Mod Rheumatol (2003) 13:35–43

ORIGINAL ARTICLE

Susumu Sakurai · Takeshi Hayashi · Shinichi Iwasaki
Toshihiko Kohno · Minoru Kohno

Expression of *wnt* signaling molecules in the synovial membranes of rabbit ankle joints injected with *Enterococcus faecalis* cell fractions

Received: January 15, 2002 / Accepted: July 9, 2002

Abstract The cell adhesion molecule β-catenin mediates the transduction of *wnt* signals to various downstream events such as gene expression, cell proliferation, and cell adhesion. In this study, the results of reverse transcriptase polymerase chain reaction (RT–PCR) amplification showed that *wnt1* and β-catenin expression increased in response to *E. faecalis*, and that the increases in *wnt1* and β-catenin activated transcription of cyclin D1. Immunohistochemistry also showed that stimulating *wnt1* with *E. faecalis* cell fractions leads to the stabilization and accumulation of β-catenin in the synovial membrane. On the other hand, the results of RT–PCR showed overexpression of various inflammatory cytokines, including IL-1-β, TGF-β, TNF-α, and IL-6, in the synovial membrane of joints injected with *E. faecalis* cell fractions. These findings suggest that expression of cyclin D1 is strongly dependent on β-catenin/Tcf and has a direct effect on the proliferation of synoviocytes, such as IL-1-β and TGF-β, that leads to inhibition of Fas-antigen-mediated apoptosis of synovial cells.

Key words Cytokine · β-Catenin · *Enterococcus faecalis* · Synovium · *wnt*

Introduction

The development of rheumatoid arthritis (RA) is associated with infiltration by blood leukocytes, angiogenesis, and the proliferation of fibroblasts in the synovial lining and connective tissue, leading to the formation of a pannus, which destroys articular cartilage.[1–4] Evidence based on the chronic arthropathic activity of whole-cell sonicates of several gram-positive bacteria[5–7] in rats after a single intraperitoneal (i.p.) or intraarticular (i.a.) injection suggests that a peptidoglycan–polysaccharide fragment of the cell wall of several species of streptococci possesses arthropathic activity.[8–10] The severity of the arthritis varies from one rat to another, but in most animals, recurrent or persistent arthritis leads to destruction of the distal joints after several months.[9,10] Moreover, direct injection of cell wall fragments from sonicated group A streptococci into the skin or joints of rabbits has also been reported to cause severe, recurrent, localized inflammation and injury.[11]

Cytokines have been shown to be capable of stimulating the proliferation of immature mesenchymal cells, and the inflammatory cytokines TNF-α and IL-1 are said to activate synovial fibroblasts and induce them to produce other cytokines, chemokines, matrix metalloproteinases (MMPs), and inflammatory mediators.[12–15] *wnt* signaling plays an important role in both embryonic development and tumorigenesis. The *wingless* (*wnt*) and *frizzled* (*fz*) family genes of *Drosophila* specify tissue patterning and cell fate during embryogenesis,[16] and homologous *wnt* and *fz* family members have been reported to function in tissue specification during mammalian development, as well as to influence cell proliferation and the response to activating stimuli.[17]

The *wnt1* gene stabilizes cytosolic β-catenin, which regulates both cell proliferation and cell adhesion via signal transduction,[18] and β-catenin is a key component of the *wnt1* signaling pathway. It interacts with the TCF/LEF family of transcription factors and activates transcription of *wnt* target genes.[19] Products of *fz* genes have been identified as receptors for secreted wnt proteins,[20] which are secreted glycoproteins that bind to frizzled 7-transmembrane-span receptors,[21] and expression of *fz2* has been linked to tissue regeneration and hyperplasia in an animal model of atherosclerosis.[22] Thus, it is important to identify the various factors that play a role in synoviocyte proliferation and pannus formation as well as in joint inflammation. The experiments

S. Sakurai (✉)
Department of Molecular Genetics, Kohno Clinical Medicine Research Institute, 3-4-4 Kitashinagawa, Shinagawa-ku, Tokyo 140-0001, Japan
Tel./Fax +81-3-3474-1833
e-mail: sakurai-s@kcmi.or.jp

T. Hayashi · T. Kohno
Tomidahama Hospital Research Division, Yokkaichi, Japan

S. Iwasaki
Second Department of Pathology of Aichi Medical University, Aichi, Japan

M. Kohno
Kohno Clinical Medicine Research Institute, Tokyo, Japan

in rabbits reported in this paper demonstrate the expression of *wnt1* signaling cascades and inflammatory cytokines in joints chronically inflamed by injection with *Enterococcus faecalis* cell fractions.

Materials and methods

Animals

Thirteen-week-old female Japanese White rabbits (SLC, Hamamatsu, Shizuoka, Japan) were used in each of the experiments.

Bacterial strains

Enterococcus faecalis ATCC 29505 was used to prepare the cell fractions. Bacteria were inoculated into 10 ml heart infusion (HI) broth (Eiken Chemical, Tokyo, Japan) and incubated for 15 h at 37°C. A 2-ml volume of the culture was inoculated into 1-l HI broth and stirred with a magnetic stirrer during the incubation. The cells were then centrifuged for 20 min at 6000 × *g* in a No. 8 rotor (KCF62, Kubota, Tokyo, Japan) for 20 min and washed twice with distilled water before preparing the cell fractions.

Preparation of *E. faecalis* ATCC 29505 cell fractions

The *E. faecalis* cell fractions were prepared by the method described previously,[5] with slight modifications. Washed, packed cells were resuspended in 30 ml distilled water and then sonicated on ice in an ultrasonic disruptor, UD 201 (Tomy Seiko, Tokyo, Japan), containing 30 g glass beads (0.17–0.18 mm diameter) for complete disruption. After allowing the beads to settle, the supernatant was centrifuged at 450 × *g* for 20 min at 4°C to remove unbroken cells. The supernatant was then removed from the unbroken cells and centrifuged at 4000 × *g* for 60 min at 4°C. The pellet obtained is referred to as the "crude cell-wall preparation." The supernatant was centrifuged at 20 000 × *g* for 60 min at 4°C, and the resultant pellet is referred to as the "crude cytoplasmic membrane fraction." The supernatant removed from the cytoplasmic membrane fraction is referred to as the "crude cell extract fraction." The crude cell-wall preparation and cytoplasmic membrane fraction were each resuspended separately by sonication in 30 ml distilled water. Next, 7 ml 0.2 M Na_2HPO_4–KH_2PO_4 buffer, pH 7.5, and 0.4 mg each of bovine pancreas DNase 1 and RNase A (Sigma Chemical, St. Louis, MO USA) were added to each suspension. After incubating the suspensions at 37°C for 2 h, the cell-wall suspension was centrifuged at 4000 × *g* for 60 min at 4°C. After washing the pellet obtained twice by centrifugation with 30 ml distilled water, it was lyophilized to obtain the cell-wall fraction (740 mg dry wt.). The crude cytoplasmic membrane fraction that had been incubated with DNase 1 and RNase A was centrifuged at 20 000 × *g* for 60 min at 4°C. After washing the pellet twice by centrifugation with 30 ml distilled water, it was lyophilized to obtain the cytoplasmic membrane fraction (260 mg dry wt.). A 30-ml volume of the crude-cell extract fraction was saturated by adding 24 g ammonium sulfate while stirring on ice, and then centrifuged at 8000 × *g* for 20 min at 4°C. The ammonium sulfate-precipitated cell extract fraction was dialyzed overnight at 4°C against 21 distilled water with seamless cellulose tubing UC 30-32-100 (Viskase Sales, Chicago, IL USA) while stirring with a magnetic stirrer. The dialysate was then lyophilized to obtain the cell extract fraction (670 mg dry wt.), and 250 mg of the lyophilized sample was suspended in 20 ml distilled water in a sterile dish and exposed to UV irradiation (1200 × 100 μJ/cm^2 × 40 s) 20 times using UV crosslinker FS-1500 (Funakoshi, Tokyo, Japan). Sterility was confirmed by streaking 0.1 ml of the undiluted suspension onto a heart infusion agar plate.

