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【Ⅴ】研究成果の刊行物・別刷

ARTHRITIS & RHEUMATISM
Vol. 64, No. 10, October 2012, pp 3355–3363
DOI 10.1002/art.34555

Interleukin-1β Induces Differentiation of Human Mesenchymal Stem Cells Into Osteoblasts via the Wnt-5a/Receptor Tyrosine Kinase–like Orphan Receptor 2 Pathway

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Objective. **Mesenchymal stem cells (MSCs) are considered to be a novel tool for the treatment of rheumatoid arthritis (RA) because of their multipotency to differentiate into osteoblasts and chondrocytes, their immunosuppressive effects, and availability. The aim of this study was to assess the mechanisms of human MSC differentiation into osteoblasts under inflammatory conditions.**

Methods. **Human MSCs were cultured in commercialized osteogenic induction medium with inflammatory cytokines for up to 10 days. Osteoblast differentiation was detected by alkaline phosphatase staining and messenger RNA (mRNA) expression of multiple osteoblast markers. Mineralization was assessed by alizarin red S staining.**

Results. **Among the various cytokines tested, interleukin-1β (IL-1β) induced differentiation of human MSCs into osteoblasts, which was confirmed by alkaline phosphatase activity, expression of *RUNX2* mRNA, and strong alizarin red S staining. Among various molecules of the Wnt family, Wnt-5a and receptor tyrosine kinase–like orphan receptor 2 (Ror2), a major receptor of Wnt-5a, were significantly induced in human MSCs by IL-1β. Silencing of either *WNT5A* or *ROR2* by small interfering RNA with 2 different sequences reduced alkaline phosphatase activity, *RUNX2* expression, and alizarin red S staining of human MSCs induced by IL-1β.**

Conclusion. **IL-1β effectively and rapidly induced human MSC differentiation into osteoblasts and mineralization, mainly through the noncanonical Wnt-5a/Ror2 pathway. These results suggest potential benefits of IL-1β–treated human MSCs in the treatment of damaged bone as well as in the induction of self-renewal and self-repair of damaged tissue, including osseous tissue.**

Rheumatoid arthritis (RA) is a common inflammatory disease characterized by synovial hyperplasia and chronic inflammation at the joint. Although the cause of RA is still uncertain, it is well known that inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), IL-6, and IL-17 play key pathogenetic roles. Sustained inflammation causes activation of osteoclasts that will produce joint deformation, which critically affects daily activities. Treatment with biologic agents targeting cytokines improves disease activity, leading to clinical remission and remission of structural changes (1). More recently, some studies have shown the ability of biologic agents to repair bone erosions (2). However, once a joint is damaged and/or

Supported in part by the Ministry of Health, Labor and Welfare of Japan (Research Grants-In-Aid for Scientific Research), by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the University of Occupational and Environmental Health, Japan (UOEH Grant for Advanced Research).

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Dr. Tanaka has received consulting fees, speaking fees, and/or honoraria from Eisai, Takeda, Astellas, and Abbott (less than $10,000 each) and from Mitsubishi Tanabe and Chugai (more than $10,000 each) and has received research grants from Bristol-Myers Squibb, Mitsubishi Tanabe, MSD, Takeda, Astellas, Eisai, Chugai, Pfizer, and Daiichi Sankyo.

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Submitted for publication August 20, 2011; accepted in revised form May 17, 2012.

deformed, repair is challenging. Therefore, a novel treatment strategy aimed at bone regeneration is a prerequisite.

Mesenchymal stem cells (MSCs) reside in the bone marrow, peripheral blood, adipose tissue, and synovium and are characterized by pluripotency to differentiate into various mesenchymal lineages such as osteoblasts, chondrocytes, and adipocytes (3). Furthermore, recent studies have shown antiinflammatory properties of MSCs (4,5), suggesting their usefulness in both organ transplantation and treatment of autoimmune diseases. In fact, several preclinical and clinical studies in diseases such as multiple sclerosis (6), diabetes (7), stroke (8), osteogenesis imperfecta (9), and cartilage defect (10) have already demonstrated the therapeutic potential of these cells. Furthermore, systemic injection of MSCs in patients with graft-versus-host disease (11,12) and patients with myocardial infarction (13) has resulted in recovery with no serious adverse effects.

MSCs are also considered to be involved in self-renewal and self-repair processes in damaged/injured tissues and organs. Both short-term and long-term exposure to inflammatory stimuli such as cytokines often result in tissue damage. However, such damage is often reversible and repaired mainly by cells of mesenchymal lineages that differentiate from MSCs, which either migrate from the bone marrow or are resident in various organs (14). Interestingly, inflammation may also be involved in the processes of tissue repair. In fact, although several inflammatory cytokines enhance osteoclast differentiation, resulting in bone resorption followed by bone damage, the same cytokines also cause chronic enthesitis and excess abnormal bone formation, as seen in ankylosing spondylitis (15).

The precise molecular mechanisms involved in the differentiation of human MSCs into osteoblasts and the effects of inflammatory cytokines in the induction of cells of mesenchymal lineages remain unclear. To clarify the molecular mechanism of differentiation of human MSCs into osteoblasts in inflamed tissue and to establish a new treatment strategy for RA, we assessed the molecular mechanism of differentiation of human MSCs into osteoblasts in the presence of inflammatory cytokines by focusing on Wnt molecules that play important roles during osteoblast differentiation (16).

MATERIALS AND METHODS

Cell culture and osteoblast differentiation assay. Human MSCs were purchased from Lonza, plated in cell culture flasks, and expanded in MSC growth medium (MSCGM BulletKit; Lonza) at 37°C in an atmosphere of 5% CO_2 for 7–10 days. After the cells were allowed to grow adequately, adherent cells were trypsinized and then seeded at a density of 5,000 cells/cm^2 on a 24-well plastic plate. After 24 hours, MSC growth medium was removed, and osteogenic induction medium (human MSC differentiation BulletKit osteogenic; Lonza) was added to induce osteoblast differentiation (day 0). Recombinant human TNFα (R&D Systems), recombinant human IL-1β (ReliaTech), recombinant human IL-6 (Miltenyi Biotec), and human soluble IL-6 receptor (sIL-6R) (R&D Systems) or recombinant human IL-17 (PeproTech) was added to the osteogenic induction medium. IL-6 and sIL-6R were added sequentially throughout the experiments. During the experiments, the medium was changed every 2–3 days.

Cell viability. The number of viable cells in each well was determined with a water-soluble tetrazolium assay, using a TetraColor One Kit (Seikagaku) according to the manufacturer's instructions.

