Expression and function of wingless and frizzled homologs in rheumatoid arthritis

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Rheumatoid arthritis (RA) is accompanied by synovial inflammation, proliferation, and cartilage destruction. The reasons the activation of synovial fibroblasts often persists despite antiinflammatory therapy are not known. One possibility is that the synovial membrane becomes gradually repopulated with immature mesenchymal and bone marrow cells with altered properties. To explore this hypothesis, we have investigated the expression in RA synovial tissues of various embryonic growth factors from the wingless (*wnt*) and frizzled (*fz*) families, which have been implicated in cell-fate determination in both bone marrow progenitors and limb-bud mesenchyme. Reverse transcriptase–PCR analysis revealed expression of five *wnt* (*wnt1*, *5a*, *10b*, *11*, and *13*) and three *fz* (*fz2*, *5*, and *7*) isoforms in RA synovial tissues. Osteoarthritis synovial tissues expressed much less *wnt5a* and *fz5*. Northern blotting confirmed the overexpression of *wnt5a* and *fz5* in RA synovial tissues, in comparison to a panel of normal adult tissues. Compared with normal synovial fibroblasts, cultured RA fibroblast-like synoviocytes expressed higher levels of IL-6, IL-8, and IL-15. Transfection of normal fibroblasts with a *wnt5a* expression vector reproduced this pattern of cytokine expression and stimulated IL-15 secretion. These results suggest that the unusual phenotypic properties of RA fibroblasts may be attributable partly to their replacement with primitive fibroblast-like synoviocytes with characteristics of immature bone marrow and mesenchymal cells. Clear delineation of the signaling pathway(s) initiated by the *wnt5a*/*fz5* ligand–receptor pair in the RA synovium may yield new targets for therapeutic intervention.

synovium | fibroblast | cytokine | *wnt*

The normal synovium contains both bone marrow-derived macrophages and mesenchymal fibroblasts. The development of rheumatoid arthritis (RA) is associated with infiltration of blood leukocytes, angiogenesis, and activation/proliferation of fibroblasts in the synovial lining and underlying connective tissue to form a pannus, which destroys articular cartilage (1–4). Inflammatory stimuli, especially tumor necrosis factor α (TNF-α) and IL-1, have been reported to activate synovial fibroblasts and induce them to produce other cytokines, chemokines, proteolytic enzymes, and inflammatory mediators (5–8). However, rheumatoid synovial fibroblast activation sometimes continues despite antiinflammatory therapy. It has been suggested that in the inflammatory stages of RA, channels between the bone marrow and the synovium might allow migration into the synovium of bone marrow stem cells with different properties (9). Cytokines can stimulate the proliferation of such immature mesenchymal cells. The gradual replacement of mature fibroblasts with more primitive cells could alter the regulation of synoviocyte activation. It is important, therefore, to know whether the fibroblast-like synoviocytes from long-standing RA express growth modulators characteristic of immature bone marrow or mesenchyme, and to determine whether such factors could play a role in synovial fibroblast activation and pannus formation.§

The wingless (*wnt*) and frizzled (*fz*) family genes were first characterized in *Drosophila*, where they specify tissue patterning and cell-fate determination during embryonic development (10, 11). The homologous *wnt* and *fz* family members in mammals have also been reported to function in tissue specification (12, 13). The secreted *wnt* family proteins are ligands for the *fz* family gene products, which resemble classical G protein-coupled cell surface receptors (14, 15). All *wnt* and *fz* family members, however, have not been biochemically characterized as ligand–receptor pairs. Mammals express at least 13 different *wnt* genes and eight or more *fz* genes. Besides being essential for cell-fate determination, various *wnt* and *fz* family members also influence cell proliferation and the response to activating stimuli. For example, *wnt1* causes stabilization of cytosolic β-catenin, which regulates both cell proliferation and cell adhesion (16). *wnt10b* has been suggested to influence hematopoiesis and cell-growth regulation (17, 18). Elevated expression of *fz2* has been linked to tissue regeneration and hyperplasia in an animal model of atherosclerosis (19). Overexpression of *fz7* has been reported to contribute to cell proliferation in esophageal cells (20). In long-standing RA, *wnt5a* may be of special interest because of its reported role in bone marrow stem cell development and in protein kinase C (PKC) activation (21, 22). In the present experiments, we report the expression of the *wnt5a-fz5* ligand–receptor pair (23) in the rheumatoid synovium and present evidence suggesting its involvement in fibroblast activation.

Materials and Methods

Tissue Collection and Processing. Surgically removed synovial tissue specimens were obtained frozen from the Arthritis and Rheumatism Branch, National Institutes of Health (Bethesda, MD) and the University of California at San Diego Multipurpose Arthritis and Musculoskeletal Disease Center and processed quickly for RNA extraction. Transfer of material was approved by the appropriate Human Subjects Committees.