Preparation of peptidoglycan (PG)

The PG was prepared by the method described previously,[23] with slight modifications. PG was extracted from the cell-wall fraction by adding 5% trichloroacetic acid and incubating at 90°C for 6 min, followed by centrifugation at 4000 × *g* for 10 min at 4°C. After washing the pellet twice by centrifugation with 30 ml distilled water, it was resuspended in 9.5 ml 0.05 M NH_4HCO_3–0.005 M NH_4OH buffer, pH 8, and 0.5 mg bovine pancreas trypsin (Roche Diagnostics, Mannheim, Germany) was added to the suspension. After incubating at 37°C for 2 h, the PG suspension was centrifuged at 4000 × *g* for 10 min at 4°C, and the pellet obtained was washed twice by centrifugation with 30 ml distilled water and then lyophilized to obtain PG (220 mg dry wt.). The PG obtained was suspended in 10 ml distilled water in a sterile dish and exposed to UV irradiation 20 times.

Induction of arthritis

Three rabbits were used per group. Arthritis was induced by injecting both ankle joints with 1 ml sterile suspension containing 1 mg of the cell-wall fraction, the cytoplasmic membrane fraction, the cell extract, a mixture of the three cell fractions (1 mg of each), or PG. The lateral diameter of six ankle joints was measured with digital calipers (Komatsu, Tokyo, Japan), and the average of three measurements of the six joints was recorded. The data are shown as the means ± SE of the increases in joint diameter of the three rabbits in each group.

RT–PCR

The synovial membrane was removed 35 days after injection, and a motor-driven homogenizer was used to homogenize the tissue immediately in 15 ml 2% sodium dodecyl sulfate (SDS)–200 mM Tris-HCl (pH 8.0)–200 mM NaCl–1.5 mM $MgCl_2$ containing protein/RNase Degrader (Invitrogen, Carlsbad, CA, USA). The mRNA was isolated with a FastTrack 2.0 kit (Invitrogen) according to the manufacturer's instructions and reverse transcribed into

complementary DNA (cDNA) with a ProSTAR first strand RT–PCR kit (Stratagene, La Jolla, CA, USA), also according to the manufacturer's instructions. The following primer pairs were used: IL-**1**-β, 5′-tacaacaagagcttccggca and 3′-ggccacaggtatcttgtcgt; TNF-α, 5′-ggctcagaatcagacctcag and 3′-gctcccattgcagagaaga; TGF-β, 5′-cggcagctgtacattgactt and 3′-agcgcacgatcatgttggac; MMP-1, 5′-tcagttcgtcctcactccag and 3′-ttggtccacctgtcatcttc; COX-2, 5′-tcagccacgcagcaaatcct and 3′-gtgatctggatgtcagcacg; β-actin, 5′-atggatgacgatatcgct and 3′-atgaggtagtctgtcaggt; IL-6, 5′-ttcctctctgcaagagact and 3′-tgtatctctctgaaggact; iNOS, 5′-cgcccttccgcagtttct and 3′-tccaggaggacatgcagcac; TIMP-1, 5′-gcaactccgaccttgtcatc and 3′-agcgtaggtcttggtgaagc; TIMP-2, 5′-gtagtgatcagggccaag and 3′-ttctctgtgacccagtccat; *wnt1*, 5′-tgccgagaaacagcgttcatc and 3′-gttgccgtaaaggacgcggga; *fz2*, 5′-ctagcgcc gctcttcgtgtacctg and 3′-cagcgtcttgcccgaccagatcca; Axin, 5′-ttggacctcggagcaagtttc and 3′-caggctgatcccatcttggtc; GSK-3-β, 5′-tcatttggtgtggtatatca and 3′-ggaacatagtccagcacca; β-catenin, 5′-gcaggcact and 3′-gacggtgcgtac; cyclin D1, 5′-atggaacaccagctcctgtgc and 3′-cggtccaggtagttcatggcc; *fz5*, 5′-ttcatgtgcctggtggtgggc and 3′-tacacgtgcgacagggacacc; *wnt5a*, 5′-tggctttggccatatttttc and 3′-ccgatgtactgcatgtggtc.

To perform the PCR assay, 2.5 μl first-strand reaction solution, 5 μl 10× Tth polymerase buffer (Toyobo, Osaka, Japan), pH 8, 5 μl 2 mM dNTPs, 0.5 μl the 5′- and 3′-oligonucleotide primers (100 p mol/μl), 0.5 μl Tth polymerase (1.25 U) (Toyobo) and sterile distilled water were added to achieve a final volume of 50 μl.

PCR

Amplification of IL-1-β was performed by 30 cycles at 93°C for 1 min, 60°C for 2 min, and 72°C for 1 min with autoextension in a thermal cycler (MiniCycler, MJ Research, Watertown, MA, USA). For TNF-α amplification, PCR was performed by 30 cycles at 90°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplification of MMP-1, COX-2, and β-actin as internal controls was carried out by 30 cycles at 93°C for 1 min, 55°C for 2 min, and 72°C for 1 min. Amplification of TGF-β was performed by 35 cycles at 90°C for 30 s, 60°C for 1 min, and 72°C for 1 min. Amplification of IL-6 and iNOS was performed by 30 cycles at 93°C for 30 s, 52°C for 2 min, and 72°C for 1 min. Amplification of β-catenin and cyclin D1 was carried out by 30 cycles at 94°C for 30 s, 55°C for 1.5 min, and 72°C for 1.5 min. Amplification of *wnt1*, *fz*2, GSK-3-β, and Axin was performed by 30 cycles at 94°C for 30 s, 50°C for 1.5 min, and 72°C for 1.5 min. A 5-μl sample of the reaction mixture was separated on a 1.5% agarose gel, and the amplified products were detected by ethidium bromide staining and UV transillumination.