Osteoblast differentiation and mineralization assay. Alkaline phosphatase–positive cells were detected using a Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich). Briefly, cells were fixed with 10% formaldehyde for 15 minutes and rinsed with deionized water. Each well was treated with 350 μl of alkaline phosphatase staining solution containing sodium nitrite solution, fast blue base alkaline solution, and naphthol AS-BI solution in deionized water. After incubation at room temperature without direct light for 15 minutes, each well was washed with deionized water. Cells positive for alkaline phosphatase stained blue. Quantitative analysis of alkaline phosphatase activity was performed using a LabAssay *p*-Nitrophenylphosphate Detection Kit according to the protocol provided by the manufacturer (Wako), and the values were normalized according to cell viability, as measured using a TetraColor One assay.

Cell mineralization was evaluated by alizarin red S staining. Briefly, cells were fixed with 10% formaldehyde for 15 minutes and rinsed with deionized water prior to the addition of 350 μl of 1% alizarin red S solution (pH 4.1) per well. After incubation at room temperature for 15 minutes, the cells were washed again with deionized water.

Real-time polymerase chain reaction (PCR). Gene expression was assessed by real-time PCR. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer. The gene-specific primers (Applied Biosystems) were as follows: for runt-related transcription factor 2 (*RUNX2*), Hs01047978_m1; for bone sialoprotein (*BSP*), Hs00913377_m1; for osteopontin (*OPN*), Hs00960942_m1; for osteocalcin (*OC*), Hs01587814_g1; for *RANKL*, Hs01092186_m1; for *WNT3A*, Hs01055707_m1; for *WNT5A*, Hs00998537_m1; for *WNT7B*, Hs00536497_m1; for *WNT10B*, Hs00559664_m1; for receptor tyrosine kinase–like orphan receptor 2 (*ROR2*), Hs00171695_m1. The expression levels of the tested genes were normalized to expression of the housekeeping gene *ACTB* (Hs99999903_m1) and calculated using the $\Delta\Delta C_t$ method.

Western blotting. Cells were washed twice with cold phosphate buffered saline and dissolved with lysis buffer containing 50 m*M* Tris HCl (pH 8.0), 150 m*M* NaCl, protease inhibitor, and 10% Nonidet P40. Equal amounts of proteins (20 μg) were electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto nitro-

cellulose membranes, and blotted with antibodies against β-catenin (Cell Signaling Technology) or β-actin (Sigma-Aldrich) followed by secondary antibodies (GE Healthcare). Densitometry was performed using ImageJ software (NIH Image, National Institutes of Health; online at http://rsbweb.nih.gov/ij/), and protein expression was standardized to β-actin expression.

Small interfering RNA (siRNA). The following siRNAs were purchased from Invitrogen: *WNT5A* siRNA-1 (5′-ACAAACUGGUCCACGAUCUCCGUGC-3′), *WNT5A* siRNA-2 (5′-CUAGGAAGAACUUGGAAGACAUUGC-3′), *ROR2* siRNA-1 (5′-AACACGAAGUGGCAGAAGGAUGGGA-3′), *ROR2* siRNA-2 (5′-UAGACUUUCCCAAACCGGUCCUCUC-3′), *IRAK1* siRNA-1 (5′-CAGAGCCACCGCAGAUUAUCAUCAA-3′), *IRAK1* siRNA-2 (5′-CCCGAAGAAAGUGAUGAAUUUCAGA-3′), *IRAK4* siRNA-1 (5′-GCUGCAAGAGAUGACAGCUUCUUAA-3′), *IRAK4* siRNA-2 (5′-GGGAGGAUUUGGAGUUGUAUAUAAA-3′). Negative control siRNA (Stealth RNA: negative control Low GC [catalog no. 12935-200], Med GC [catalog no. 12935-112], and Higher GC [catalog no. 12935-400]) was also obtained from Invitrogen. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen). Briefly, 1 day before transfection, human MSCs (10,000 cells) were plated on a 24-well plastic plate in 500 μl of MSC growth medium free of antibiotics. The next day, transfection reagents containing 6 pmoles of siRNA and 1 μl of Lipofectamine RNAiMAX in a final volume of 100 μl with Opti-MEM I (Invitrogen) were added to each well and incubated for 24 hours prior to osteoblast differentiation assay.

Overexpression of Wnt-5a. Vector pCMV-Entry or vector pCMV-Entry carrying *WNT5A* complementary DNA (cDNA) (OriGene) was transfected using Lipofectamine LTX with Plus reagent (Invitrogen). Briefly, 1 day before transfection, human MSCs (10,000 cells) were plated on a 24-well plastic plate in 500 μl of MSC growth medium free of antibiotics. The next day, transfection reagents containing 500 ng of cDNA, 0.75 μl of Lipofectamine LTX, and 1 μl of Plus reagent in a final volume of 100 μl with Opti-MEM I (Invitrogen) were added to each well and incubated for 24 hours prior to osteoblast differentiation assay.

Statistical analysis. Data are expressed as the mean ± SEM. Differences between 2 groups were tested for significance by Student's *t*-test. Analysis of variance (ANOVA) was used for comparison of ≥3 groups, followed by Dunnett's multiple comparison post-test if the results were significant. *P* values less than 0.05 were considered significant.

RESULTS

Enhancement of osteoblast differentiation and mineralization by IL-1β. We first examined the effects of inflammatory cytokines on differentiation of human MSCs into osteoblasts. Among the tested cytokines, IL-1β efficiently induced the expression of messenger RNA (mRNA) for *RUNX2*, a key regulator of osteoblast differentiation, when undifferentiated human MSCs were cultured in osteogenic induction medium for 10 days (Figure 1A). TNFα, IL-6, and IL-17 also tended to induce *RUNX2* expression in human MSCs, especially at high concentrations, although their effects tended to be weaker than those of IL-1β. Concurrently, on day 10, alkaline phosphatase–positive cells (i.e., osteoblasts) were also detected in the presence of these cytokines (Figure 1B). Next, we confirmed that the differentiated human MSCs were osteoblasts, as determined by marked mineralization with alizarin red S staining. In contrast to TNFα, IL-6, and IL-17, the addition of IL-1β to human MSCs induced their differentiation into alizarin red S–positive cells within 10 days, even at the lowest concentration (0.1 ng/ml) (Figure 1C), whereas

