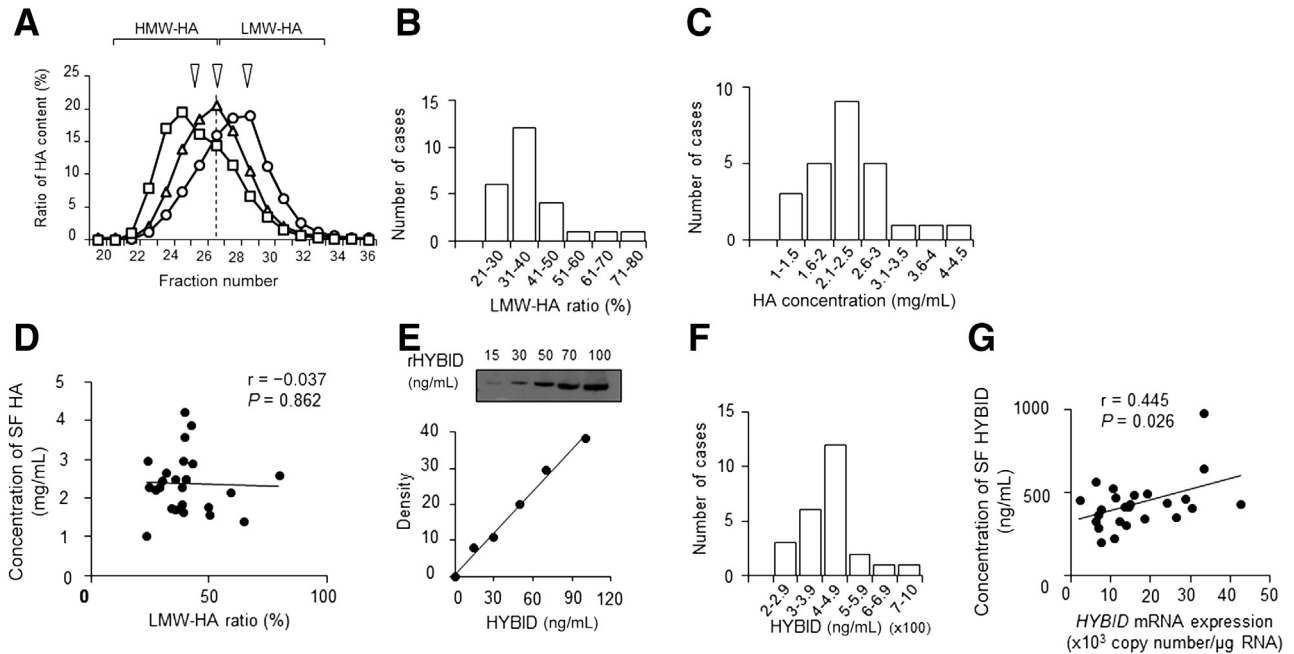
Figure 2 Measurement of molecular weight distribution and concentrations of hyaluronan (HA) and concentrations of hyaluronan (HA)-binding protein involved in HA depolymerization (HYBID) in osteoarthritic (OA) synovial fluids. **A:** Three representative cases of size distribution pattern of HA in OA synovial fluids. Molecular weights are determined by size-exclusion chromatography, and amount of HA in each fraction is shown as the ratio (%) to total HA amount (100%). High-molecular-weight (HMW)-HA and lower-molecular-weight (LMW)-HA were defined as HA species above and below 1000 kDa (**dashed line**), respectively. **Arrowheads** indicate elution peaks of sodium HA species with 2500 kDa, 1000 kDa, and 150 kDa from left to right. **B:** Distribution of OA cases with different ratios (%) of LMW-HA in synovial fluids. **C:** Distribution of OA cases with different HA concentrations in synovial fluids. **D:** Correlation between ratios of LMW-HA and HA concentrations in OA synovial fluids (SF). Pearson correlation coefficient analysis. **Diagonal line** shows the estimated regression line superimposed on the scatter diagram. **E:** The standard curve for HYBID protein. Different amounts of recombinant HYBID (rHYBID) were subjected to immunoblotting for HYBID, and densitometric analysis of the immunoreactive bands was performed. The line represents the trendline of the calibration curve. **F:** Distribution of OA cases with different HYBID concentrations in OA synovial fluids. HYBID concentrations in the synovial fluids were determined by immunoblotting according to the standard curve. **G:** Correlation between the HYBID mRNA expression levels (mRNA copy numbers per µg RNA) in OA synovial tissue and the HYBID protein concentrations in OA synovial fluids (SF) by Pearson correlation coefficient analysis. Diagonal line shows the estimated regression line superimposed on the scatter diagram. $n = 25$ OA cases (**B**, **C**, and **F**).