Histopathology

Rabbits were killed by carbon dioxide inhalation, and one of their ankle joints was removed, skinned, and fixed in formalin. After decalcification and embedding in paraffin, it was sagittally sectioned and stained with hematoxylin–eosin.

Results

Arthritis induced in rabbit ankle joints by i.a. injection of *E. faecalis* cell fractions and PG

As shown in Fig. 1d, e, the cell fraction mixture and PG both induced severe prolonged inflammation which peaked around day 8 after the injection, and its severity did not begin to subside until 30 days later. The cell-wall fraction induced moderately severe acute arthritis between day 2 and day 10, which gradually increased in severity and persisted throughout the entire period of the experiment (Fig. 1a). The cell-extract and the cytoplasmic membrane fraction induced moderately acute arthritis, which peaked around day 8 to day 15, and gradually decreased in severity thereafter (Fig. 1b, c).

Histopathology

Histological sections were prepared from the ankle joints of all experimental groups at the end of the experiments. Tissue was collected 35 days after a single i.a. injection of each cell fraction and of PG. The articular cavity of the rabbit ankles injected with the cell-wall fraction or PG (data not shown) expanded and filled with exudate. The synovial membrane proliferated, and villous proliferation was seen. Diffuse inflammatory cells had infiltrated the surface layer of the synovial membrane and the underlying tissue, and sporadic follicular lymphocyte aggregation had increased (Fig. 2A, B). Increased pannus (inflammatory granulation tissue) and erosion of the cartilage surface were observed (Fig. 2C), and numerous osteoclasts had induced bone resorption (Fig. 2D, E). After injection with the cell extract or cell membrane fraction, fibrous scars were observed under the surface layer of the synovial membrane, and although some inflammatory cells were visible, no synovial proliferation was detected, suggesting that the scars represented healing of the acute exudative inflammation (data not shown).

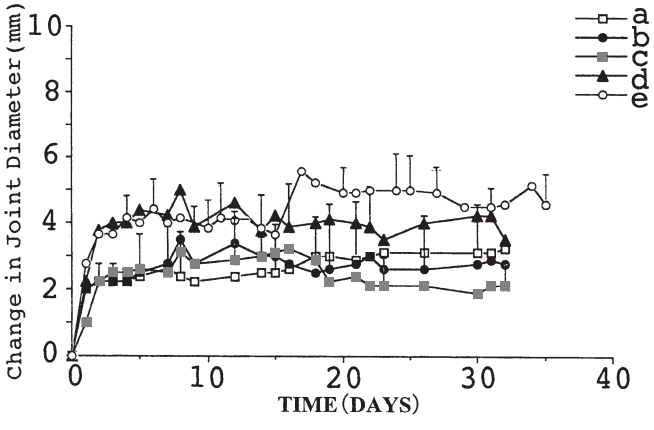

Fig. 1. Time-course studies of the development of arthritis in rabbit ankle joints after intraarticular (i.a.) injection of the ankle joints of each rabbit with cell fractions isolated from *E. faecalis*. Arthritis induced by (*a*) the cell-wall fraction, (*b*) the cell membrane fraction, (*c*) a cell extract fraction, (*d*) a mixture of cell fractions, and (*e*) PG

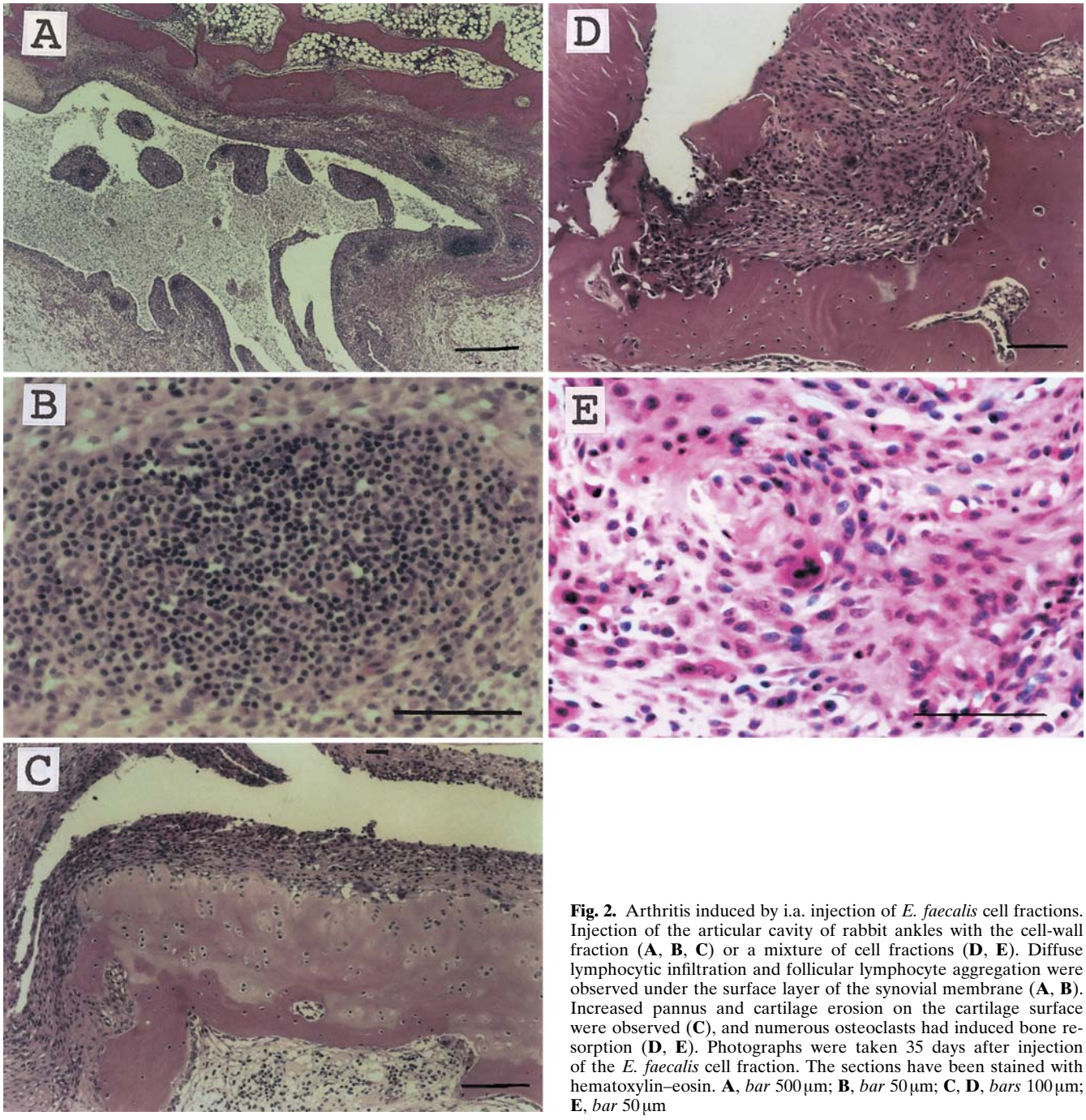

Fig. 2. Arthritis induced by i.a. injection of *E. faecalis* cell fractions. Injection of the articular cavity of rabbit ankles with the cell-wall fraction (**A**, **B**, **C**) or a mixture of cell fractions (**D**, **E**). Diffuse lymphocytic infiltration and follicular lymphocyte aggregation were observed under the surface layer of the synovial membrane (**A**, **B**). Increased pannus and cartilage erosion on the cartilage surface were observed (**C**), and numerous osteoclasts had induced bone resorption (**D**, **E**). Photographs were taken 35 days after injection of the *E. faecalis* cell fraction. The sections have been stained with hematoxylin–eosin. **A**, *bar* 500 μm; **B**, *bar* 50 μm; **C**, **D**, *bars* 100 μm; **E**, *bar* 50 μm