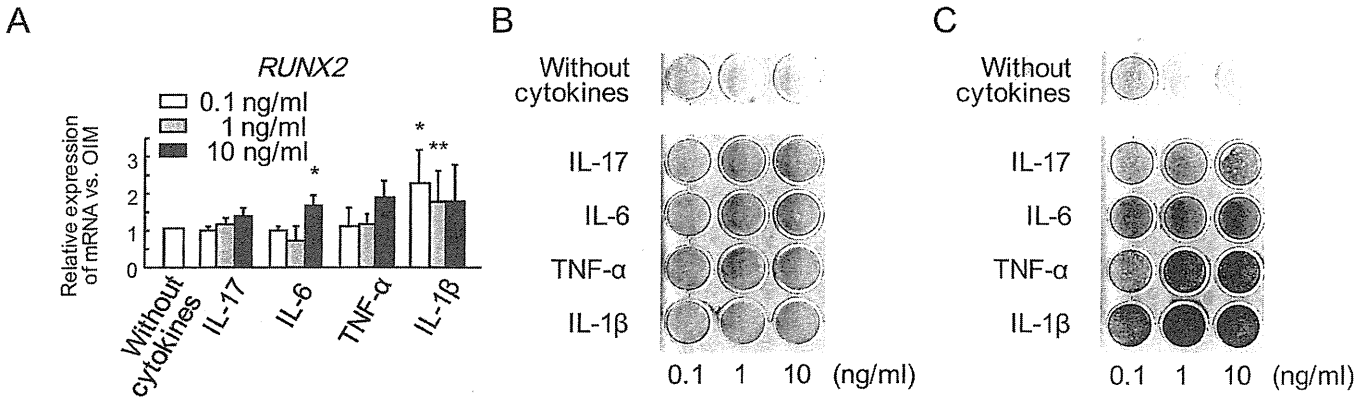

Figure 1. Effects of inflammatory cytokines on osteoblast differentiation. Human mesenchymal stem cells were cultured in osteogenic induction medium (OIM) with interleukin-17 (IL-17), IL-6, tumor necrosis factor α (TNFα), or IL-1β for 10 days. *RUNX2* expression was determined by real-time polymerase chain reaction (**A**), alkaline phosphatase staining (**B**), and alizarin red S staining (**C**). Soluble IL-6 receptor (100 ng/ml) was added together with IL-6. In **A**, Bars show the mean ± SEM of 3 experiments. * = $P < 0.05$; ** = $P < 0.01$ versus without cytokines, by analysis of variance followed by Dunnett's post-test. In **B** and **C**, results are representative of 3 experiments with similar findings.

differentiation and mineralization were delayed by at least 2 weeks when the cells were cultured with osteogenic induction medium alone (data not shown). Staining with both alkaline phosphatase and alizarin red S was limited in the presence of low concentrations of IL-17, IL-6, and TNFα but increased in a dose-dependent manner, reflecting the results of *RUNX2* expression in the cells (Figure 1C).

Kinetic analyses showed apparent mineralization on day 7, with further enhancement up to day 10, in the presence of IL-1β during the culture of human MSCs (Figure 2A). Alkaline phosphatase staining (Figure 2B) and *RUNX2* expression (Figure 2C) were also observed as early as culture day 4 and day 7, respectively. Although changes in osteoblast-specific genes such as *BSP*, *OPN*, *OC*, and *RANKL* were not significant, the expression of these genes tended to be increased by the addition of IL-1β, in a time-dependent manner (Figure 2C). These results indicated that IL-1β promotes the differentiation of human MSCs into osteoblasts.

Specific up-regulation of the Wnt-5a/Ror2 pathway by IL-1β. To assess the molecular mechanism of the induction of osteoblasts from human MSCs, we evaluated the expression of Wnt molecules, which are known to be crucial regulators of osteogenesis (16–19). Wnt ligands have been shown to activate various intracellular signals through either β-catenin–dependent (canonical) or β-catenin–independent (noncanonical) pathways (20). The canonical pathway inactivates glycogen synthase kinase 3β and enhances expression and nuclear translocation of β-catenin, which results in induction of the target genes (21). First, we evaluated the effect of IL-1β on β-catenin expression. Culture of human MSCs with osteogenic induction medium alone resulted in

