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Expression and regulation of WISP2 in rheumatoid arthritic synovium

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Abstract

Numbers of growth factors expressed in the synovium deeply impact on the pathology of rheumatoid arthritis (RA). The WISP family was identified as growth factors, which are upregulated by WNT signaling. In the present study, we investigated expression pattern and regulatory mechanisms of WISPs in the synovium in patients with RA and osteoarthritis (OA). Among three members of WISP family, WISP2 mRNA was only preferentially detected in RA synovium by RT-PCR. WISP2 expression was immunohistochemically identified in RA fibroblasts in an extensive fibrotic area. WNT signaling-activated (s/aβ-catenin-expressing) synovial fibroblasts upregulated WISP2 at 2.9-fold, but -inactivated (Δβ-catenin-expressing) cells downregulated the expression. Quantitative RT-PCR demonstrated that WISP2 expression was increased upon 17-β-estradiol stimulation and synergistically enhanced by WNT signaling. These data demonstrate that the expression of WISP2 is synergistically upregulated in RA synovial fibroblasts by estrogen and WNT pathways, and suggest an involvement in the pathology of the disease.

Keywords: Rheumatoid arthritis; Osteoarthritis; Synovium; Synovial fibroblast; CCN; WISP-1; WISP-2; Estrogen; WNT; β-catenin

Rheumatoid arthritis (RA) is a systematic inflammatory and destructive arthropathy that affects 0.5–1% of the population, in a female/male ratio of 2.5/1. RA is characterized by the pathological changes of synovium, which include initial vasculitis of the joint, followed by edema, infiltration of inflammatory cells into the synovium, hyperplasia of the synovial lining, and development of the pannus. RA synovial cells take a central role in progression of the disease as they proliferate, promote angiogenesis, and degrade the extracellular matrix macromolecules. These aspects of RA synovial cells distinguish the disease from other arthritic conditions such as osteoarthritis (OA) [1–3]. Numbers of growth factors and cytokines are expressed in the RA synovium and documented to play a role in the pathology. However, no information is available about expression of the WISP family, which belongs to the CCN growth factor family and is upregulated by a signaling pathway of WNT. Recently, it is reported that certain members of the WNT family are expressed at higher levels than others RA versus OA tissue [4]. We also observed that β-catenin-mediated signaling pathway downstream of WNTs is activated in RA synovium (Imai K, et al., manuscript in preparation). Since the WNT signaling pathway activates many aspects of cells, including proliferation, angiogenesis, and extracellular matrix remodeling [5], it is intriguing to explore the expression pattern of WNT target genes in the RA synovium.

The CCN family member, named after the first three members discovered (CTGF, CYR61, and NOV), encodes four distinct domains, insulin-like growth factor binding protein domain, von Willebrand factor type C domain, thrombospondin type I domain, and cysteine

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knot (CK) domain. WISP1 (CCN4) and WISP2 (CCN5, rCop1, CTGF-L, and CTGF3) were identified as CCN members downstream of the WNT-β-catenin signaling pathway using subtractive hybridization between WNT1-transformed mammary epithelial cells and parental cells [6]. WISP2 lacks the C-terminal CK domain, which is considered to be important for the mitogenic activity of the family, thus, suggests that its function in pathophysiological process is different from other CCN proteins [7]. WISP3 (CCN6) is another member highly related to WISP1 and WISP2 in amino acid sequence and the genetic mutation causes progressive pseudorheumatoid dysplasia [8], but the biological role is a controversial issue [9,10]. CCN proteins are multidisciplinary growth factors and expressed in various pathological conditions. They stimulate cell proliferation and migration, angiogenesis, and tissue fibrosis [11]. These molecular activities appear likely to be compatible with the histological changes in the RA synovium. Therefore, WISPs and CCNs seem likely to be involved in many aspects of the disease if they are expressed in the synovium. However, the expression in the synovium of arthritic patients is not currently reported. In the present study, we examined the expression of WISPs and the regulatory pathway for the expression in RA synovium.

Materials and methods

Synovial tissues. Arthritic synovial tissues were obtained from patients undergoing operations for total knee joint or hip joint replacement at Keio University Hospital under informed consent to patients that was reviewed and approved by the Institutional Review Boards of Keio University and Nippon Dental University. Synovial tissues were subjected to experiments as follows: five knee joints of RA patients and four of OA patients for RNA isolation; 16 knee joints of RA, 14 knee joints of OA, and five hip joints of patients with femoral neck fracture for immunohistochemistry.

Synovial fibroblasts isolation and treatments. Fibroblasts isolated from two RA synovium by a standard method [12] were cultured in DMEM supplemented with 10% FBS for 4–6 passages. For estrogen treatments of cells, synovial fibroblasts (SFs) were maintained in phenol red-free medium containing charcoal-stripped serum to neglect the effect of endogenous estrogen and estrogen-like materials within culture medium [13]. After 5 days of culture in this medium, SFs stopped proliferation and permitted the small effects of 17-β-estradiol (E2) to be detected (10^{-11}–10^{-6} M). Stock solutions of E2 were prepared in ethanol with a final concentration of ethanol below 0.1%. To check the specificity and directivity of E2 on *WISP1* and *WISP2* transcription, SFs were pretreated with 10 μM estrogen antagonist (ICI 182,780, Tocris, Bristol, UK), 10 μg/ml of cycloheximide (Calbiochem, Carmstadt, Germany), or 5 μg/ml of actinomycin D (Sigma–Aldrich, St. Louis, MA) for 1 h, and cultured for 24 h in the presence of E2.