RNA Analysis from Tissue Samples. Total RNA was extracted from fresh rapidly thawed tissue samples by using RNA-zol (GIBCO). Because it was difficult to obtain sufficient normal synovial tissue as a negative control, we compared osteoarthritis (OA) and RA surgical specimens. Different pairs of gene-specific primers based on sequences of cloned human isoforms of the *wnt* and *fz* genes were used for reverse transcriptase–PCR (RT-PCR) analysis. One microgram of RNA was used from each specimen, and 30 cycles of PCR were

Abbreviations: RA, rheumatoid arthritis; TNF-α, tumor necrosis factor α; OA, osteoarthritis; RT-PCR, reverse transcriptase–PCR; HA, hemagglutinin; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C.

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carried out. Expression of β-actin was used to normalize the expression of the different *wnt* and *fz* isoforms. The following list summarizes the primer pairs used:

fz2: 5′-cagcgtcttgcccgaccagatcca-3′(reverse); 5′-ctagcgccgctcttcgtgtacctg-3′ (forward). *fz5*: 5′-ttcatgtgcctggtggtgggc-3′ (forward); 5′-tacacgtgcgacagggacacc-3′ (reverse)

wnt1: 5′-cacgacctcgtctacttcgac-3′ (forward); 5′-acagacactcgtgcagtacgc-3′ (reverse). *wnt5a*: 5′-acacctctttccaaacaggcc-3′ (forward); 5′-ggattgttaaactcaactctc-3′ (reverse)

wnt10b: 5′-gaatgcgaatccacaacaacag-3′ (forward); 5′-ttgcggttgtgggtatcaatgaa-3′(reverse). *wnt13*: 5′-aagatggtgccaacttcaccg-3′ (forward); 5′-ctgccttcttgggggctttgc-3′(reverse) β-actin: 5′-caggtgatcacgataggtaacga-3′ (forward); 5′-catccacatttgttggaacgt-3′(reverse).

The expression of the *wnt5a* and *fz5* isoforms in RA tissues was confirmed by Northern blotting by using labeled gene-specific fragments corresponding to specific *wnt* and *fz* isoforms. The probes were obtained by RT-PCR and then labeled with [^{32}P]-dCTP by using a nick translation kit (GIBCO). The same radioactive probes were used for hybridization to a commercially available multiple tissue Northern blot (CLONTECH). The intensities of *wnt* and *fz* hybridization were compared with β-actin hybridization intensity.

Transfection of Synovial Fibroblasts with a *wnt5a* Expression Vector. The human *wnt5a* cDNA was subcloned into the expression vector pcDNA3 (Invitrogen) at the *Eco*RI and *Eco*RV restriction sites. Lipofectamine (GIBCO) was used for transfecting synovial fibroblasts with the *wnt5a* expression construct and the empty vector. Briefly, synovial fibroblasts were plated 1 day before transfection in 6-well tissue culture plates (≈2×10^5 cells/ml, 1 ml each well) and incubated at 37^0 in 5% CO_2. Plasmid DNA (≈1 μg) was complexed with 6 μl of Lipofectamine in 200 μl of serum-free antibiotic-free culture medium in each sterile tube for 30° min, after which 800 μl of medium containing 5% FBS was added. The cells in each well of the 6-well plate were washed with sterile PBS and then incubated with 1 ml of the transfection mix for 7–12 h. One milliliter of culture medium with 10% FBS was added to each well after 12 h. The transfection mix was replaced by fresh culture medium containing 10% FBS after 24 h. For transient transfection, cells were collected for assay after 48 h. For obtaining stable transfectants, 48 h after transfection, cells were washed, trypsinized, suspended in culture medium with 10% serum and 400 μg/ml of G418 (geneticin; selects for neomycin resistance gene) and plated in a 24-well plate with 500 μl in each well at a density of 2–6 cells per well. These cultures were maintained for 4–6 wk, fresh medium being added every other week. Cells from two wells of a 24-well plate were subsequently passaged ≈4 times separately in selective medium containing G418. One stable cell line was subsequently used for experiments.