significantly 5.5-fold higher in OA synovium ($20.2 \pm 12.1 \times 10^3$ copies per µg of total RNA) than in normal control synovium ($3.7 \pm 1.8 \times 10^3$ copies per µg of total RNA) ($P < 0.001$) (Figure 1A). By contrast, the expression level of TMEM2 did not differ between OA ($13.0 \pm 8.1 \times 10^3$ copies per µg of total RNA) and control synovium ($11.8 \pm 7.1 \times 10^3$ copies per µg of total RNA) (Figure 1B). Immunohistochemistry showed that HYBID is localized mainly to synovial lining cells and also sublining cells and endothelial cells of capillaries in the OA synovium (Figure 1C), whereas only background staining was detected by immunostaining with nonimmune IgG (Figure 1C). Double immunofluorescence staining of HYBID and CD68 or that of HYBID and fibroblast-specific protein 1 in OA synovial tissue demonstrated that HYBID is positively immunostained in CD68-negative and fibroblast-specific protein 1−positive synovial lining cells and sublining cells (Figure 1, D and E). Because synovial lining cells are composed of macrophage-like type A cells and fibroblast-like type B cells[27] and sublining cells contain fibroblasts and macrophages in OA synovium,[28,29] the data suggest that HYBID could be expressed mainly by fibroblast-like type B

cells and fibroblasts in the lining and sublining cell layers, respectively.

Measurement of Molecular Weight Distribution of HA and HYBID Concentration in OA Synovial Fluids

The molecular weight distribution of HA in OA synovial fluids was determined by size-exclusion chromatography. The peak of HA molecular weight and the distribution of HA species varied among the samples (Figure 2A). Because a previous study has demonstrated that the weight % of HA below 1000 kDa is associated with progression of knee OA,[8] the ratios (%) of HA species below 1000 kDa (ie, LMW-HA) to total HA, which is composed of LMW-HA below 1000 kDa and HMW-HA over 1000 kDa, were calculated. The LMW-HA ratios ranged from 23% to 80% in the samples, and the median value was 38.6% (mean $\pm$ SD, $39.7 \pm 13.1\%$) (Figure 2B). HA concentrations in OA synovial fluids determined by ELISA ranged from 1.0 mg/mL to 4.2 mg/mL, showing the median value of 2.3 mg/mL (mean $\pm$ SD, 2.4 ± 0.8 mg/mL) (Figure 2C). There was no correlation between the LMW-HA ratios and

the HA concentrations in OA synovial fluids (Figure 2D). To evaluate concentrations of HYBID in OA synovial fluids, the samples of synovial fluid were subjected to immunoblotting for HYBID, and band densities determined by densitometric analysis was compared with the standard curve obtained using recombinant HYBID (Figure 2E). The concentrations were distributed from 204 ng/mL to 982 ng/mL with a median value of 420 ng/mL (mean ± SD, 432 ± 152 ng/mL) (Figure 2F). Importantly, a direct correlation was obtained between the concentrations of HYBID protein in OA synovial fluid and the HYBID mRNA expression levels (copy numbers per μg of total RNA) in OA synovium (Figure 2G).

Correlation of HYBID and TMEM2 Expression with HA Depolymerization in OA Synovial Fluids

The mRNA expression levels of HYBID (copy numbers per μg of total RNA) in OA synovial tissue directly correlated with the LMW-HA ratios in synovial fluids (r = 0.511; P = 0.009) (Figure 3A), although there was no positive correlation between the mRNA expression levels of HYBID and the HA concentrations in synovial fluids (r = −0.386; P = 0.057) (Figure 3B). Similarly, the concentrations of HYBID protein in synovial fluids directly correlated with the LMW-HA ratios in synovial fluids (r = 0.764; P < 0.001) (Figure 3C), but not with the HA concentrations in synovial fluids (r = 0.012; P = 0.956) (Figure 3D). Neither the mRNA nor protein expression levels of HYBID showed positive correlations with the serum concentrations of HA (Figure 3, E and F). In addition, the serum HA concentrations did not correlate with the HA concentrations or the LMW-HA ratios in synovial fluids (Figure 3, G and H). When correlations of the mRNA expression levels of TMEM2 (copy numbers per μg of total RNA) in OA synovial tissue with the LMW-HA ratios in synovial fluids, the HA concentrations in synovial fluids, or the serum concentrations of HA were examined, no positive correlations were obtained (Supplemental Figure S1).