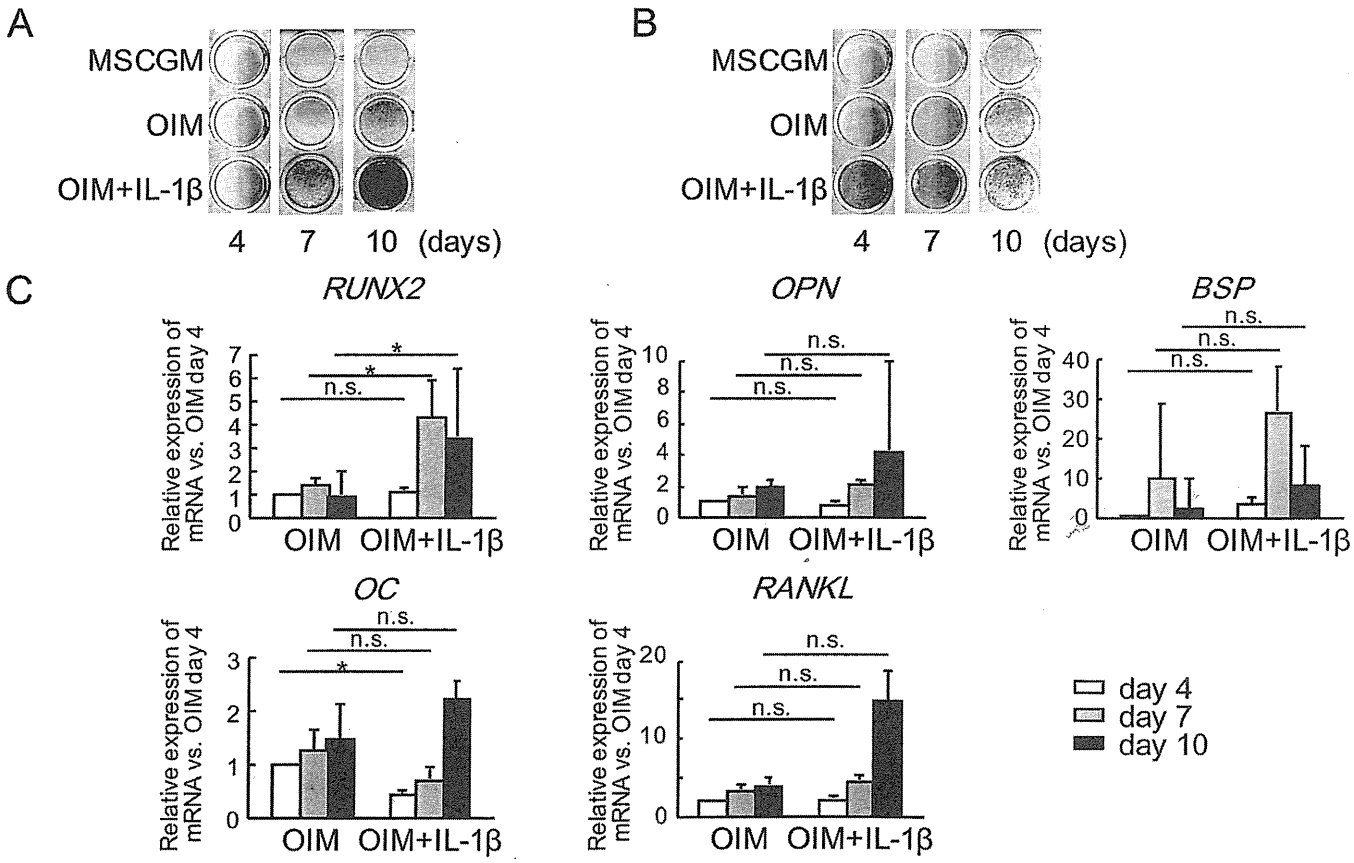

Figure 2. IL-1β enhances osteoblast differentiation. Human mesenchymal stem cells (MSCs) were cultured in osteogenic induction medium with or without IL-1β (0.1 ng/ml) for the indicated time periods. Osteoblast differentiation was analyzed by alizarin red S staining (**A**), alkaline phosphatase staining (**B**), and osteoblast-specific gene expression (**C**). In **A** and **B**, results are representative of 3 experiments with similar findings. In **C**, bars show the mean ± SEM of 3 experiments. * = $P < 0.05$ by unpaired *t*-test. MSCGM = MSC growth medium; NS = not significant (see Figure 1 for other definitions).

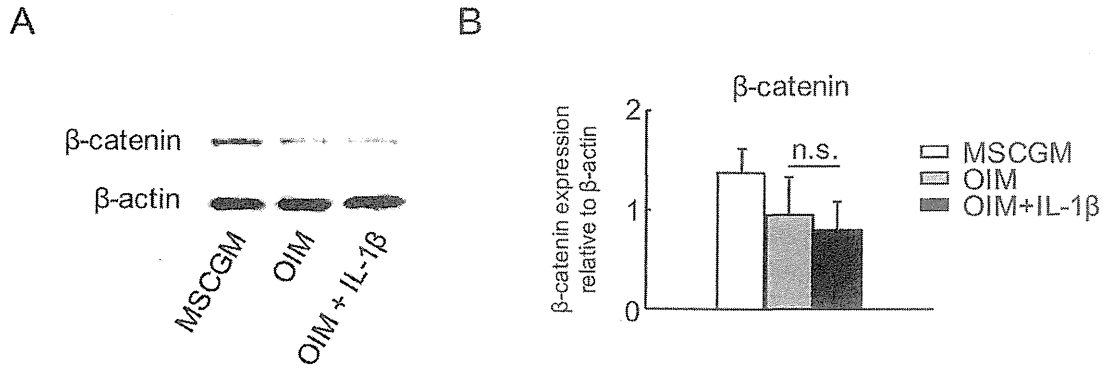

Figure 3. IL-1β has no effect on β-catenin expression. Human mesenchymal stem cells (MSCs) were cultured with or without IL-1β (0.1 ng/ml) for 7 days. β-catenin expression was detected by Western blotting (**A**) and analyzed by densitometry (**B**). In **A,** results are representative of 3 experiments with similar findings. In **B,** bars show the mean ± SEM of 3 experiments. MSCGM = MSC growth medium; NS = not significant (see Figure 1 for other definitions).

down-regulation of β-catenin expression, and the addition of IL-1β to the culture did not alter β-catenin expression (Figures 3A and B), indicating that IL-1β does not activate the canonical pathway in human MSCs.

Next, we investigated the effect of IL-1β on the expression of various Wnt molecules involved in osteogenesis (Wnt-3a, Wnt-5a, Wnt-7b, and Wnt-10b). Interestingly, only *WNT5A*, which is involved in the noncanonical pathway (20), was expressed in human MSCs