Expression of cytokine mRNA in the synovial membrane of the ankle joint following i.a. injection of the cell fractions and PG

Preferential expression of IL-1-β, TNF-α, IL-6, and TGF-β mRNA was detected in the synovial membrane collected on day 35 from ankle joints injected with the cell fractions and PG. By contrast, very little or no cytokine mRNA was detected in the synovial membrane from uninjected joints (control group) (Fig. 3a, b, d, e). Expression of β-actin transcripts was the same in all experimental groups and in the control group (Fig. 3c).

Analysis of the function of *wnt1* in the synovial membrane of joints injected with cell fractions and PG

RT–PCR studies were carried out to determine the expression of *wnt1* in the synovial membrane of the joints injected with cell fractions. *wnt1* signaling has been reported to promote the activation of dsh, and the active form of dsh leads to the inactivation of GSK-3-β, resulting in an accumulation of β-catenin. There were significant increases in *wnt1*, β-catenin, and cyclin D1 mRNA in the synovial membrane of the joints injected with the cell fractions and PG compared with control synovial membrane (Fig. 4b, e, f). However,

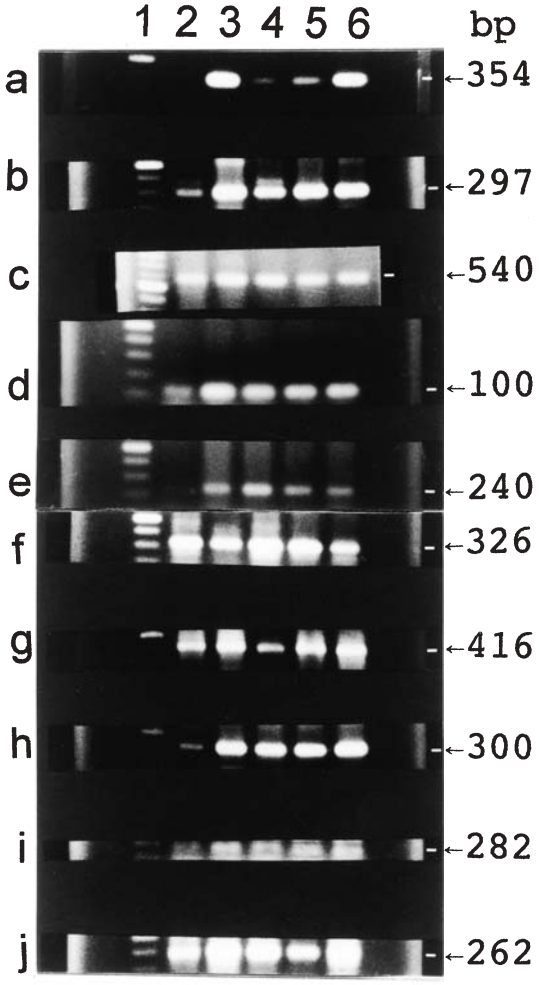

Fig. 3. Expression of cytokines, MMP-1, COX-2, iNOS, β-actin, and TIMP-1 and -2 mRNA in the synovial membrane of ankle joints injected with *E. faecalis*: *3*, cell wall fraction; *4*, cytoplasmic membrane fraction; *5*, cell extract fraction; or *6*, a mixture of cell fractions (ethidium bromide stain). *Lane 1*, m.w. standards (100 bp DNA ladder); *lane 2*, controls. *a*, IL-1-β; *b*, TNF-α; *c*, β-actin; *d*, TGF-β; *e*, IL-6; *f*, TIMP-1; *g*, TIMP-2; *h*, MMP-1; *i*, COX-2; *j*, iNOS

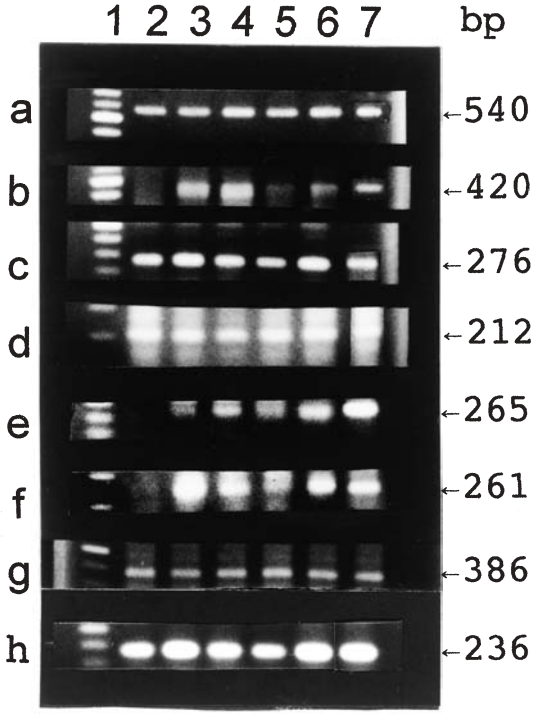

Fig. 4. Expression of the mRNA of *wnt1*, *fz2*, Axin, GSK-3-β, β-catenin, cyclin D1, and *fz5* in the synovial membrane of ankle joints injected with *E. faecalis* cell fractions or PG: *3*, cell-wall fraction; *4*, cell membrane fraction; *5*, cell extract fraction; *6*, mixture of cell fractions; *7*, PG from the *E. faecalis* cell-wall fraction. *Lane 1*, m.w. standards (100 bp DNA ladder); *lane 2*, controls. *a*, β-actin; *b*, *wnt1*; *c*, Axin; *d*, GSK-3-β; *e*, β-catenin; *f*, cyclin D1; *g*, *fz2*; *h*, *fz5*

the levels of *fz2*, GSK-3-β, and Axin mRNA were nearly the same in both the experimental groups and the control group (Fig. 4c, d, g). Expression of β-actin transcripts was the same in both the experimental groups and the control group (Fig. 4a).