Effects of Factors on HYBID and TMEM2 Expression in OA Synovial Fibroblasts

Because HYBID was originally discovered by microarray analysis of dermal fibroblasts as a gene that is up-regulated and down-regulated by histamine and TGF-β1, respectively,[17] this study first examined the expression by quantitative real-time PCR in OA synovial fibroblasts at P3 treated with these factors, and confirmed up-regulation by histamine and down-regulation by TGF-β1 (Figure 4A). By treating OA synovial fibroblasts with 10 other factors including IL-6, IL-1α, IL-8, TNF-α, VEGF, bFGF, PGE2, IGF-1, TNF-β, and CCL5, all of which are known to exist in joint tissues in arthritides such as OA,[12,30,31] this investigation demonstrated that only IL-6 significantly

promotes the HYBID mRNA expression (Figure 4A). Immunoblotting analysis confirmed the stimulative effect of IL-6 on HYBID protein expression (Figure 4B), and this stimulation was abrogated by treating them with anti−IL-6R antibody (tocilizumab) (Figure 4C), indicating that the stimulative effect is due to IL-6. In addition, the basal expression levels of HYBID mRNA and protein in non-treated OA synovial fibroblasts at P1 were significantly reduced by treatment with tocilizumab to about 45% of the original levels (Figure 4, D and E). When production levels of IL-6 by the cells (P0) directly isolated from OA synovial tissues were measured by ELISA, they produced IL-6 at a concentration of 262.2 ± 164.3 ng/mL for 24 hours (n = 3).

This study also examined effects of these factors on the TMEM2 expression in OA synovial fibroblasts, and found that the expression is slightly up-regulated by TNF-β and down-regulated by PGE2 and IGF-1 (Supplemental Figure S2). However, no changes in the expression of TMEM2, HYAL1, and HYAL2 was observed by treatment with IL-6 (Supplemental Figure S2). HA-degrading activity of OA synovial fibroblasts completely disappeared by knocking down the HYBID expression by siRNAs (Figure 5A), whereas the activity was up-regulated by treatment with IL-6 (Figure 5B). Under stimulation of OA synovial fibroblasts with IL-6, HA production tended to be slightly increased (Figure 5C), and the expression of HAS2, but not HAS1 or HAS3, was significantly enhanced by treatment with IL-6 (Figure 5D). When the molecular size distribution of newly produced HA was analyzed by the gel filtration chromatography, LMW-HA species appeared to be increased by IL-6−treated fibroblasts compared with those without treatment (Figure 5E).

Expression and Tissue Localization of IL-6 in OA Synovium, and Positive Correlation between the IL-6 and HYBID Expression Levels

Immunohistochemistry showed that IL-6 is produced mainly by synovial lining cells and sublining cells, whereas nonimmune IgG showed only background staining (Figure 6A). Double immunofluorescence staining of IL-6 and CD68 indicated that CD68-positive cells (macrophage-like type A cells) and CD68-negative cells (fibroblast-like type B cells) in the synovial lining cell layer and CD68-positive macrophages and CD68-negative fibroblasts in the sublining cell layer are positively immunostained (Figure 6B), suggesting that IL-6 is produced by both cell types of macrophages and fibroblasts. The IL-6 production levels in OA synovial tissue by ELISA were first measured, and their production levels were compared with the HYBID mRNA expression levels. As shown in Figure 6C, the HYBID expression levels (copy numbers per μg of total RNA) directly correlated with IL-6 concentrations.
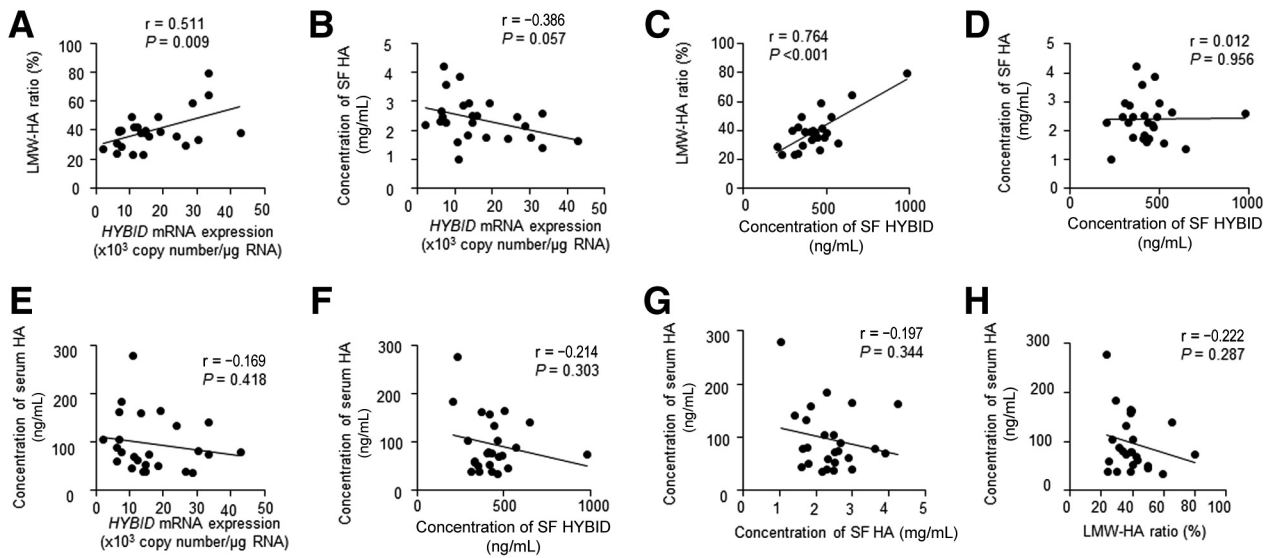
Figure 3 Correlations of hyaluronan (HA)-binding protein involved in HA depolymerization (HYBID) expression with hyaluronan (HA) depolymerization in osteoarthritic (OA) synovial fluids. **A** and **B:** Correlations of the HYBID mRNA expression levels (mRNA copy numbers per μg RNA) in OA synovial tissue with lower molecular weight (LMW)-HA ratios or HA concentrations in synovial fluids (SF). **C** and **D:** Correlations of the concentration of HYBID protein with LMW-HA ratios or HA concentrations in OA SF. **E** and **F:** Correlations of the HYBID mRNA expression levels (mRNA copy numbers per μg RNA) in synovial tissue or HYBID protein expression in OA SF with the concentrations of serum HA. **G:** Correlation between the HA concentrations in SF and the concentrations of serum HA. **H:** Correlation between the LMW-HA ratios in OA SFs and the concentrations of serum HA. Pearson correlation coefficient analysis was used for all of the analyses. **Diagonal lines** show the estimated regression lines superimposed on the scatter diagrams. r, correlation coefficient. n = 25 (**A**−**H**).
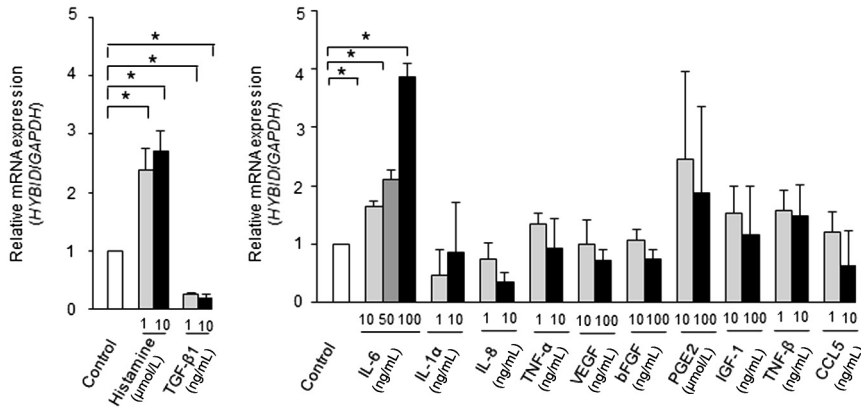
Discussion