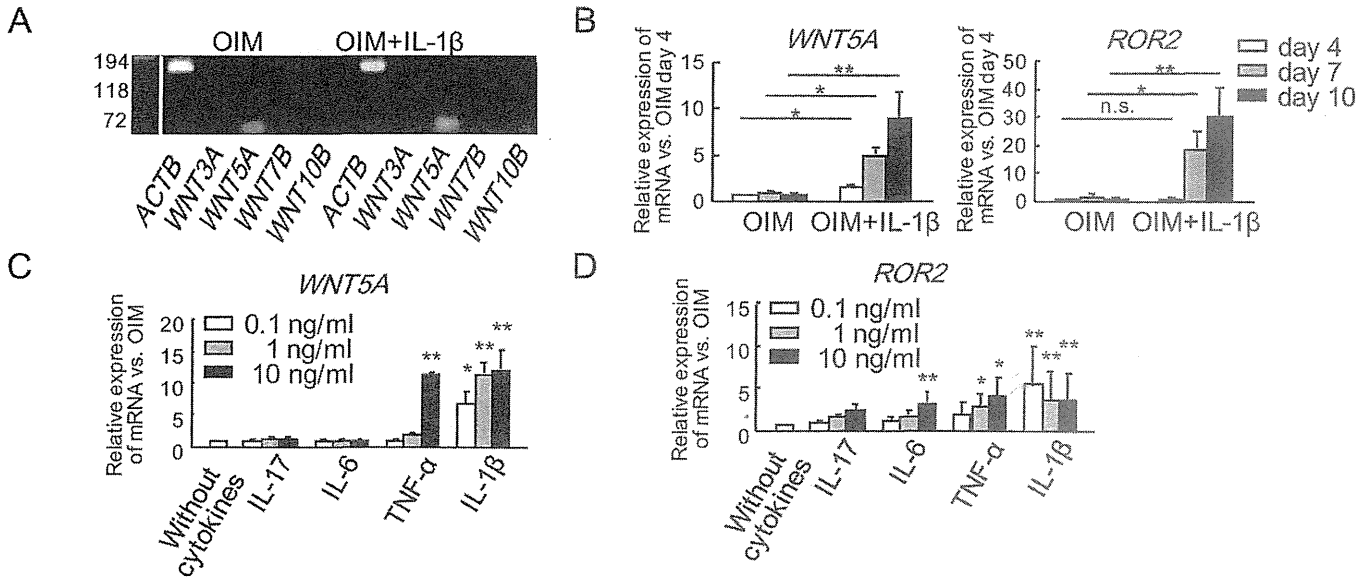

Figure 4. IL-1β specifically increases Wnt-5a and receptor tyrosine kinase–like orphan receptor 2 (Ror2) expression in human mesenchymal stem cells (MSCs). Human MSCs were cultured in osteogenic induction medium with or without inflammatory cytokines for the indicated time periods, and mRNA expression was analyzed by real-time polymerase chain reaction (PCR). **A,** Complementary DNA from human MSCs cultured for 10 days was amplified for 32 cycles and electrophoresed (for *ACTB*, 171 kbp; for *WNT3A*, 72 kbp; for *WNT5A*, 61 kbp; for *WNT7B*, 79 kbp; for *WNT10B*, 63 kbp). Results are representative of 3 experiments with similar findings. **B,** Human MSCs were cultured in osteogenic induction medium with or without IL-1β (0.1 ng/ml) for the indicated time periods, and the expression levels of *WNT5A* and *ROR2* were assessed by real-time PCR. * = $P < 0.05$; ** = $P < 0.01$, by unpaired *t*-test. **C** and **D,** Human MSCs were cultured in osteogenic induction medium with IL-17, IL-6, TNFα, or IL-1β (0.1 ng/ml, 1 ng/ml, 10 ng/ml) for 10 days, and *WNT5A* (**C**) and *ROR2* (**D**) expression was assessed by real-time PCR. Soluble IL-6 receptor (100 ng/ml) was added together with IL-6. Bars show the mean ± SEM of 3 experiments. * = $P < 0.05$; ** = $P < 0.01$ versus without cytokines, by analysis of variance followed by Dunnett's post-test. NS = not significant (see Figure 1 for other definitions).

throughout the experiments, and its expression was significantly up-regulated by IL-1β (Figures 4A and B), on culture day 4 and thereafter. The up-regulation of *WNT5A* was highly specific for IL-1β even at the lowest concentration (Figure 4C). However, when human MSCs were cultured with a high concentration of cytokines, both TNFα and IL-1β increased *WNT5A* expression to a similar level, whereas IL-6 and IL-17 had only a limited effect (Figure 4C). In contrast, *WNT3A*, *WNT7B*, and *WNT10B* were not detected in cultured human MSCs, regardless of the presence of IL-1β (Figure 4A).

Wnt-5a is known to bind to its cell surface receptors Frizzled and Ror2 in order to transduce intracellular signals. It is noteworthy that at low concentrations, IL-1β significantly up-regulated *ROR2* expression in human MSCs, but TNFα, IL-6, and IL-17 did not (Figure 4D). In contrast, at the higher concentration, TNFα and IL-6 induced *ROR2*, and IL-17 did not up-regulate *ROR2* expression. The pattern of *WNT5A* and *ROR2* expression after culture with both high and low cytokine concentrations and the pattern of alizarin red S staining were similar (Figure 1C and Figures 4C and D). These results suggest involvement of the IL-1β–induced Wnt-5a/Ror2 pathway in the differentiation of human MSCs into osteoblasts.

Role of the Wnt-5a/Ror2 pathway in accelerated osteoblast differentiation by IL-1β. To further evaluate the roles of Wnt-5a and Ror2, we performed gene knockdown to inhibit the Wnt-5a/Ror2 pathway in human MSCs. Silencing of either *WNT5A* or *ROR2* using 2 different siRNA sequences caused efficient suppression of *WNT5A* and *ROR2* expression (Figure 5A), resulting in prominent inhibition of mineralization on culture day