Expression of the mRNAs of the tissue inhibitor of metalloproteinase (TIMP), COX-2, and inducible nitric oxide synthase (iNOS) in the synovial membrane of the joints injected with the cell fractions

TIMP-1 mRNA levels decreased in the joints injected with the cell-wall fraction and the joints injected with the mixture of cell fractions (see Fig. 3f), but the TIMP-2 transcript levels did not differ significantly from the control levels (see Fig. 3g). The MMP-1 mRNA levels, on the other hand, increased significantly in all experimental groups compared with the control group (Fig. 3h). As shown in Fig. 3i, after injection of the cell-wall fraction or mixture of cell fractions, the COX-2 mRNA levels were higher than the control levels, and iNOS transcripts were slightly increased (Fig. 3j).

Immunostaining of synovial membrane with anti-β-catenin monoclonal antibody

Synovial membrane collected 35 days after a single i.a. injection of the mixture of cell fractions strongly stained with anti-β-catenin antibody. β-Catenin had infiltrated the surface layer of the synovial membrane, and numerous immature macrophages were filled with β-catenin (Fig. 5B). By contrast, no β-catenin was detectable in the control synovial membrane, suggesting that GSK-3β induces β-catenin degradation in the absence of the *wnt1* signal (Fig. 5A).

Discussion

Several animal disease models have been developed to elucidate the pathogenesis of RA in humans. A number of substances, including type II collagen,[24] mycobacteria,[25] streptococcal cell-wall components,[5,6,8–11,26] and *Lactobacillus casei* cell wall fragments,[7] are known to induce arthritis in rabbits, rats, and mice. As shown in this study, the find-

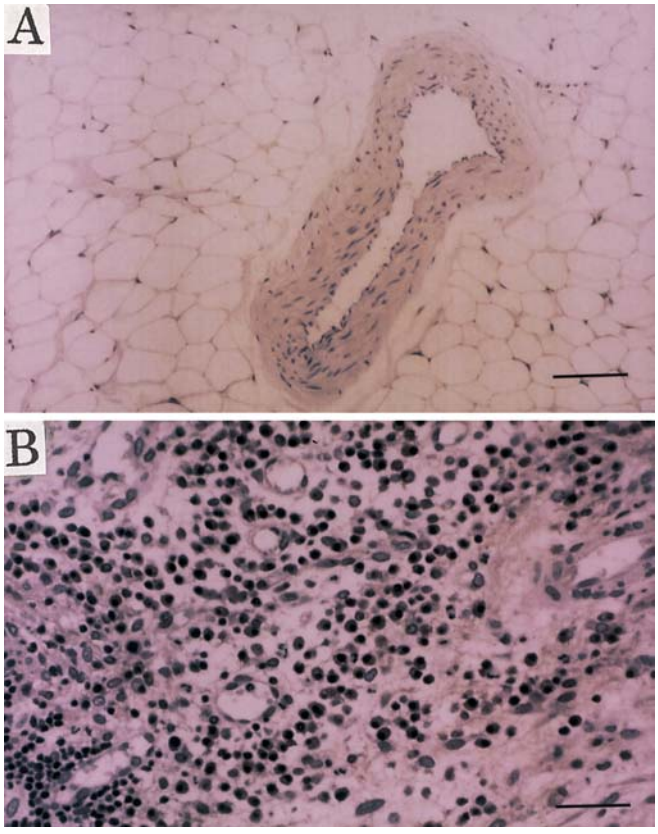

Fig. 5. Immunostaining of synovial membrane with anti-β-catenin monoclonal antibody. **A**, control; **B**, synovial membrane of ankle joint injected with mixture of cell fractions from *E. faecalis*. *Bars* 50 μm

ings in rabbits injected with *E. faecalis* cell fractions closely resemble those of RA, not only histopathologically, but also in terms of increased expression of various inflammatory cytokines. Several studies have found that the penicillin-treated avirulent Su strain of *Streptococcus pyogenes* induces many cytokines, including IL-1, IL-2,[27] IFN-γ,[28] and TNF,[29] both in vivo and in vitro. It has been well documented that a variety of inflammatory cytokines are often detected in both the synovial fluid and the serum of RA patients, implicating them in the pathogenesis of RA,[30–32] and, indeed, i.a. injection of IL-1 induces arthritis in rabbits.[33] In the present study, higher IL-1-β expression was observed in rabbits injected with the cell-wall fraction and mixture of cell fractions of *E. faecalis*, and expression of various inflammatory cytokines, including IL-6, TNF-α, and TGF-β, and of *wnt1* also increased, suggesting that overexpressed inflammatory cytokines are involved in the pathogenesis of RA. In this study, we showed that *E. faecalis* cell fractions and PG, and particularly the mixture of cell fractions and PG, play an important role in this process, as evidenced by the induction of severe prolonged inflammation, which peaked around day 8 and did not begin to decrease in severity until 38 days later. RA is accompanied by villus proliferation of synovial membranes, infiltration by lymphocytes, aggregation of follicular lymphocytes under the surface layer of synovial membrane, pannus formation, cartilage and subchondral bone destruction by osteoclasts, and fibrous ankylosis of joints.[1–4] In our experiment, tissue collected 35 days after a single i.a. injection of *E. faecalis* cell fractions showed villus proliferation of synovial membranes (Fig. 2A), infiltration by lymphocytes and aggregation of follicular lymphocytes under the surface layer of synovial membrane (Fig. 2A, B), pannus formation (Fig. 2C), and bone resorption by osteoclasts (Fig. 2D, E). Our experiment revealed overexpression of IL-1-β, TNF-α, TGF-β, and IL-6 transcripts in the joints injected with *E. faecalis* cell fractions and PG, especially the cell-wall fraction and the mixture of cell fractions. Based on the results of our experiments, we concluded that the PG in the cell wall of the mixture of cell fractions of *E. faecalis* possesses potent arthropathic activity. Fuseler et al.[34] reported finding that a single intraperitoneal injection of bacterial-cell-wall-derived PG–polysaccharide (PG–PS) produced acute joint inflammation in female rats, and they also detected high levels of TNF, IL-1, IL-6, and nitric oxide in the serum of the rats intraperitoneally injected with PG–PS. We recently discovered the presence of a specific 53-kDa protein in *E. faecalis* cell fractions by immunoblot analysis which was not detected in cell fractions from *E. coli* or *Staphylococcus aureus*. It showed high immunogenicity in mice, rats, and rabbits, and high immunoreactivity by Western blot with mouse, rabbit, and rat anti-*E. faecalis* antibody. The anti-*E. faecalis* antibody reacted strongly with 53-kDa protein from *E. faecalis* cell fractions, i.e., the cell-wall fraction, a cytoplasmic membrane fraction, and a cell extract fraction, and reacted slightly with other proteins from *E. faecalis* cell fractions (data not shown). Based on these findings, we hypothesized that the 53-kDa protein plays an important role in the pathogenesis of the arthritis induced by the cell fractions of *E. faecalis*. To test this hypothesis, we are now cloning and sequencing the full length of the gene coding the 53-kDa protein to analyze the homology between its amino acid sequences and those of heat-shock protein.