This study provides the first evidence that HYBID is overexpressed in OA synovium compared with control normal synovium, whereas the TMEM2 expression is constitutively expressed in normal and OA synovia. Although the expression of murine TMEM2 was reported higher than that of HYBID in most mouse normal tissues including the brain, eye, heart, lung, liver, gastrointestinal tract, kidney, bladder, articular cartilage, and synovium,[32] only limited information was available for the expression and role of TMEM2 in human tissues under various pathophysiological conditions. The study data on the expression of HYBID and TMEM2 indicate the possibility that differently from mouse normal synovium, HYBID, but not TMEM2, may play a key role in HA degradation in OA.

Band et al[8] have recently reported that in a 3-year follow-up study of OA patients, the risk of knee OA progression is associated with the preponderance of LMW-HA (ie, the weight % of HA below 1000 kDa), but not average HA molecular weight, in synovial fluid. This study did not provide information about the mechanism on HMW-HA degradation in OA synovial fluids, although it was speculated that LMW-HA may be generated by the action of reactive oxygen species, which are known to randomly digest HA.[33] The present study demonstrated that the LMW-HA ratios in OA synovial fluid directly correlate with the expression levels of HYBID mRNA and protein in OA synovial tissue and synovial fluids, respectively. The double-immunofluorescence study on OA synovium has indicated that CD68-negative and fibroblast-specific protein
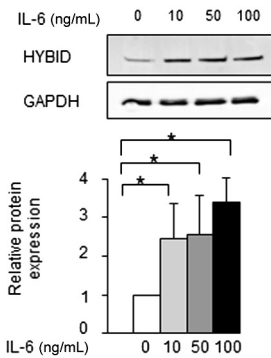
1−positive synovial lining and sublining cells express HYBID protein. It is previously shown that HYBID-mediated HA degradation in fibroblasts occurs through the clathrin-coated pit pathway without intracytoplasmic accumulation, releasing HA fragments into the extracellular milieu.[17] Altogether, HYBID overexpressed by synovial fibroblast-like type B cells and sublining fibroblasts in OA synovial membrane seems to play a central role in degradation of HMW-HA and subsequent accumulation of LMW-HA in OA synovial fluid, although implication of reactive oxygen species in HA degradation is not completely excluded.