Transfection of β-catenin mutants. Full-length cDNAs of *S37A-β-catenin* (*s/aβ-catenin*) or *Δ19-β-catenin* (*Δβ-catenin*) were subcloned into pcDNA3 expression plasmid. s/aβ-catenin was generated by site-directed mutagenesis of an S^{37} to A and encodes a stable mutant accelerating the β-catenin-dependent signaling [14]. An internal deletion mutant of aa 346–364 of β-catenin (*Δβ-catenin*) disrupts binding to transcription factors and abrogates the activities [15]. Fibroblasts isolated from RA synovium were transfected plasmids using Lipofectamine-Plus (Invitrogen, San Diego, CA), and the stably expressing cells were selected by 100 μg/ml of G418.

Reverse transcription (RT)-PCR. Total RNA was isolated from RA or OA synovium using TRIzol (Invitrogen) and reverse transcribed by SuperScript II (Invitrogen) with oligo(dT) primer. After RT reaction, PCR was performed with gene-specific primer sets for *WISPs* or *GAPDH*, and with AmpliTaq DNA polymerase (ABI, Brunchburg, NJ). After 5 min denaturation at 94 °C, 30 cycles of amplification (94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min) followed by a final extension at 72 °C for 5 min. Amplicons were analyzed on a 2% agarose gel. Specific primer sets to each gene were used: 5′-GAGCCTGCC AAGAGGTACTG-3′ (forward) and 5′-CAAGCAGGACAAGGGA GAAG-3′ (reverse) for *WISP1*, 5′-TTTCTGGCCTTGTCTCTTCC-3′ (forward) and 5′-GTGTGTGTAGGCAGGGAGTG-3′ (reverse) for *WISP2*, 5′-ATCTGTGCCAAGCAACCAGG-3′ (forward) and 5′-AT GTTCTGGAGCAGGGAGTC-3′ (reverse) for *WISP3*, and 5′-GTC AGTGGTGGACCTGACCT-3′ (forward) and 5′-AGGGGAGCTTC AGTGTGGTG-3′ (reverse) for *GAPDH*.

Immunohistochemistry. Formalin-fixed and paraffin-embedded synovial tissue specimens were subjected to immunohistochemistry as previously described [18]. A primary antibody specific to WISP2 (4 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) and biotinylated secondary rabbit anti-goat IgG (Vector Laboratory, Burlingame, CA) was used. Immunostaining of WISP2 in arthritic synovial specimens was graded by measuring the ratio (%) of immunoreactive fibroblasts, where 0 is not staining, 1+ is <10%, 2+ is 11–30%, 3+ is 31–50%, and 4+ is >51% staining of cells. One-way analysis of variance or Mann–Whitney *U* test was used for analyses of relationship between WISP2 immunoreactivity and histological scores of synovial tissues.

Real-time quantitative RT-PCR. For quantitative analysis of *WISP1* and *WISP2*, and *GAPDH* expression levels, total RNA extracted was subjected to real-time RT-PCR using AmpliTaq Gold DNA polymerase (ABI) and SYBR Green dye 1 (ABI), and quantified the expression levels by the ABI 7000HT sequence detection system (Applied Biosystems, Foster City, CA) in duplicate [16]. The computer-assisted program was used for primer design (Primer Express version 1.5a, Applied Biosystems). Nucleotide sequences of the primers were as follows: for WISP1, 5′-AGGTATGGCAGAGGTGCAAG-3′ (forward) and 5′-GTGTGTGTAGGCAGGGAGTG-3′ (reverse); for *WISP2*, 5′-AGTTTTCTGGCCTTGTCTCT-3′ (forward) and 5′-AGAAGCGGTTCTGGTTGGAC-3′ (reverse); and for *GAPDH*, 5′-AATCCCATCACCATCTTCCAG-3′ (forward) and 5′-CC TTCTCCATGGTGGTGAAGAC-3′ (reverse). Samples with high starting copy number of the target gene show an increase in fluorescence early in the PCR process, resulting in a low threshold cycle (C_t) number when standardized with the internal control *GAPDH* (ΔC_t).

Results

Expression of WISPs in synovium

To dissect expression pattern of the *WISP* family (*WISP1–3*) in arthritic synovial tissues, we examined the expression by RT-PCR (Fig. 1). Although no differential expression of *WISP1* between RA and OA synovium was observed (2/5 and 1/4 cases, respectively), *WISP2* mRNA was preferentially detected in tissues of RA patients. A 415 bp of single band was amplified 5/5 RA and 1/4 OA synovial tissue samples. Expression of *WISP3* was limited to one sample of