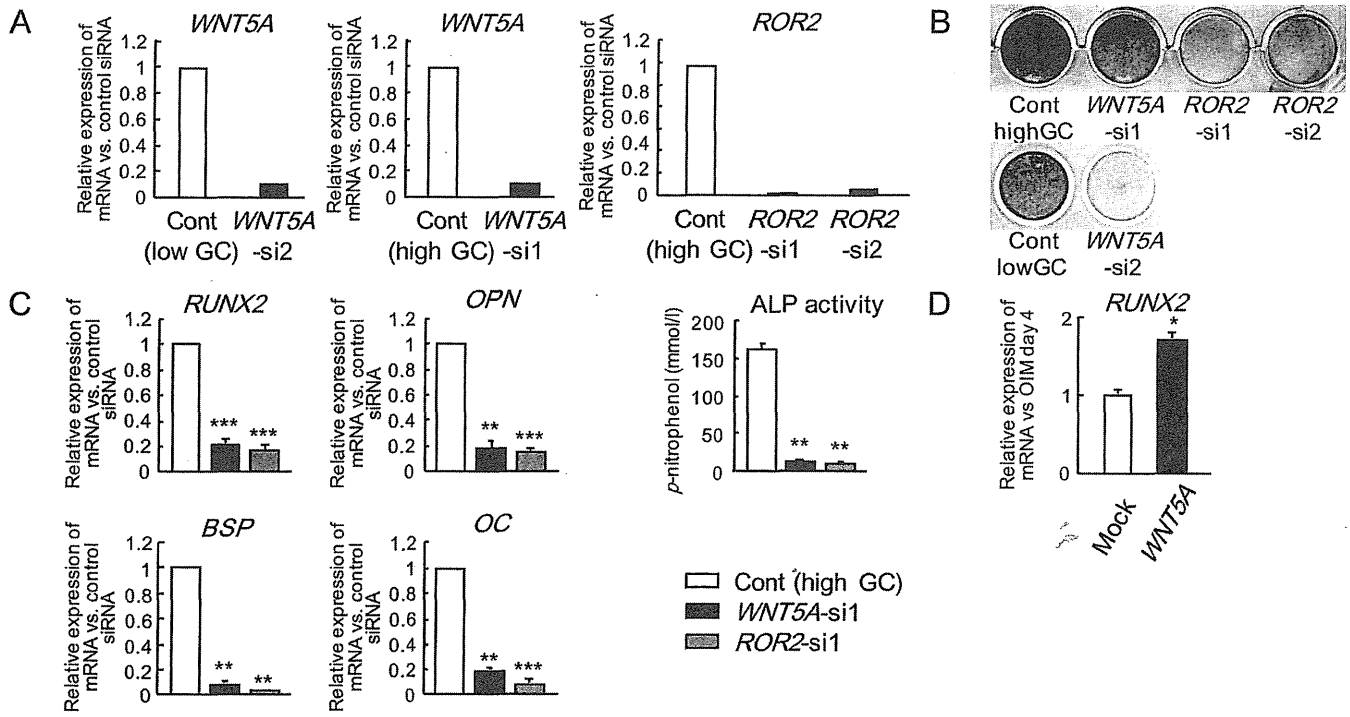

Figure 5. Wnt-5a/Ror2 pathway activated by interleukin-1β (IL-1β) plays an important role in enhancement of osteoblast differentiation. Human mesenchymal stem cells (MSCs) were transfected with control (Cont), *WNT5A*, or *ROR2* small interfering RNA (siRNA) (**A**) and cultured in osteogenic induction medium with IL-1β (0.1 ng/ml) for 7 days (**B** and **C**). **A** and **B**, Silencing of either *WNT5A* or *ROR2* using 2 different siRNA sequences caused efficient suppression of *WNT5A* and *ROR2* expression (**A**), resulting in prominent inhibition of mineralization on culture day 7, as analyzed by alizarin red S staining (**B**). In **B**, results are representative of 3 experiments with similar findings. **C**, Silencing of *WNT5A* or *ROR2* expression markedly reduced alkaline phosphatase (ALP) activity and also reduced the expression of various osteoblast markers (*RUNX2*, *BSP*, *OPN*, and *OC*) in human MSCs cultured with osteogenic induction medium and IL-1β for 7 days. **D**, When *WNT5A* was overexpressed in human MSCs and cultured for 4 days in osteogenic induction medium, *RUNX2* expression increased. Two different sequences of siRNA with high GC content (*WNT5A*-si1, *ROR2*-si1, and *ROR2*-si2) and low GC content (*WNT5A*-si2), respectively, were used for the experiments. Values in **A** are representative of 3 independent experiments. Values in **C** and **D** are the mean ± SEM of 3 independent experiments. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$, by unpaired *t*-test.

7, as analyzed by alizarin red S staining, compared with control siRNA (Figure 5B). In addition, silencing of either *WNT5A* or *ROR2* expression markedly reduced alkaline phosphatase activity and also reduced the expression of various osteoblast markers (*RUNX2*, *BSP*, *OPN*, and *OC*) in human MSCs cultured with osteogenic induction medium and IL-1β for 7 days (Figure 5C). Furthermore, when *WNT5A* was overexpressed in human MSCs and cultured for 4 days in osteogenic induction medium, *RUNX2* expression increased (Figure 5D), and the positive correlation between *WNT5A* and *RUNX2* expression and osteoblast differentiation was confirmed. These results also support the notion that there is a sequence of events induced by IL-1β; *WNT5A* was up-regulated by day 4, and subsequently, up-regulation of *RUNX2* was detected beginning on day 7 during the differentiation of human MSCs (Figures 2C and 4B). These findings confirm the commitment of the IL-1β–induced Wnt-5a/Ror2 pathway to the differentiation of human MSCs into osteoblasts.

Effect of IL-1β signal transduction through IL-1 receptor–associated kinase (IRAK) on osteoblast differentiation. The IL-1β–mediated signaling pathway is known to consist of IRAK-dependent and IRAK-independent pathways. IRAK plays an essential role in IL-1–mediated signaling pathways for optimal induction of cellular responses (22). To assess the direct involvement of IL-1β signaling in enhanced osteoblast differentiation, we performed gene knockdown to inhibit the IRAK pathway. Silencing of either *IRAK1* or *IRAK4* by siRNA with 2 different sequences resulted in efficient reduction of IL-1β–induced mineralization during the differentiation of human MSCs into osteoblasts (Figure 6).

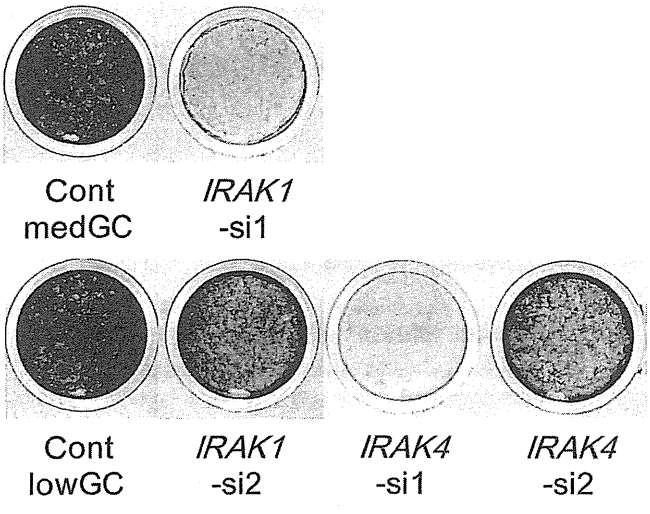

Figure 6. Involvement of interleukin-1 (IL-1) receptor–associated kinase 1 (IRAK-1)/IRAK-4 in IL-1β–induced osteoblast differentiation. Human mesenchymal stem cells were transfected with control (Cont), *IRAK1*, or *IRAK4* small interfering RNA (siRNA), cultured in osteogenic induction medium with IL-1β (0.1 ng/ml) for 7 days, and stained with alizarin red S. Two different sequences of siRNA with medium GC content (IRAK1-si1) and low GC content (*IRAK1*-si2, *IRAK4*-si1, *IRAK*-si2), respectively, were used for the experiments.

DISCUSSION

Among the 4 inflammatory cytokines tested in this study, IL-1β was the most potent in inducing differentiation of human MSCs into osteoblasts. This was confirmed by mRNA expression of osteoblast markers, alkaline phosphatase activity, and strong alizarin red S staining within 7 days of culture. Although the effects of TNFα and IL-6 on the induction of osteoblast differentiation were similar, these effects were inferior to those of IL-1β. As shown in Figure 2C, IL-1β up-regulated *RUNX2* expression; the expression continued to increase up to day 7, although it started to decrease on day 10. This was in sharp contrast to cultures in osteogenic induction medium alone, in which the expression level did not change even after 10-day culture. Because *RUNX2* functions as an enhancer in the early stage and as a suppressor in the late stage of osteoblast differentiation, we speculate that IL-1β enhanced the functions of *RUNX2* at the early phase of human MSC differentiation. Although culture with IL-1β did not up-regulate the expression of *BSP*, *OPN*, or *OC*, it resulted in enhanced mineralization, which is essential for bone formation. In contrast, the expression of *OC* was rather suppressed on day 4 (Figure 2C). This phenomenon is consistent with the findings of a previous study showing that IL-1β suppresses *OC* synthesis (23). IL-1β first suppressed transcription of *OC* mRNA and subsequently increased the *RUNX2*-promoted transcription of *OC* mRNA.

Although our results confirmed enhanced osteoblast differentiation, the expression of *RANKL*, an osteoblast marker that is also known to cause bone resorption by inducing osteoclast differentiation, tended to increase in the presence of IL-1β (Figure 2C). This result leads to a concern that the induced differentiation of osteoblasts might cause inadequate bone resorption. However, the expression of *RANKL* was comparable between human MSCs and normal osteoblasts in the presence or absence of IL-1β (data not shown). In addition, constitutive production of osteoprotegerin by MSCs was preserved after culture in osteogenic induc-

tion medium for 28 days (data not shown). These results also imply that human MSCs can differentiate into osteoblasts without inducing bone resorption, even in an environment with inflammatory stimuli.