HYBID expression was stimulated by IL-6. Similar to skin fibroblasts,[17] the HYBID expression was also promoted by histamine, but the up-regulation rate was higher by treatment with IL-6 than histamine. A recent study indicated that HYBID expression in OA chondrocytes is up-regulated by TNF-α,[34] but TNF-α exerted no influence in OA synovial fibroblasts. The different responses to these cytokines suggest that regulation of HYBID gene expression may be cell-type specific, although detailed mechanisms remain unclear. Because human anti−IL-6R antibody (tocilizumab) abrogated the IL-6−stimulated HYBID expression in OA synovial fibroblasts at P3 to the basal level and reduced the expression by about 45% of the original level in synovial fibroblasts at P1, HYBID expression in OA synovial fibroblasts can be ascribed, at least in part, to the IL-6/IL-6R signaling pathways. In contrast to HYBID, modulation of TMEM2 expression by these factors was minimal, showing only a slight increase by TNF-β and a decrease by PGE2 or IGF-1. The data seem to be in accord with the constitutive
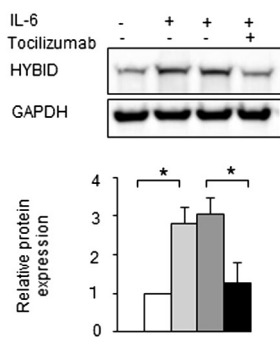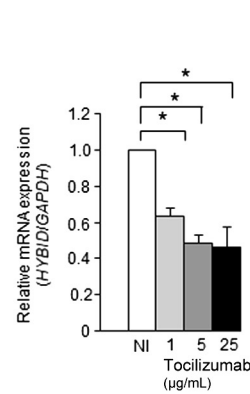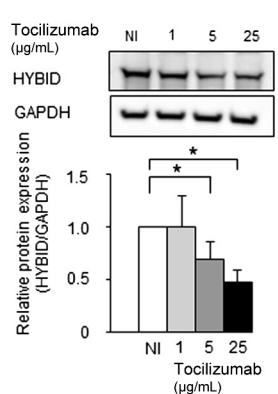
Figure 4 Promotion of the hyaluronan (HA)-binding protein involved in HA depolymerization (HYBID) expression by IL-6 in osteoarthritic (OA) synovial fibroblasts. **A:** Effects of histamine, transforming growth factor-β1 (TGF-β1), IL-6 (+100 ng/mL sIL-6R), IL-1α, IL-8, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), prostaglandin E2 (PGE2), insulin-like growth factor-1 (IGF-1), tumor necrosis factor β (TNF-β), and C-C motif chemokine ligand 5 (CCL5) on the *HYBID* mRNA expression in OA synovial fibroblasts. OA synovial fibroblasts (P3) were treated with these factors or vehicle alone for 24 hours, and the relative mRNA expression of *HYBID* to *GAPDH* was measured by quantitative real-time PCR using the ΔΔC$_T$ method. The average *HYBID*:*GAPDH* ratio in control OA synovial fibroblasts treated with vehicle was set at 1. **B:** Immunoblotting analysis of HYBID expression in OA synovial fibroblasts (P3) treated with IL-6. The cells were stimulated with IL-6 (0, 10, 50, and 100 ng/mL) in the presence of sIL-6R (100 ng/mL) for 48 hours, and cell lysates were subjected to immunoblotting with anti-HYBID antibody, followed by densitometric analysis. **C:** Effect of anti−IL-6R antibody (tocilizumab) on the IL-6−stimulated HYBID protein expression in OA synovial fibroblasts (P3). The cells were stimulated with IL-6 (0 or 100 ng/mL) in the presence of sIL-6R (100 ng/mL) with nonimmune IgG (25 μg/mL) or tocilizumab (25 μg/mL) for 48 hours, and cell lysates were subjected to immunoblotting with anti-HYBID antibody, followed by densitometric analysis. **D:** Effect of anti−IL-6R antibody (tocilizumab) on the HYBID mRNA expression in OA synovial fibroblasts (P1). The cells were treated with nonimmune human IgG (NI; 25 μg/mL) or tocilizumab (1, 5 and 25 μg/mL) for 24 hours, and the HYBID expression was determined by quantitative real-time PCR using the ΔΔC$_T$ method. **E:** Effect of anti−IL-6R antibody (tocilizumab) on the HYBID protein expression in OA synovial fibroblasts (P1). The cells were treated as described above for 48 hours and then subjected to immunoblotting with anti-HYBID antibody, followed by densitometric analysis. Data are expressed as means ± SD. n = 25 (experiments). *P < 0.05.

expression pattern of TMEM2 in normal and OA synovial tissues. In addition, no changes in the *HYAL1* and *HYAL2* expression in response to IL-6 suggest negligible effect of these enzymes on the IL-6−stimulated HA-degrading activity in OA synovial fibroblasts.