Cytokine Assays. RNA was extracted from RA fibroblasts and *wnt5a* and empty vector transfected synovial fibroblasts, by using RNA-zol. IL-6-, IL-8-, IL-15-, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH)-specific primers were used for estimating the level of expression of the corresponding messages in the different cell populations by RT-PCR. G3PDH expression was used as an internal control for all cell types. The following list summarizes the primer pairs corresponding to the IL-6, IL-8, IL-15, and G3PDH genes used for PCR analysis:

IL-6: 5′-cagatgagtacaaaagtcctga-3′(forward); 5′-ctacatttgccgaagagccc-3′(reverse). IL-8: 5′-cagttttgccaaggagtgctaa-3′(forward); 5′-aacttctccacaaccctctgc-3′(reverse)

IL-15: 5′-gagttacaagttatttcacttgag-3′(forward); 5′-caagaagtgttgatgaacatttgg-3′(reverse). G3PDH: 5′-accacagtccatgcccatcac-3′(forward); 5′-tccaccaccctgttgctgta-3′(reverse).

Analysis of *wnt5a* Protein [Hemagglutinin (HA)-Tagged] Expression in Transfected Cells by Western Blotting. Protein extracts were prepared from synovial cells transfected with the HA-tagged *wnt5a* expression construct, 48 h after transfection. After removal of medium and washing with PBS, 1 ml of lysis buffer (20 mM Tris·Hcl, pH 7.5/500 mM NaCl/1% Triton X-100/1 mM EDTA/50 mM DTT/2 mM PMSF) was added directly to the adherent cells in a 6-well tissue culture plate (150–160 μl was added to each well). The lysed mix was collected into a microfuge tube and assayed for total protein by using Bradford's reagent (Bio-Rad). Approximately 36 μg of protein from the *wnt5a*-HA transfectants and untransfected cells was used for Western blotting. A rabbit polyclonal anti-HA (Upstate Biotechnology, Lake Placid, NY) was used as a primary antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG (Transduction Laboratories, Lexington, KY) was used as a secondary antibody for the analysis, followed by visualization with a chemiluminescence system (Amersham Pharmacia Biotech).

Quantitation of IL-15 Protein Expression by ELISA. ELISA was performed with supernatants from *wnt5a*-transfected synovial fibroblasts (stable transfectants), RA synovial fibroblasts, and normal synovial fibroblasts. About 3 μg supernatant protein was added to microtiter plates that had been coated previously with an anti-human IL-15 mAb (2 μg/ml). After overnight incubation and washing, about 1 μg/ml of a goat anti-human IL-15 polyclonal antibody was added to each well (R & D Systems). The secondary antibody used was horseradish peroxidase-conjugated donkey anti-goat IgG (The Jackson Laboratory).

Construction of *wnt5a* Expression Construct. The *wnt5a* cDNA was amplified from human fetal brain cDNA (CLONTECH) by using the primers 5′-aaccttgaattcagttgctttggggatggctgg-3′(forward) and 5′-aaccttgatatccacccactacttgcacacaaac-3′ (reverse) for PCR. The amplified cDNA was subcloned into pcDNA3 into the *Eco*RI and *Eco*RV restriction enzyme sites. This expression vector was used for all functional studies. For the purpose of protein expression studies, an HA tag was introduced in-frame with the *wnt5a* coding sequence into the *Eco*RV and *Xho*I sites of a modified *wnt5a* expression construct. First, a modified reverse primer without the stop codon and the original forward primer were used to isolate by PCR the *wnt5a* cDNA from the fetal brain cDNA template. The modified *wnt5a* cDNA was then introduced into the *Eco*RI and *Eco*RV sites of pcDNA3. The HA tag with a stop codon incorporated at the end was then introduced in-frame with the *wnt5a* into the *Eco*RV and *Xho*I sites of the modified expression construct. The amino acid sequence of the HA-tag used was KAFSNCYPYDVPDYASLRS (24).

Results

Expression Pattern of *wnt* and *fz* mRNAs in RA vs. OA Tissue. The levels of expression of different *wnt* and *fz* isoforms in RA vs. OA synovial tissues were compared first by RT-PCR. Table 1 summarizes the results, and Fig. 1 depicts some of the amplified *wnt* and *fz* isoform products. Of the five *fz* isoforms tested, we detected the expression of *fz2*, *fz5*, and *fz7* in all five RA tissue specimens. The same specimens also expressed *wnt1*, *wnt5a*, *wnt11*, and *wnt13*. *wnt10b* was found in three of five RA specimens. A comparison of the RA and OA tissue specimens revealed that the isoforms preferentially expressed in the RA tissues comprised the *wnt5a* and *fz5* ligand–receptor pair. All of the tissue specimens expressed comparable levels of the housekeeping gene β-actin. The *wnt* and *fz* PCR products were sequenced, confirming their identities.