Next, we assessed the signaling mechanisms of IL-1β–induced differentiation of human MSCs into osteoblasts and demonstrated involvement of the Wnt-5a/Ror2 pathway. The Wnt molecules are known to play important roles in various developmental processes including osteogenesis (16–19) and are induced by a variety of cytokines (25,26). Of the Wnt family molecules, Wnt-3a is involved in the canonical pathway and is known to inhibit the noncanonical pathway, resulting in the suppression of differentiation of cells of multiple lineages (18,27). Paradoxically, Wnt-5a stimulates the noncanonical pathway and thus enhances the proliferation of fibroblasts in RA (28) and is involved in sepsis, inflammatory bowel disease (29), malignant melanoma (30), non–small cell lung cancer (31), and gastric cancer (32). The results of the current study demonstrated that IL-1β induced the expression and function of Wnt-5a in human MSCs during their differentiation into osteoblasts, suggesting that this pathway is a potentially suitable target to induce activation by therapeutic agents for the treatment of RA. Takada et al reported the importance of the balance of Wnt-5a–peroxisome proliferator–activated receptor γ (PPARγ) in the fate of MSCs (27). Considering the molecular mechanism of the interaction between IL-1β and Wnt-5a, transrepression of PPARγ by IL-1β might result in activation of Wnt-5a transcription (33).

The results of the present study also showed that IL-1β (even at low concentrations) markedly induced the expression of *ROR2*, a receptor for Wnt-5a, in human MSCs, whereas TNFα, IL-6, and IL-17 did not. Furthermore, knockdown of *ROR2* or *WNT5A* by siRNA completely suppressed the expression of various osteoblast markers as well as alkaline phosphatase activity and mineralization. These results indicate that IL-1β–induced human MSC differentiation into osteoblasts is mediated through the Wnt-5a/Ror2 pathway. Wnt5a activates at least 3 different pathways—the Wnt5a/Ca^{2+}, Wnt-5a/Rho, and Wnt-5a/JNK pathways, which manage the cytoskeleton, and the importance of JNK in osteoblast differentiation has been reported (34). Because IL-1β is known to activate JNK, a sequence of events comprising IL-1β, Wnt-5a/Ror2, JNK, and RUNX-2 is potentially involved in the differentiation of human MSCs into osteoblasts.

It is well known that chronic inflammatory stimulation often results in tissue and organ damage. However, such damage is usually reversible and is repaired spontaneously through unknown mechanisms. It is postulated that cells of mesenchymal lineages that differentiate from MSCs or those resident in various organs might be involved in self-renewal and/or self-repair of tissue. Our results imply that IL-1β rapidly and efficiently induces differentiation of MSCs into osteoblasts, resulting in sufficient tissue mineralization. This phenomenon could be an example of an MSC-based bone self-repair process or a nonimmunologic self-defense mechanism.

In RA, bone resorption may overcome bone formation, probably in association with defective MSC-related self-repair mechanisms. Regeneration of the damaged joints of patients with RA could potentially be induced by MSCs, based on the following: 1) MSCs can differentiate into osteoblasts in the presence of inflammatory cytokines, which are abundantly present in the inflamed synovium, 2) we have observed that MSCs produce high amounts of osteoprotegerin, which efficiently suppresses RANKL/RANK pathway–mediated osteoclastogenesis (35), and 3) MSCs have immunosuppressive properties, making them further suitable for the treatment of RA (5).

Our findings indicate that the presence of normal MSCs at the site of local inflammation can enhance the self-repair ability, especially in bone- and joint-destructive disease. Taken together, the results of this study suggest that IL-1β–mediated differentiation of MSCs into osteoblasts could be beneficial not only for the self-renewal system in injured organs and inflamed tissue, including bone, joints, and many others, but also for regenerative therapy of damaged organs such as the joints of patients with RA.

ACKNOWLEDGMENT

We thank Ms T. Adachi for excellent technical assistance.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Tanaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sonomoto, Yamaoka, Oshita, Fukuyo, Nakano, Okada, Tanaka.

Acquisition of data. Sonomoto, Oshita, Zhang.

Analysis and interpretation of data. Sonomoto, Yamaoka, Oshita, Fukuyo.

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ARTHRITIS & RHEUMATISM
Vol. 64, No. 6, June 2012, pp 1790–1798
DOI 10.1002/art.34329

The JAK Inhibitor Tofacitinib Regulates Synovitis Through Inhibition of Interferon-γ and Interleukin-17 Production by Human CD4+ T Cells

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Objective. **Tofacitinib (CP-690,550) is a novel JAK inhibitor that is currently in clinical trials for the treatment of rheumatoid arthritis (RA). The aim of this study was to examine the effects of tofacitinib in vitro and in vivo in RA, in order to elucidate the role of JAK in the disease process.**

Methods. **CD4+ T cells, CD14+ monocytes, and synovial fibroblasts (SFs) were purified from the synovium and peripheral blood of patients with RA and were evaluated for the effect of tofacitinib on cytokine production and cell proliferation. For in vivo analysis, synovium and cartilage samples obtained from patients with RA were implanted in immunodeficient mice (SCID-HuRAg mice), and tofacitinib was administered via an osmotic minipump.**

Results. **Tofacitinib treatment of CD4+ T cells originating from synovium and peripheral blood inhibited the production of interleukin-17 (IL-17) and interferon-γ (IFNγ) in a dose-dependent manner, affecting both proliferation and transcription, but had no effect on IL-6 and IL-8 production. Tofacitinib did not affect IL-6 and IL-8 production by RASFs and CD14+ monocytes. However, conditioned medium from CD4+ T cells cultured with tofacitinib inhibited IL-6 production by RASFs and IL-8 production by CD14+ monocytes. Treatment of SCID-HuRAg mice with tofacitinib decreased serum levels of human IL-6 and IL-8 and markedly suppressed invasion of synovial tissue into cartilage.**

Conclusion. **Tofacitinib directly suppressed the production of IL-17 and IFNγ and the proliferation of CD4+ T cells, resulting in inhibition of IL-6 production by RASFs and IL-8 production by CD14+ cells and decreased cartilage destruction. In CD4+ T cells, presumably Th1 and Th17 cells, JAK plays a crucial role in RA synovitis.**

The importance of inflammatory cytokines in the pathogenesis of rheumatoid arthritis (RA) has become apparent based on the clinical efficacy of biologic agents targeting tumor necrosis factor α (TNFα), interleukin-1 (IL-1) receptor, and IL-6. For such cytokines to exert their biologic activities, the appropriate intracellular signaling pathways must be activated via their specific receptors on the cell surface. Tyrosine kinases are the first intracellular signaling molecules to be activated following receptor binding in a cytokine response. Therefore, various tyrosine kinases are involved at the sites of inflammation (1,2). Several recent studies have focused on tyrosine kinases as potential targets for the treatment of RA. Among these, the JAK family, consisting of JAK-1, JAK-2, JAK-3, and tyrosine kinase 2

Supported in part by the Ministry of Health, Labor, and Welfare of Japan (Research Grant-In-Aid for Scientific Research), by the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by the University of Occupational and Environmental Health, Japan.