IL-6 is overproduced in OA synovium and increased in serum samples from OA patients.[35−37] IL-6 levels in OA synovial fluids (396 ± 508 pg/mL) are reported to be approximately 6.2-fold higher than that in healthy subjects (64 ± 120 pg/mL).[38] Although information about production levels at local tissues such as OA synovium was limited, data in the present study showed that primary

OA synovial cells (P0), which were directly isolated from OA synovial tissue containing heterogeneous cells including fibroblasts and inflammatory cells, produce a high level of IL-6 (262.2 ± 164.3 ng/mL) in the culture medium within 24 hours. Similar levels of IL-6 production (more than 100 ng/mL) are also reported in conditioned medium of OA synovial tissue organ culture.[39] Therefore, it seems likely that 100 ng/mL IL-6, which was utilized for stimulation of OA synovial fibroblasts in the present study and also commonly used for stimulation of cultured cells,[40−42] is an appropriate concentration to recapitulate the stimulation of HYBID expression in
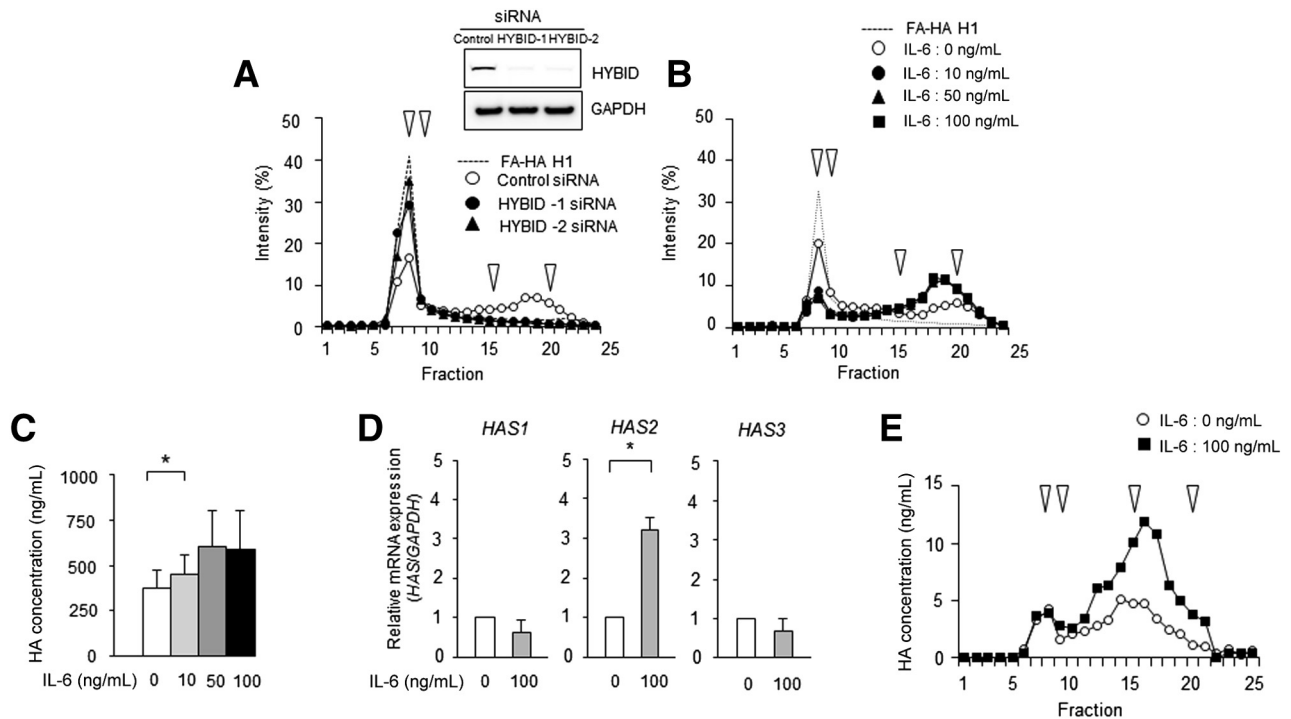
Figure 5 Hyaluronan (HA)-binding protein involved in HA depolymerization (HYBID)-mediated HA-degrading activity of osteoarthritic (OA) synovial fibroblasts and effects of IL-6 on HA-degrading activity and synthesized HA species. **A:** Abrogation of HA-degrading activity by knocking down HYBID expression with two different siRNAs. OA synovial fibroblasts transfected with siRNA for HYBID or nonsilencing RNA (Control) were cultured with FA-HA H1 for 48 hours, and HA-degrading activity was determined by gel filtration chromatography. Immunoblot analysis for HYBID and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (a loading control) is shown. **B:** HA-degrading activity in OA synovial fibroblasts treated with IL-6. OA synovial fibroblasts were stimulated with IL-6 (0, 10, 50, and 100 ng/mL) and sIL-6R (100 ng/mL), and cultured with FA-HA H1 for 48 hours. HA-degrading activity was determined by gel filtration chromatography. Enhanced HA-degrading activity appears to be saturated by treatment with 10 ng/mL IL-6 in this assay. **C:** HA content in the culture medium of OA synovial fibroblasts stimulated with IL-6. Cells were cultured in the absence or presence of IL-6 for 48 hours, and concentrations of HA produced in the medium were quantified by enzyme-linked immunosorbent assay. **D:** Quantitative real-time PCR analysis of the HA synthase *HAS1*, *HAS2*, and *HAS3* gene expression in OA synovial fibroblasts treated without or with 100 ng/mL IL-6 and sIL-6R. Relative expression levels were determined by normalizing to the expression level of *GAPDH* transcripts using the $\Delta\Delta C_T$ method. **E:** The size distribution of newly synthesized HA in OA synovial fibroblasts. Cells were cultured in the absence or presence of 100 ng/mL IL-6 and sIL-6R for 48 hours. The conditioned medium was subjected to gel filtration chromatography, and HA concentrations in each fraction were determined by enzyme-linked immunosorbent assay. **Arrowheads** indicate elution peaks of FA-HA with 1800 kDa, 1000 kDa, 200 kDa, and 50 kDa from left to right. Data are expressed as means ± SD. $n = 3$ (**C** and **D**). *$P < 0.05$.