Northern blotting studies confirmed the preferential expression of *wnt5a* and *fz5* in RA synovial tissues compared with other human adult tissues (Fig. 2). Whereas three different RA tissues

Table 1. Differential expression of *wnt* and *fz* isoforms in synovial tissue specimens

	OA	RA
fz2	+/−	+
fz3	nd	−
fz5	−/+	+
fz6	nd	−
fz7	+	+
wnt1	+/−	+
wnt2	nd	−
wnt5a	−/+	+
wnt10b	+/−	+/−
wnt11	+	+
wnt13	+/−	+

Results shown are based on RT-PCR analysis of RNA obtained from five RA and five OA tissue specimens. −/+, detected in one to two specimens. +/−, detected in three to four specimens. +, detected in all. −, detected in none. nd, not determined.

expressed high levels of the characteristic 5-kb *fz5* and *wnt5a* mRNAs, 12 different human adult tissues expressed either much less or almost no detectable mRNAs of the same size. The latter results are consistent with previous data showing that *wnt5a* message in adult tissues is difficult to detect because of short half-life. As a positive control for *wnt5a* expression, we tested RNA from human fetal fibroblasts. The levels of *wnt5a* message were nearly equivalent in the RA synovial tissue and the embryonic cells when normalized to the housekeeping gene β-actin (Fig. 2).

Analysis of the Function of *wnt5a* in Rheumatoid Synovium. To determine whether isolated fibroblast-like cell lines established from the rheumatoid synovium continued to express *wnt5a*, RT-PCR studies were carried out. Compared with normal fibroblasts, the RA fibroblast cell lines expressed more *wnt5a* message after 6–10 passages in tissue culture (Fig. 3).

wnt5a signaling has been reported to stimulate PKC (22). The PKC pathway can modulate the transcription of NFκB-dependent genes, which include the cytokines IL-6, IL-8, and IL-15 (25–28). Indeed, the same RA synovial fibroblasts that overexpressed *wnt5a* also produced more IL-6, IL-8, and IL-15, compared with the normal synovial fibroblasts (Fig. 3). Moreover, transient transfection of normal synovial fibroblasts with a *wnt5a* expression vector, but not with the pcDNA3 control vector, increased IL-6, IL-8, and IL-15 mRNA to levels approximately equivalent to those observed in the RA cells (Fig. 4*A*). Both the *wnt5a*-transfected and empty vector-transfected synovial fibroblasts expressed similar levels of the G3PDH message (Fig. 4*A*). In Fig. 4*B*, each vertical bar graphically represents the fold difference (average of five different experiments) between the intensities of a specific IL gene product (IL-6, IL-8, or IL-15) expressed in *wnt5a*- and empty vector-transfected synovial fibroblasts. Fold difference was calculated after evaluating the intensities of the different gene products by using Kodak IMAGE ANALYSIS Software, Ver. 2.0.2, for Macintosh. The transfection of the fibroblasts was confirmed by Western blotting by using a polyclonal antibody against an HA tag fused to the carboxyl-terminal end of the *wnt5a* protein (Fig. 4*C*).

Results obtained from transient transfection studies were reproduced by stable transfection of normal synovial fibroblasts with the *wnt5a* expression vector (Fig. 5*A*). After four passages in culture, the *wnt5a* stable transfectants of normal synovial fibroblasts continued to express more IL-6, IL-8, and IL-15 (Fig. 5*A*, lanes 2–4) than did the empty vector (pcDNA3) stable transfectants (Fig. 5*A*, lanes 6–8). Both the *wnt5a* and