wnt genes are defined by their sequence homology to the original *wnt1* members in the mouse (*int-1*),[35] and they encode secreted glycoproteins that are 350–400 amino acids long. Frizzled proteins, encoded by the *frizzled* gene family, are members of the fz family of cell-surface proteins and act as receptors for Wnt proteins[20,21] to promote activation of the cytoplasmic phosphoprotein dishevelled (Dsh).[16,36] Dsh is a phosphoprotein that is predominantly localized in the cytoplasm, and Yanagawa et al.[37] reported that Wnt stimulation in cells or embryos leads to hyperphosphorylation of Dsh. The hyperphosphorylated form of Dsh is an active form, and as a repressor of β-catenin activity it leads to the inhibition of serine kinase glycogen synthase kinase (GSK-3-β (homologous to *zest white* in *Drosophila*)).[38] In this experiment, we found that *wnt1* and β-catenin transcripts were overexpressed in the synovial membrane of joints injected with *E. faecalis* cell fractions compared with control synovial membrane, although the levels of Axin, *fz2*, *fz5*, and GSK-3-β mRNA were nearly the same in both the experimental and control groups.

In the presence of a *wnt1* signal, the GSK-3-β function is suppressed in response to activation of Dsh, thereby decreasing the phosphorylation of β-catenin and APC. It has been shown that colon cancer cells containing mutant APC have abnormally high levels of intracellular β-catenin, and that the addition of full-length APC destabilizes and eliminates the cytoplasmic β-catenin pool.[39] Although we did not examine expression of the APC transcript, Kawahara et al.[40] reported that APC forms a complex with β-catenin, Axin,[41] and GSK-3-β and induces the degradation of β-catenin by GSK-3-β. However, a *wnt* signal inhibits the activity of the GSK-3 β-APC–Axin complex, resulting in the stabilization of cytoplasmic β-catenin.[42,43] Thus, the inhibition of GSK-3-β in response to the *wnt1* signal results in activation of β-catein function,[38,43] leading to suppression of β-catenin degradation, and, in turn, β-catenin accumulation. RT–PCR analysis in the present study showed higher β-catenin expression levels in the synovial membrane of joints injected with *E. faecalis* cell fractions, and this finding was confirmed immunohistochemically. It has been shown that nuclear localization of endogenous β-catenin requires the presence of both lymphoid-enhancer factor-1 (Lef-1) and the *wnt* signal,[44] and that *wnt* signaling, which increases the stability of β-catenin, results in transcriptional activation by Lef-1/T cell factor (Tcf) proteins in association with β-catenin. Behrens et al.[45] showed that the nuclear transcription factor Lef-1 binds directly to β-catenin and translocates β-catenin to the nucleus. It has been shown that despite the binding of Lef-1 proteins to DNA, inducing a sharp bend in the DNA helix,[46] they are poor transcriptional activators. However, complexes of Tcf proteins and β-catenin act as potent transcriptional activators of reporter gene constructs containing the DNA element recognized by Tcf.[19,47] Tetsu and McCormick[48] showed that β-catenin activates the transcription of cyclin D1 through Tcf-binding sites within the promoter, and that cyclin D1 expression is strongly dependent on β-catenin/Tcf and has a direct effect on cell proliferation. They also reported that the dominant–negative Tcf causes cells to arrest in the G1 phase of the cell cycle, and that this phenotype can be rescued by expression of cyclin D1. In our experiment, increased expression of cyclin D1 transcript was observed in the synovial membrane of ankle joints injected with *E. faecalis* cell fractions and PG, suggesting that cyclin D1 may play an important role in synovial membrane proliferation.

Various cytokines, including IL-1, IL-2, IL-6, TNF-α, TGF-β, and IFN-γ, have been reported to be overexpressed in the joints of RA patients.[31,49] In our experiment, increased expression of various inflammatory cytokines, including IL-1-β, IL-6, TNF-α, and TGF-β, was found in the synovial membrane of joints injected with the *E. faecalis* cell fraction. TGF-β may actually suppress abnormal immune reactions while promoting inflammation in the initial stage of RA.[50] It has been reported that IL-1-β and TGF-β both directly activate the proliferation of human synovial cells[51] and suppress Fas-antigen-mediated apoptosis of synovial cells.[52,53] The results of our experiment suggest that, as with β-catenin and cyclin D1, increased expression of IL-1-β and TGF-β in the presence of a *wnt1* signal may cause proliferation of synovial membrane repopulated with immature mesenchymal and bone marrow cells which have altered properties in the synovial membrane of joints injected with *E. faecalis* cell fractions and PG. Sen et al.[54] reported expression of *fz2*, *fz5*, and *fz7*, as well as of *wnt1*, *wnt5a*, *wnt11*, *wnt10b*, and *wnt13*, in RA synovial tissue, and that synovial tissue in osteoarthritis expresses much less *wnt5a* and *fz*5. It has been discovered that *wnt5a* is an activator of the PKC signaling cascade,[55] that activated PKC enhances NF-κ-B activation,[56] and that after translocation to the nucleus,[57] activated NF-κ-B causes transcriptional activation of the IL genes encoding IL-6, IL-8, and IL-15.[57] Sen et al.[54] also found overexpression of the *wnt5a–fz5* ligand–receptor pair in the rheumatoid synovium, and higher levels of IL-6, IL-8, and IL-15 transcripts in cultured RA fibroblast-like synoviocytes compared with control synovial fibroblasts. We detected expression of both *fz2* and *fz5* in our experiment (see Fig. 4), but no expression of *wnt5a*.

RA is accompanied by synovial inflammation, proliferation, and cartilage destruction. In this study, expression of MMP-1, COX-2, and iNOS transcripts was increased in every experimental group compared with the controls. MMP activity is inhibited by TIMP.[58] TIMP-1 and TIMP-2 preferentially form complexes with the proenzymes of MMP-2 and MMP-9, and they both inhibit the activity of other MMPs.[58] Since TIMP-1 mRNA levels were slightly decreased in the synovial membrane of joints injected with the cell-wall fraction or the mixture of cell fractions, if transcript levels are a reflection of protein and biological activity, these results suggest that the TIMP synthesis–MMP balance is disrupted. IL-4, IL-8, IL-10, and TGF-β have been reported to inhibit the induction of nitric oxide synthase,[59–62] whereas the cytokines IL-1, TNF-α, and IFN-γ induce nitric oxide synthase gene expression.[63,64] Despite increased expression of TGF-β, elevated iNOS mRNA expression in the synovial membrane of joints injected with the cell-wall fraction and the mixture of cell fractions may be involved in the development of inflammation in the synovial membrane via increased peroxinitrite, which damages DNA.[65,66]

Further experiments will be needed to determine the levels of expression of the hyperphosphorylated forms of Dsh and GSK-3-β proteins in inflamed synovial membranes in experimental arthritis and in RA in humans. Injection of antisense *wnt1*, Axin, or *fz2* into the ankle joints of animals with experimental arthritis or humans with RA may have a therapeutic effect.