synovial fibroblasts under pathological conditions including OA synovitis.

IL-6 is known to be secreted by many cell types including monocytes/macrophages, endothelial cells, smooth muscle cells and fibroblasts such as synovial fibroblasts.[38,39,43,44] Actually, the current immunohistochemical study showed that IL-6 is localized to CD68-positive macrophage-like type A cells and CD68-negative fibroblast-like type B cells in synovial lining cell layer, and also to CD68-positive macrophages and CD68-negative fibroblasts in sublining cell layer. These data suggest the IL-6 production by both macrophages and fibroblasts in the lining and sublining cell layers of OA synovium. The expression levels of IL-6 in OA synovial tissue homogenates directly correlated with the HYBID expression levels. Therefore, these studies support the hypothesis that IL-6 produced by the synovial cells stimulates synovial fibroblast-like type B cells in the lining cell layer and sublining fibroblasts to overexpress HYBID through an autocrine and/or paracrine pathway, and HYBID

depolymerizes HMW-HA, leading to accumulation of LMW-HA species in OA synovial fluid (Figure 6D).

There are several limitations in this study. One is that the OA and normal control synovial samples were not age-matched. Because no information is available on the effect of aging on the expression of HYBID and TMEM2 in human cells such as synovial fibroblasts, this study cannot exclude the possible involvement of aging in HYBID up-regulation in OA synovium. However, the expression profiles of HYBID and TMEM2 in synovial tissues and OA synovial fibroblasts treated with various factors suggest that the expression is differently controlled, showing the cytokine-inducible and constitutive expression of HYBID and TMEM2, respectively. The current study has shown that the concentrations of HYBID protein in OA synovial fluid directly correlate with the LMW-HA ratios in the synovial fluid, and considered that this finding suggests the possible involvement of HYBID in HA degradation. However, because the HA-degrading activity
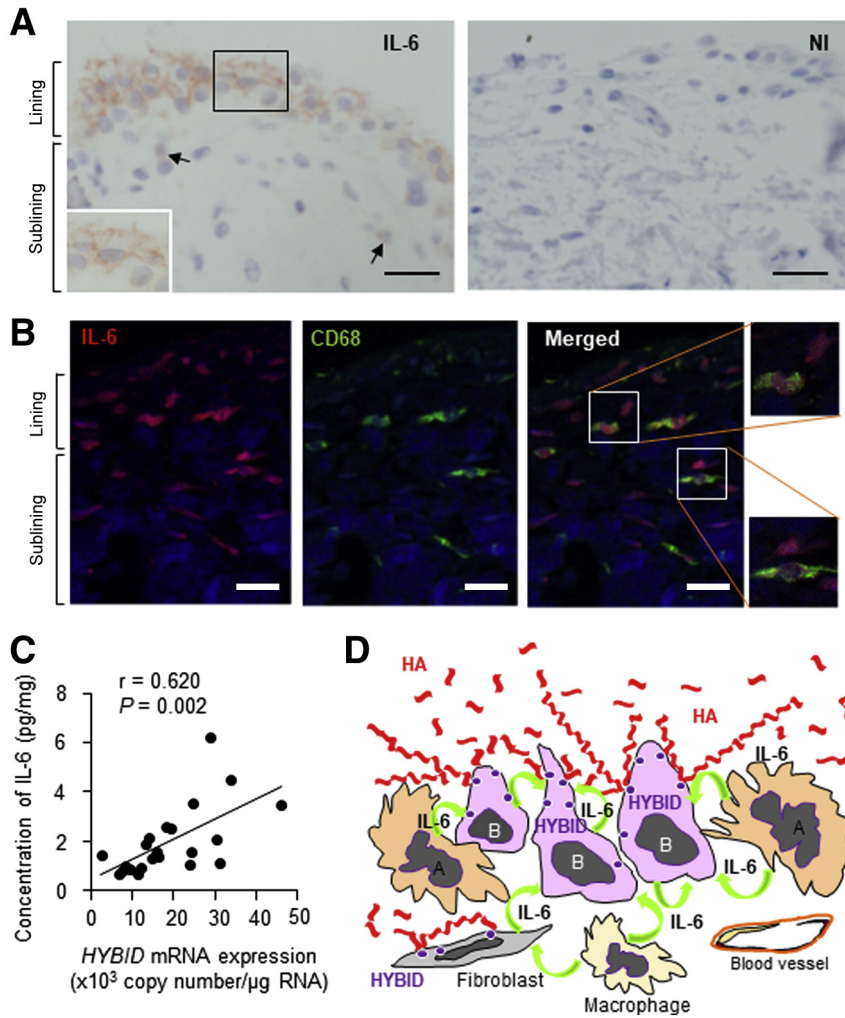
Figure 6 Tissue localization of IL-6 and correlation between the IL-6 concentrations and the hyaluronan (HA)-binding protein involved in HA depolymerization (HYBID) expression in osteoarthritic (OA) synovial tissue. **A:** Immunohistochemistry of IL-6 in OA synovial tissue. Paraffin sections of OA synovium was immunostained with anti—IL-6 antibody (IL-6) or nonimmune IgG (NI). **Boxed area** is shown at higher magnification in the **inset**. **Arrows** denote IL-6-positive cells in sublining cell layer. **B:** Double immunofluorescence staining of IL-6 and HYBID. Paraffin sections of OA synovial tissue were subjected to double-immunofluorescence staining for IL-6 (red) and CD68 (green). **C:** Correlation between the IL-6 concentrations and the HYBID mRNA expression (mRNA copy numbers per μg RNA) in OA synovial tissue. **Diagonal line** shows the estimated regression line superimposed on the scatter diagram. **D:** Schematic diagram showing the hypothesis on the HYBID expression and the HYBID-mediated HA degradation by an autocrine/paracrine pathway of IL-6 and HYBID in OA synovial tissue. $n = 22$ (**C**). Scale bars = 25 μm (**A** and **B**).