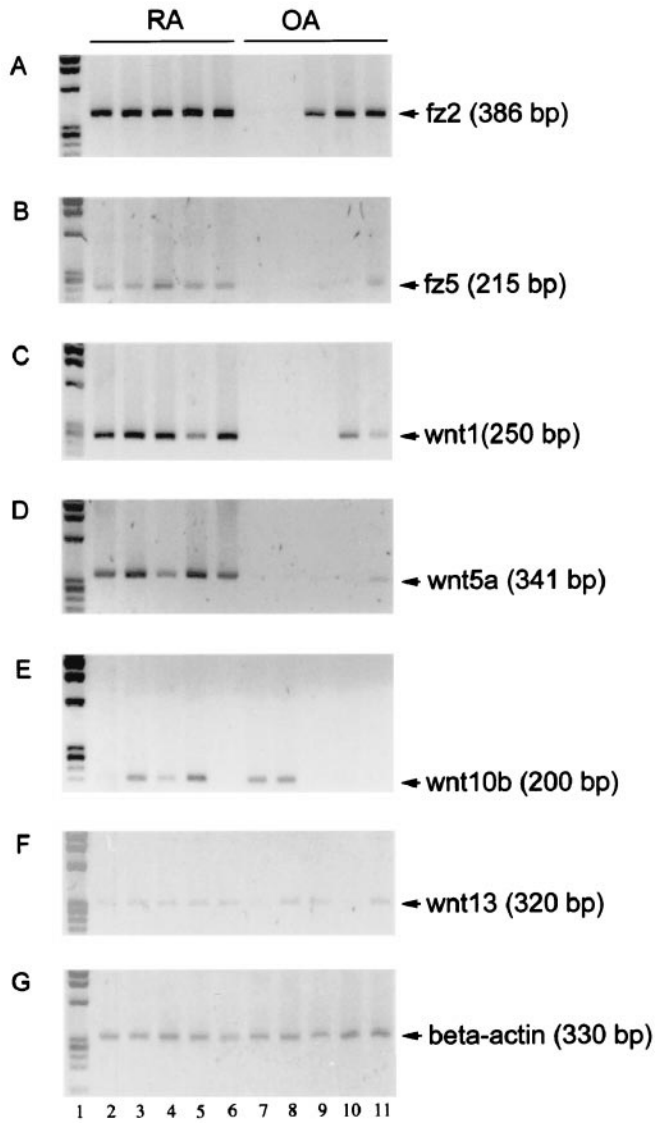

Fig. 1. RT-PCR analysis of various *wnt* and *fz* isoforms in RA and OA tissue specimens. Total RNA was extracted from five RA and five OA synovial tissue samples. RT-PCR was performed by using 1 μg of RNA from each sample with different primers for the specified *wnt* and *fz* isoforms as well as β-actin. (*A*) *fz2*, (*B*) *fz5*, (*C*) *wnt1*, (*D*) *wnt5a*, (*E*) *wnt10b*, (*F*) *wnt13*, and (*G*) β-actin. The PhiX 174 DNA standard is shown in the first lane of each gel.

empty vector stable transfectants expressed comparable levels of G3PDH (Fig. 5*A*, lanes 5 and 9). The specific PCR product obtained by RT-PCR on RNA of *wnt5a* stable transfectants by using *wnt5a* gene-specific and pcDNA3 vector-specific primers shows that *wnt5a* RNA is transcribed off the expression construct (Fig. 5*A*, lane 10). That *wnt5a* protein is expressed in *wnt5a* stable transfectants was confirmed by Western blotting with antibodies to the HA tag fused to the carboxyl-terminal end of *wnt5a* (data not shown).

Association of *wnt5a* Overexpression with IL-15 Protein Synthesis in Synovial Fibroblasts. Among the cytokines tested, the most pronounced effect of *wnt5a* overexpression was on IL-15 gene transcription. Therefore, we compared IL-15 protein levels in RA and *wnt5a* transfected synovial fibroblasts (stable transfectants) with normal synovial fibroblasts (Fig. 5*B*). Initial Western blotting studies with a monoclonal anti-IL-15 antibody showed

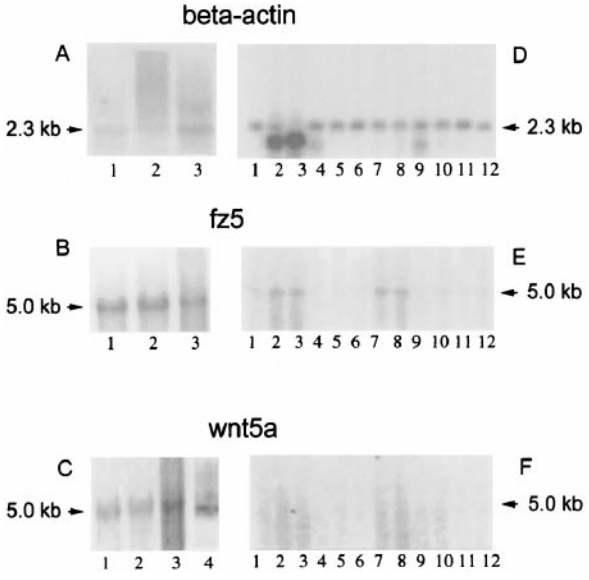

Fig. 2. Expression of *fz5*, *wnt5a*, and β-actin in RA tissue samples and adult tissues by Northern analysis. *A–C* show the expression of β-actin, *fz5*, and *wnt5a* in RA tissue specimens. *D–F* show the expression of the same genes in 12 adult tissues that are peripheral blood leukocyte, lung, placenta, small intestine, liver, kidney, spleen, thymus, colon, skeletal muscle, heart, and brain (lanes 1–12, respectively, in *D–F*). (*A*) Northern blot showing 2.3-kb β-actin-specific band in three RA tissue specimens; (*B*) 5-kb *fz5*-specific band in three RA tissue specimens; (*C*) lanes 1–3, 5-kb *wnt5a*-specific band in three RA specimens; lane 4, *wnt5a*-specific band in fetal fibroblast as a positive control. (*D*) Northern blot showing 2.3-kb β-actin-specific band in 12 different adult tissue specimens; (*E*) 5-kb *fz5*-specific band in the 12 adult tissue Northern blot; (*F*) result of *wnt5a* probe hybridization by using the same Northern blot. Specific activity of the probes used for hybridization and RNA concentrations of the tissue samples was the same for each analysis. The adult multiple tissue Northern blot was stripped and reprobed during the course of the experiments, and β-actin hybridization was performed last of all.