References

1. Koch AE. Review. Angiogenesis: implications for rheumatoid arthritis. Arthritis Rheum 1998;41:951–62.
2. Goronzy JJ, Zettl A, Weyand CM. T cell receptor repertoire in rheumatoid arthritis. Int Rev Immunol 1998;17:339–63.
3. Ohba T, Takase Y, Ohhara M, Kasukawa R. Thrombin in the synovial fluid of patients with rheumatoid arthritis mediates proliferation of synovial fibroblast-like cells by induction of platelet-derived growth factor. J Rheumatol 1996;23:1505–11.
4. Bresnihan B. Pathogenesis of joint damage in rheumatoid arthritis. J Rheumatol 1999;26:717–9.

5. Cromartie WJ, Craddock JG, Schwab JH, Anderle SK, Yang CH. Arthritis in rats after systemic injection of streptococcal cells or cell walls. J Exp Med 1977;146:1585–602.
6. Schwab JH, Cromartie WJ, Ohanian SH, Craddock JG. Association of experimental chronic arthritis with the persistence of group D streptococcal cell walls in the articular tissue. J Bacteriol 1967;94:1728–35.
7. Lehman TJA, Allen JB, Plotz PH, Willeer RL. Poly-arthritis in rats following the systemic injection of *Lactobacillus casei* cell walls in aqueous suspension. Arthritis Rheum 1983;26:1259–65.
8. Fox A, Brown RR, Anderle SK, Chetty C, Cromartie WJ, Gooder H, et al. Arthropathic properties related to the molecular weight of peptidoglycan–polysaccharide polymers of streptococcal cell walls. Infect Immun 1982;35:1003–10.
9. Cromartie WJ. Arthropathic properties of peptidoglycan–polysaccharide complexes of microbial origin. In: Deicher H, Schulz LCI, editors. Arthritis: models and mechanisms. Berlin: Springer; 1981. p. 24–38.
10. Stimpson SA, Brown RR, Anderle SK, Klapper DG, Clark RL, Cromartie WJ, et al. Arthropathic properties of cell wall polymers from normal flora bacteria. Infect Immun 1986;51:240–9.
11. Ohanian SH, Schwab JH. Persistence of group A streptococcal cell walls related to chronic inflammation of rabbit darmal connective tissue. J Exp Med 1967;125:1137–48.
12. Monier S, Reme T, Cognot C, Gao QL, Travaglio-Encinoza A, Cuchacovich M, et al. Growth factor activity of IL-6 in the synovial fluid of patients with rheumatoid arthritis. Clin Exp Rheumatol 1994;12:595–602.
13. Badolato R, Oppenheim JJ. Role of cytokines, acute-phase proteins, and chemokines in the progression of rheumatoid arthritis. Semin Arthritis Rheum 1996;26:526–38.
14. Peichl P, Pursch E, Broll H, Lindley IJ. Anti-IL-8 autoantibodies and complexes in rheumatoid arthritis: polyclonal activation in chronic synovial tissue inflammation. Rheumatol Int 1999;18:141–5.
15. Harada S, Yamamura M, Okamoto H, Morita Y, Kawashima M, Aita T, et al. Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. Arthritis Rheum 1999;42:1508–16.
16. Klingensmith J, Nusse R. Signaling by wingless in *Drosophila*. Dev Biol 1994;166:396–414.
17. Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. Genes Dev 1997;11:3286–305.
18. Miller JR, Moon RT. Signal transduction through β-catenin and specification of cell fate during embryogenesis. Genes Dev 1996;10:2527–39.
19. Molenaar M, van de Wetering M, Oosterwegel M, Petersonmaduro J, Godsave S, Korinek V. Xtcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. Cell 1996;86:391–99.
20. Bhanot P, Brink M, Samos CH, Hsieh J-C, Wang Y, Macke JP, et al. A new member of the frizzled family from *Drosophila* functions as a wingless receptor. Nature 1996;382:225–30.
21. Vinson CR, Conover S, Adler PN. A *Drosophila* tissue polarity locus encodes a protein containing seven potential transmembrane domains. Nature 1989;338:263–4.
22. Blankesteijn WM, Essers-Janssen YP, Ulrich MM, Smits JF. Increased expression of a homologue of *Drosophila* tissue polarity gene "*frizzled*" in left ventricular hypertrophy in the rat, as identified by subtractive hybridization. J Mol Cell Cardiol 1996;28:1187–91.
23. Takada H, Tsujimoto M, Kotani S, Kusumoto S, Inage M, Shiba T, et al. Mitogenic effects of bacterial cell walls, their fragments and related synthetic compounds on thymocytes and splenocytes of guinea pigs. Infect Immun 1979;25:645–52.
24. Trentham DE, Townes AS, Kaug AH. Autoimmunity to type II collagens: an experimental model of arthritis. J Exp Med 1977;146:857–68.
25. Pearson CM, Waksman BH, Sharp JT. Studies of arhritis and other lesions induced in rats by injection of mycobacterial adjuvant. J Exp Med 1961;113:485–90.
26. Koga T, Kakimoto K, Hirofuji T, Kotani S, Ohkuni H, Watanabe K, et al. Acute joint inflammation in mice after systemic injection of the cell wall, its peptidoglycan, and chemically defined peptidoglycan subunits from various bacteria. Infect Immun 1985;50:27–34.
27. Ichimura O, Suzuki S, Saito M, Sugawara Y, Ishida N. Augmentation of interleukin-1 and interleukin-2 production by OK-432. Int J Immunopharmacol 1985;7:263–70.
28. Saito M, Ebina T, Koi M, Yamaguchi T, Kawada Y, Ishida N. Induction of interferon-γ in mouse spleen cells by OK-432, a preparation of *Streptococcus pyogenes*. Cell Immunol 1982;68:187–92.
29. Yamamoto A, Nagamura M, Usai H, Sugawara Y, Watanabe H, Niitsu Y, et al. Release of tumor necrosis factor (TNF) into mouse peritoneal fluids by OK-432, a streptococcal preparation. Immunopharmacology 1986;11:79–86.
30. Firestein GS, Alvaro-Garcoa JM, Maki R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J Immunol 1990;144:3347–53.
31. Fontana A, Hengartner H, Weber E, Fejr K, Grob PJ, Cohen G. Interleukin-1 activity in the synovial fluid of patients with rheumatoid arthritis. Rheumatol Int 1982;2:49–53.
32. Chu CQ, Field M, Feldman M, Maini RN. Localization of tumor necrosis factor-α in synovial tissues and at the cartilage–pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 1991;34:1125–32.
33. Feige U, Karbowski A, Rordorf-Adam C, Pataki A. Arthritis induced by continuous infusion of hr-interleukin-1-α into the rabbit knee joint. Int J Tissue React 1989;11:225–38.
34. Fuseler JW, Conner EM, Davis JM, Wolf RE, Grisham MB. Cytokine and nitric oxide production in the acute phase of bacterial cell-wall-induced arthritis. Inflammation 1997;21:113–31.
35. Nusse R, Varmus HE. *wnt* genes. Cell 1992;69:1073–87.
36. Theisen H, Purcell J, Bennet M, Kansagara D, Syed A, Marsh J. Dishevelled is required during *wingless* signaling to establish both cell polarity and cell identity. Development 1994;120:347–60.
37. Yanagawa S-I, Leeuwen FV, Woderz A, Klingensmith J, Nusse R. The dishevelled protein is modified by *wingless* signaling in *Drosophila*. Genes Dev 1995;9:1087–97.
38. Siegfried E, Wilder EL, Perrimon N. Components of *wingless* signaling in *Drosophila*. Nature 1994;367:76–80.
39. Peifer M. Regulating cell proliferation: as easy as APC. Science 1996;272:974–5.
40. Kawahara K, Morishita T, Nakamura T, Hamada F, Toyoshima K, Akiyama T. Down regulation of β-catenin by the colorectal tumor suppressor APC requires association with Axin and β-catenin. J Biol Chem 2000;275:8369–74.
41. Zeng L, Fagotto F, Zhang T, Hsu W, Vasicek TJ, Perry WL, et al. The mouse fused locus encodes Axin, an inhibitor of the *wnt* signaling pathway that regulates embryonic axis formation. Cell 1997;90:181–92.
42. Papkoff J, Rubinfeld B, Schryver B, Polakis P. *Wnt-1* regulates free pools of catenins and stabilizes APC–catenin complexes. Mol Cell Biol 1996;16:2128–34.
43. Young C, Kitamura M, Mardy S, Kitajewski J. *Wnt-1* induces growth, cytosolic β-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblast. Mol Cell Biol 1998;18:2474–85.
44. Hsu S, Galceran J, Grosschedl R. Modulation of transcriptional regulation by LEF-1 in response to *Wnt-1* signaling and association with β-catenin. Mol Cell Biol 1998;18:4807–18.
45. Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, et al. Functional interaction of β-catenin with the transcription factor LEF-1. Nature 1996;382:638–42.
46. Giese K, Cox J, Grosschedl R. The HMG domain of lymphoid enhancer factor 1 bends DNA and facilitates assembly of functional nucleoprotein structures. Cell 1992;69:185–95.
47. Korinek V, Barker N, Morin PJ, van Wichen D, deWeger R, Kinzler K, et al. Constitutive transcriptional activation by a β-catenin–Tcf complex in APC(−/−) colon carcinoma. Science 1997;275:1784–7.
48. Tetsu O, McCormick F. β-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.
49. Yamagata N, Kobayashi K, Kasama T, Fukushima T, Tabata M, Yoneya I, et al. Multiple cytokine activities and loss of interleukin-2 inhibitor in synovial fluids of patients with rheumatoid arthritis. J Rheumatol 1988;15:1623–7.
50. Shull MM, Kier AB, Diebold RJ, Yin M, Doetschman T. The importance of transforming growth factor β-1 in immunological