of secreted HYBID has not been demonstrated,[17] the function of HYBID in synovial fluid remains unclear. Nonetheless, the data showing the direct correlation between the HYBID mRNA expression in OA synovial tissue and the HYBID protein expression in the synovial fluid suggest that HYBID protein originates from OA synovial fibroblasts, and reflects the overexpression of HYBID by OA synovial fibroblasts. Another question is what factors other than IL-6 contribute to the up-regulation of the HYBID expression in OA synovial fibroblasts. The present study has shown that IL-6 is a strong stimulator for HYBID expression in OA synovial fibroblasts, but complete suppression of HYBID expression was not obtained by treating them with anti—IL-6R antibody. In addition, this study did not provide the *in vivo* data directly showing that IL-6 exerts an effect on the HYBID expression in OA synovium. As expected from the authors' previous study with skin fibroblasts,[17] histamine also stimulated HYBID expression. Detailed further studies are needed to clarify the regulation mechanisms of *HYBID* expression in OA synovial tissue.

In summary, this investigation has demonstrated that HYBID overexpressed by synovial fibroblast-like type B cells and sublining fibroblasts in OA synovium plays a key role in HA degradation in OA synovial fluid. Accumulated lines of evidence have established the notion that HMW-HA plays an essential role in maintaining the viscosity and functioning as shock-absorber in synovial fluid.[1,2] In addition, HMW-HA is known to have anti-inflammatory and antiangiogenic activity, whereas LMW-HA is proinflammatory and proangiogenic.[6,7] Therefore, maintaining HMW-HA in OA synovial fluid seems to be important, not only to avoid persistent damage of articular cartilage by mechanical stress enhanced due to HA degradation, but also to slow down the OA progression by attenuating synovitis. Because this study has provided the data that IL-6 is a stimulator of HYBID expression in OA synovium, IL-6 can be a molecular target in OA patients. Thus, therapeutic strategies may include application of IL-6 or IL-6R blockade and development of inhibitors to HYBID activity and/or molecules to selectively suppress the expression. However, deliberate and detailed analyses on the

characteristics and role of HYBID in pathophysiological conditions are necessary.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2020.01.003*.

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