that the reactive IL-15 bands were much more intense in the RA- and *wnt5a*-transfected synovial fibroblasts than in the normal synovial fibroblasts (data not shown). Therefore we quantitated, by ELISA, the relative levels of IL-15 in the supernatants of the rheumatoid, *wnt5a*-transfected and normal synovial fibroblasts. By using equivalent amounts of culture supernatant proteins, the IL-15 levels were 3- to 4-fold higher in the RA- and *wnt5a*-transfected synovial fibroblast supernatants than in the normal fibroblast supernatant (Fig. 5*B*). Each horizontal bar in Fig. 5*B* represents an average of three different experiments. These results suggest that *wnt5a* overexpression can enhance IL-15 mRNA and protein expression in synovial fibroblasts.

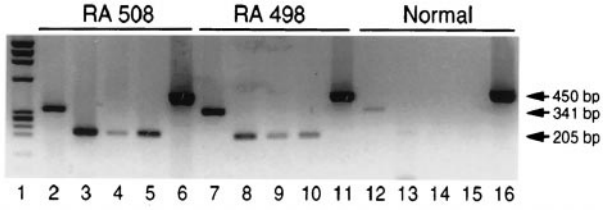

Fig. 3. RT-PCR analysis to identify expression of *wnt5a*, IL-6, IL-8, and IL-15 in RA fibroblasts compared with normal synovial fibroblasts. Lane 1, DNA standard PhiX174; lane 2, *wnt5a*-specific RT-PCR product from rheumatoid synovial fibroblast (RA 508); lanes 3–5 and 6, IL-6-, IL-8-, IL-15-, and G3PDH-specific RT-PCR products from RA 508; lanes 7–11, *wnt5a*-, IL-6-, IL-8-, IL-15-, and G3PDH-specific RT-PCR products from RA 498; lanes 12–16, *wnt5a*-, IL-6-, IL-8-, IL-15-, and G3PDH-specific RT-PCR products in normal synovial fibroblasts.

Discussion

Various members of the *wnt* and *fz* families control tissue patterning and cell-fate determination during embryogenesis. At least 13 *wnt* genes and eight or more *fz* genes have been identified. The *wnt* proteins are secreted glycoproteins that bind to the cell surface or the extracellular matrix and thus probably act locally in an autocrine or paracrine fashion. The *fz* proteins are typical G-coupled proteins that function as *wnt* receptors. However, the exact specificities of the *wnt*/*fz* ligand–receptor pairs have been difficult to determine, because some *wnt* proteins can interact with multiple *fz* receptors. We have found that both *wnt5a* and *fz5*, which have been reported to function as a ligand–receptor pair, are overexpressed in the RA synovium in comparison to OA and normal adult tissues.

wnt5a has been reported to be an activator of the PKC signaling cascade (22), and it has been shown that activated PKC enhances NFκB activation and translocation to the nucleus (25, 26). It is also well documented that activated NFκB causes transcriptional activation of IL genes like IL-6, IL-8, and IL-15 (27, 28). Our results showed that not only *wnt5a*, but also IL-6, IL-8, and IL-15, are overexpressed in cultured RA fibroblast-like synoviocytes compared with normal synovial fibroblasts. Moreover, transfection of normal synovial fibroblasts with a *wnt5a* expression construct increased IL-6, IL-8, and IL-15 mRNAs to levels equivalent to those observed in RA fibroblast-like synoviocytes. Collectively, these data suggest that activation of the IL-6, IL-8, and IL-15 genes in RA fibroblast-like synoviocytes is influenced by *wnt5a*-dependent signaling events. Future experiments will aim to investigate whether *wnt5a* mediates signaling through PKC and NFκB in the RA fibroblast-like synoviocytes. It will also be investigated whether the constitutive production of the cytokines IL-6, IL-8, and IL-15 by the RA fibroblast-like synoviocytes can be blocked by inhibiting *wnt5a*-mediated signaling. To carry out the relevant experiments, antisense *wnt5a*, a dominant negative *wnt5a*, or a soluble fragment of an *fz* receptor that binds to the signal generating motif of *wnt5a* would be useful.