homeostasis, as revealed by gene ablation in mice. In: Jacob CO, editor. Overexpression and knockout of cytokines in transgenic mice. San Diego: Academic Press; 1994. p. 135.
51. Butler DM, Piccoli DS, Hart PH, Hamilton JA. Stimulation of human synovial fibroblast DNA synthesis by recombinant human cytokines. J Rheumatol 1988;15:1463–70.
52. Kawakami A, Eguchi K, Matsuoka N, Tsuboi M, Kawabe Y, Aoyagi T, et al. Inhibition of Fas antigen-mediated apoptosis of rheumatoid synovial cells in vitro by transforming growth factor β-1. Arthritis Rheum 1996;39:1267–76.
53. Tsuboi M, Eguchi K, Kawakami A, Matsuoka N, Kawabe Y, Aoyagi T, et al. Fas antigen expression on synovial cells was down-regulated by interleukin-1-β. Biochem Biophys Res Commun 1996;218:280–5.
54. Sen M, Lauterbach K, El-Gabalawy H, Firestein GS, Corr M, Carson DA. Expression and function of wingless and frizzled homologs in rheumatoid arthritis. Proc Natl Acad Sci USA 2000;97:2791–6.
55. Sheldahl LC, Park M, Malbon CC, Moon RT. Protein kinase C is differentially stimulated by Wnt and frizzled homologs in a G-protein-dependent manner. Curr Biol 1999;9:695–8.
56. Washizu J, Nishimura H, Nakamura N, Nimura Y, Yoshikai Y. The NF-κ-B binding site is essential for transcriptional activation of the IL-15 gene. Immunogenetics 1998;48:1–7.
57. Anrather J, Csizmadia V, Soares MP, Winkler H. Regulation of NF-κ-B RelA phosphorylation and transcriptional activity by p21 (ras) and protein kinase C-ζ in primary endothelial cells. J Biol Chem 1999;274:13594–603.
58. Cawston TE. Proteinase inhibitors. In: Barret AJ, Salvesen G, editors. Metalloproteinase inhibitors. Amsterdam: Elsevier; 1986. p. 589–610.
59. al-Ramadi BK, Meissler JJ Jr, Huang D, Eisenstein TK. Immunosuppression induced by nitric oxide and its inhibition by interleukin-4. Eur J Immunol 1992;22:2249–54.
60. McCall TB, Palmer RM, Moncada S. Interleukin-8 inhibits the induction of nitric oxide synthase in rat peritoneal neutrophiles. Biochem Biophys Res Commun 1992;186:680–5.
61. Gazzinelli RT, Oswald IP, James SL, Sher A. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-γ-activated macrophages. J Immunol 1992;148:1792–6.
62. Schini VB, Durante W, Elizondo E, Scott-Burden T, Junquero DC, Schafer AI, et al. The induction of nitric oxide synthase activity is inhibited by TGF-β-1, PDGF AB and PDGF BB in vascular smooth muscle cells. Eur J Pharmacol 1992;216:379–83.
63. Tsujino M, Hirata Y, Imai T, Kanno K, Eguchi S, Ito H, et al. Induction of nitric oxide synthase gene by interleukin-1-β in cultured rat cardiocytes. Circulation 1994;90:375–83.
64. Goureau O, Lepoivre M, Coutois Y. Lipopolysaccharides and cytokines induce a macrophage-type of nitric oxide synthase in bovine retinal pigmented epitherial cells. Biochem Biophys Res Commun 1992;186:854–9.
65. Szabo C, Zingarelli B, O'Connor M, Salzman AL. DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity in macrophages and smooth muscle cells exposed to peroxynitrite. Proc Natl Acad Sci USA 1996;93:1753–8.
66. Nguyen T, Brunston D, Crespi CL, Penman BW, Winshnok JS, Tannenbaum SR. DNA damage and mutation in human cells exposed to nitric oxide in vitro. Proc Natl Acad Sci USA 1992;89: 3030–4.