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Dr. Y. Tanaka has received consulting fees, speaking fees, and/or honoraria from Eisai, Pfizer, Abbott, Janssen Pharma, Takeda, AstraZeneca, Astellas, Asahi Kasei Pharma, and GlaxoSmithKline (less than $10,000 each) and from Chugai and Mitsubishi Tanabe Pharma (more than $10,000 each).

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Submitted for publication April 28, 2011; accepted in revised form December 1, 2011.

(Tyk-2), has gathered particular attention, because JAKs are essential for the signaling pathways of various cytokines and growth factors that have been implicated in the pathogenesis of RA (e.g., IL-2, IL-6, IL-7, IL-12, IL-15, IL-17, IL-23, granulocyte–macrophage colony-stimulating factor, and interferon-γ [IFNγ]).

The importance of JAKs in development of the immune system has been demonstrated by gene deletion or mutation. According to the abundant expression of JAK-1 and JAK-2, deletion or mutation of either gene in mice has been shown to be lethal, whereas mutation of Tyk-2 or JAK-3 results in immunodeficiency in both humans and mice (3,4). In contrast to other members of the JAK family that are widely expressed, JAK-3 expression is essentially limited to hematopoietic cells, and JAK-3 constitutively binds to the common γ-chain (γ_c-chain). The γ_c-chain is a common receptor subunit for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, some of which are known to be involved in RA. In fact, tofacitinib, an orally available selective inhibitor of JAK, "dose-dependently decreased endpoints of disease" in both murine collagen-induced arthritis and rat adjuvant-induced arthritis (5). Furthermore, tofacitinib is currently in clinical trials for the treatment of RA, and satisfactory effects, acceptable safety, and, surprisingly, efficacy comparable with that of known biologic agents have been observed (6–8).

Tofacitinib is a selective inhibitor of JAKs with nanomolar potency resulting in the inhibition of transmigration of STAT molecules to the nucleus. Initially, the high specificity of tofacitinib for JAK-3 attracted attention; however, recent efforts to investigate the mechanism of action have shown that tofacitinib interacts with multiple JAKs and presumably other kinases (9–11). Tofacitinib preferentially inhibits JAK-1, JAK-3, and STAT-1 activation, resulting in potent inhibition of γ_c-chain cytokines, IL-6, and IFNγ in naive CD4+ T cells (12).

Although the biologic roles of JAK in lymphocytes are well known, its function in monocyte-lineage cells remains elusive. Previously, we reported that dendritic cells (DCs) from $Jak3^{-/-}$ mice produce increased IL-10, but not IL-6 or TNFα, compared with wild-type DCs, in response to Toll-like receptor ligands (13). Ghoreschi et al also showed increased IL-10 production in mouse plasma after the mice received an intraperitoneal injection of lipopolysaccharide (LPS) following tofacitinib pretreatment (12). Accordingly, tofacitinib may affect not only lymphoid cells but also myeloid cells and other cells that do not express JAK-3, such as mesenchymal cells involved in synovitis, as an off-target effect.

The remarkable effects of tofacitinib observed in clinical studies thus far indicate that this agent will be widely used for the treatment of RA. Although the precise action of tofacitinib on the JAK/STAT pathway in mice has been investigated, the exact mechanism of action under inflammatory conditions in humans remains unclear. Improved knowledge of the underlying mechanisms of tofacitinib would contribute to a better understanding of the pathogenesis of RA and to further application of the drug in other diseases. In this study, we used synovium from patients with RA for in vitro and in vivo experiments to evaluate the effect of tofacitinib and elucidate the role of JAKs at the sites of inflammation in RA.

PATIENTS AND METHODS

Tofacitinib (CP-690,550). Tofacitinib (kindly provided by Pfizer) was dissolved in DMSO (Wako) and kept as a 20-μ*M* stock solution at −80°C.

Cell isolation. Human synovial tissue specimens were obtained from patients undergoing joint replacement surgery or synovectomy at our university and at the National Hospital Organization Kyushu Medical Center. All patients fulfilled the 1987 American College of Rheumatology criteria for the classification of RA (14) and provided written informed consent. All patients had active RA but had never received treatment with biologic agents. The tissue was digested with collagenase (1 mg/ml; Wako) and Dispase (1,000 proteolytic units/ml; Godo Shusei) for at least 2 hours at 37°C. After being filtrated, the cells were cultured in 10-cm culture dishes with RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Following overnight culture, CD14+ cells were isolated from adherent cells by positive selection, and CD4+ T cells were isolated from nonadherent cells by negative selection using a magnetic cell separation system (Miltenyi Biotec). Purities were >90%, as determined by flow cytometry (FACSCalibur; BD PharMingen). Adherent cells were subcultured in Dulbecco's modified Eagle's medium to purify RA synovial fibroblasts (RASFs). Cells between passages 3 and 6 were used for the experiments, as a homogeneous population of RASFs. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation over Lymphocyte Separation Medium (ICN Pharmaceuticals), and CD4+ T cells and CD14+ monocytes were obtained as described above.

Cell proliferation, apoptosis, and cytokine production. Synovial and peripheral blood CD4+ T cells were plated at 2×10^5 cells/200 μl with the indicated concentrations of tofacitinib and stimulated with plate-bound anti-CD3 antibodies (100 ng/well; R&D Systems) and soluble anti-CD28 antibodies (1 μg/ml; R&D Systems) for 72 hours; supernatants were harvested to measure cytokine levels and to use as culture medium for RASFs and CD14+ cells. Peripheral blood CD4+ T cells were prestimulated with anti-CD3 and anti-CD28 antibodies for 72 hours, collected and washed, and re-plated at 2×10^5 cells/200 μl with the indicated concentrations of tofacitinib; the cells were stimulated with recombinant IL-2 (100 ng/ml; R&D Systems) for 72 hours, and supernatants