Yamaguchi and others (29, 30) found that *wnt5a* is required for limb-bud development. Austin *et al*. (17) reported that *wnt5a* could stimulate growth and colony formation of purified murine hematopoietic progenitors. Recently, Van den Berg and coworkers (21) found that *wnt5a* was expressed in human CD34+ bone marrow progenitors, and that infection of the cells with a *wnt5a*-expressing retrovirus enhanced mixed colony formation.

wnt5a has been shown to interact with the cell-surface receptors *fz5* and *fz2* (22). Detectable levels of mRNAs corresponding to both *fz* species were present in OA tissues (Table 1), and also in normal synovial fibroblasts. The stoichiometric levels of *wnt5a* and *fz5*/*fz2* required to initiate signaling are not clearly known. However, a plausible mechanism by which overexpressed *wnt5a* in the transfected synovial fibroblasts could exert its effects is via autocrine and/or paracrine loops involving cell surface *fz5*/*fz2* receptors.

Considering that *wnt5a* has been shown to influence both limb-bud mesenchyme and bone marrow stem cell development, it is conceivable that it also plays a role in chronic inflammatory diseases associated with joint destruction and attempted regeneration. In long-standing RA, the fibroblast-like synoviocytes acquire an abnormal phenotype, characterized by the synthesis of inflammatory cytokines, metalloproteinases, and the up-regulation of cell-surface adhesive molecules (8, 31–33). These properties are thought to be responsible for the destruction of articular cartilage. The activated phenotype of fibroblast-like synoviocytes in RA is at

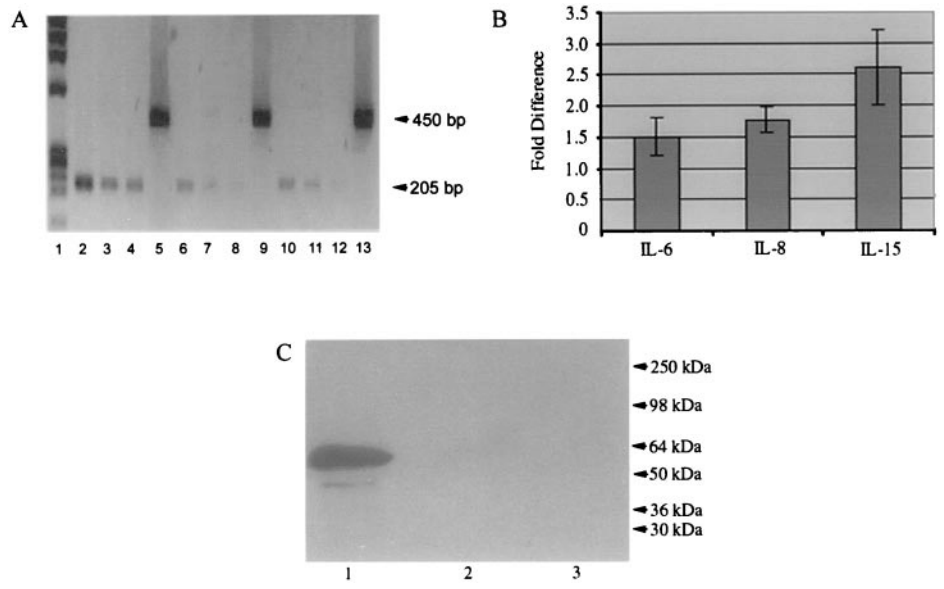

Fig. 4. RT-PCR experiment to analyze IL-6, IL-8, and IL-15 expression in *wnt5a*-transfected (transient) normal synovial fibroblasts and determination of transfection efficiency. (*A*) Lane 1, DNA standard PhiX174; lanes 2–5, IL-6-, IL-8-, IL-15-, and G3PDH-specific RT-PCR products from *wnt5a*-pcDNA3-transfected normal synovial fibroblasts; lanes 6–9, IL-6-, IL-8-, IL-15-, and G3PDH- specific RT-PCR products from empty vector transfected normal synovial fibroblasts; lanes 10–13, IL-6-, IL-8-, IL-15-, and G3PDH-specific RT-PCR products from untransfected normal synovial fibroblasts. (*B*) Bar graph showing fold increase in IL-6, IL-8, and IL-15 gene expression (measured by PCR product intensity) on transfection of normal synovial fibroblasts with *wnt5a* expression construct. Fold increase with *wnt5a* is compared with the effects produced by transfection by the empty vector. This represents an average of five different experiments. For each experiment, the same amounts of RNA were used from the *wnt5a*- and empty vector-transfected cells. G3PDH expression was used an internal control. (*C*) Western blot of lysate from *wnt5a*-HA-transfected synovial fibroblasts. Lane 1, ≈60 kDa *wnt5a*-HA protein from *wnt5a*-HA-transfected synovial fibroblasts; lanes 2 and 3, no band observed from untransfected synovial fibroblasts.

least partly autonomous, because it persists after several passages of the cells in tissue culture.

Possible explanations for the aggressive characteristics of RA fibroblast-like synoviocytes include, (*i*) genetic transformation caused by retroviral infection or somatic mutations; (*ii*) sorting and selection of variants from a preexisting heterogeneous cell population; and (*iii*) migration of new cells from the blood and bone marrow. These hypotheses are not mutually exclusive. Mutations of the p53 gene have been documented in RA and have been shown to influence the growth of RA synovial fibroblasts (34). However, the polyarticular nature of RA and the infrequent occurrence of synovial tumors argue against genetic transformation as a sole explanation for the aggressive RA fibroblast phenotype.

The major cytokines found in RA joints (TNF-α, IL-1) are potent growth stimulators of fibroblast-like synoviocytes together with osteoprotegerin (35). They may also promote the formation of osteoclast-like cells that which can enlarge channels between the bone marrow and the synovial cavity, as has been observed in a murine model of collagen-induced arthritis (9). Intense growth stimulation may cause immature mesenchymal cells, with the characteristics of RA fibroblast-like synoviocytes, to replace gradually the normal synovial lining layer of mature fibroblasts. In this context, mention should be made of pannocytes, distinctive cells observed in rheumatoid arthritis cartilage erosions, which might represent an earlier stage of mesenchymal cell differentiation (36).

The results of our investigations may have implications for the therapy of RA. Because of autocrine or paracrine signaling between *wnt* and *fz* family members, the persistent activation of immature fibroblast-like synoviocytes in longstanding RA may not require continued exposure to inflammatory cells and cytokines. Thus, control of inflammation in RA should be instituted early, before the outgrowth of the immature cells. In long-standing disease refractory to inflammation inhibitors, blockade of *wnt*/*fz* signaling could be of therapeutic benefit. The

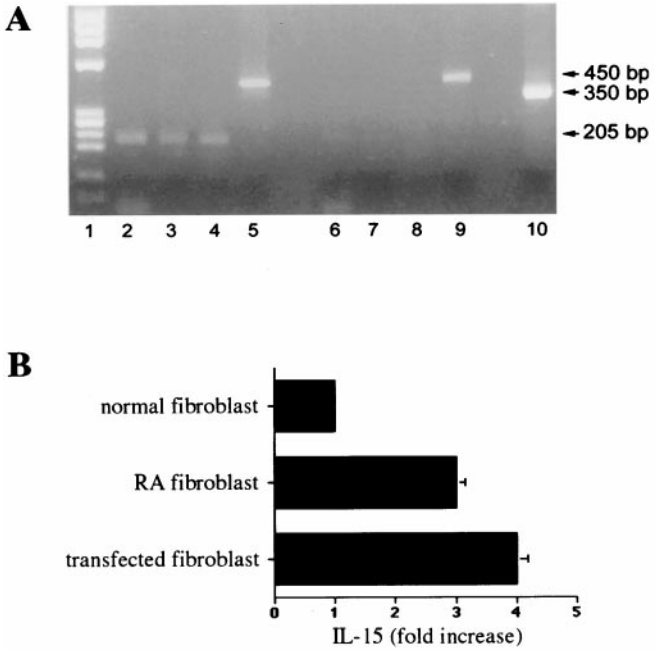

Fig. 5. (*A*) RT-PCR analysis of IL-6, IL-8, and IL-15 in *wnt5a* and pcDNA3 stable transfectants. Lane 1, DNA standard PhiX174; lanes 2–5, IL-6-, IL-8-, IL-15-, and G3PDH-specific PCR products from *wnt5a* stable transfectants; lanes 6–9, IL-6-, IL-8-, IL-15-, and G3PDH-specific PCR products obtained from pcDNA3 stable transfectants; lane10, *wnt5a*-specific PCR product (using *wnt5a*-specific and pcDNA3-bovine growth hormone-specific primers) obtained from *wnt5a* stable transfectant. (*B*) IL-15 protein expression in synovial fibroblasts. Bar graph showing fold difference in IL-15 protein levels in cell supernatants of RA, *wnt5a*-transfected, and normal synovial fibroblasts. This represents the average of three experiments. The baseline level of expression was estimated to be 60 pg/μg total protein by comparison with a known standard.

lack of availability of recombinant mammalian proteins and specific antibodies has hampered analyses of *wnt* and *fz* function in human diseases. Future studies will be needed to ascertain exactly which *wnt* and *fz* proteins are expressed in fibroblast-like synoviocytes and the effects of *wnt*/*fz* neutralization on the progression of arthritis.

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