Successful Treatment of Animal Models of Rheumatoid Arthritis with Small-Molecule Cyclin-Dependent Kinase Inhibitors[1]

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Intraarticular gene transfer of cyclin-dependent kinase (CDK) inhibitors to suppress synovial cell cycling has shown efficacy in treating animal models of rheumatoid arthritis. Endogenous CDK inhibitors also modulate immune function via a CDK-independent pathway. Accordingly, systemic administration of small molecules that inhibit CDK may or may not ameliorate arthritis. To address this issue, alvocidib (flavopiridol), known to be tolerated clinically for treating cancers, and a newly synthesized CDK4/6-selective inhibitor were tested for antiarthritic effects. In vitro, they inhibited proliferation of human and mouse synovial fibroblasts without inducing apoptosis. In vivo, treatment of collagen-induced arthritis mice with alvocidib suppressed synovial hyperplasia and joint destruction, whereas serum concentrations of anti-collagen type II (CII) Abs and proliferative responses to CII were maintained. Treatment was effective even when therapeutically administered. Treated mice developed arthritis after termination of treatment. Thus, immune responses to CII were unimpaired. The same treatment ameliorated arthritis induced by K/BxN serum transfer to lymphocyte-deficient mice. Similarly, the CDK4/6-selective inhibitor suppressed collagen-induced arthritis. Both small-molecule CDK inhibitors were effective in treating animal models of rheumatoid arthritis not by suppressing lymphocyte function. Thus, the two small-molecule CDK inhibitors ameliorated arthritis models in a distinctive way, compared with other immunosuppressive drugs. ***The Journal of Immunology*****, 2008, 180: 1954–1961.**

Rheumatoid arthritis (RA)[3] is a chronic inflammatory disease characterized by synovial hyperplasia with massive infiltration of inflammatory cells in the affected joints. This hyperplasia leads to degeneration of cartilage, erosion of bone, and ultimately functional loss of joints. Although the etiology of RA remains elusive, T cells recognizing unknown autoantigens have been proposed to initiate inflammation in synovial tissues. This mechanism is followed by local recruitment of leukocytes, which are further activated in the inflamed sites (1). Immune complexes, compliment activation, and activation of lymphocytes, macrophages, neutrophils, mast cells, and synovial fibroblasts are part of the aberrant humoral and cellular network (2). Cytokines derived from these cells induce intense proliferation in synovial fibroblasts. Activated fibroblasts become another source of inflammatory cytokines and mediators including tissue-degrading proteinases and prostaglandins. In this manner, the hyperplastic synovial tissue, called a pannus, serves as a nidus for further spread of destructive inflammation, producing cartilage-degrading enzymes and invading the bone matrix of rheumatoid joints (3).

Therapeutic agents of a biologic nature, such as mAbs against TNF-α, soluble TNFR, anti-IL-6R Ab, and IL-1R antagonist, were recently brought into clinical use with the aim of interrupting inflammatory cytokine circuits in joints affected by RA. Although these agents often are more effective than conventional antirheumatic drugs, quite a few patients fail to obtain expected clinical benefit from these medications. Other new drugs such as abatacept, tacrolimus, and rituximab are given to suppress upstream targets such as T and B lymphocytes. These anti-inflammatory agents share similar limitations in clinical response (4). More seriously, these medications often render patients immunocompromised, making them susceptible to serious infections (4).

As mentioned earlier, a salient characteristic in rheumatoid pathology is overgrowth of synovial fibroblasts to form a hyperplastic pannus. This feature has led us to explore cell cycle regulation as a new therapeutic approach to treatment of RA. This antiproliferative treatment focuses on cyclin-dependent kinase (CDK) inhibitors (CDKI) as primary cell cycle regulators. These endogenous nucleoproteins inhibit CDKs that bind to cyclins to initiate catalytic activation (5). CDKs phosphorylate a retinoblastoma (RB) gene product, which results in release of E2F transcription factors for cell cycle progression (5). Based on structural and functional characteristics, CDKIs have been grouped into two distinct families, INK4 and Cip/Kip (5, 6). The INK4 proteins, consisting of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, form binary complexes with CDK4 or CDK6, which otherwise would promote

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; smCDK, small-molecule CDK; CIA, collagen-induced arthritis; CII, type II collagen; FLS, fibroblast-like synoviocyte; MFLS, mouse fibroblast-like synoviocyte; RB, retinoblastoma; LN, lymph node.

G_1/S phase transition in the cell cycle. In contrast, the Cip/Kip proteins, consisting of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, bind to all cyclin-CDK complexes.

We have reported that $p16^{INK4a}$ and $p21^{Cip1}$ were induced readily in vitro in cultured RA fibroblast-like synoviocytes (FLSs). Intra-articular gene transfer of $p16^{INK4a}$ or $p21^{Cip1}$ ameliorated disease in animal models of RA, including collagen-induced arthritis (CIA) in mice and adjuvant arthritis in rats (7–9). These studies have indicated that induction of $p16^{INK4a}$ and $p21^{Cip1}$ expression in synovial tissue should hold promise as a new therapeutic strategy for RA treatment. Notably, because cell cycle regulation therapy suppresses a phase of rheumatoid pathology distinct from that suppressed by anti-inflammatory drugs, combination with another antirheumatic drug might act synergistically.

To apply gene transfer to treatment of patients with RA, vectors and protocols need be optimized to avoid unexpected tragic complications (10). An alternative to gene therapy as a way to inhibit CDK activity is the use of small-molecule (sm) compounds that inhibit CDK. Because uncontrolled CDK activity drives neoplastic cell cycling in some tumor cells, CDK activity represents an attractive target in development of anti-cancer therapeutics (11). In response to these smCDK inhibitors, some tumor cells stop proliferation and then also undergo apoptotic cell death (12–14). Thus, many smCDK inhibitors have been developed as candidate oncostatic drugs with a prototype being alvocidib (formerly called flavopiridol) (11). This synthetic flavone, the first CDKI to undergo clinical trials (11), inhibits the kinase activity of multiple CDKs such as CDK1, 2, 4, 6, and 7 (15). Structure-activity studies have shown that alvocidib interferes with binding of ATP to the adenine-binding pocket of CDK2 (16). However, although alvocidib generally proved safe in clinical trials, its efficacy was limited except for a subtype of malignant cells (11, 17). Other smCDK inhibitors, such as staurosporine, R-roscovitine, and BMS-387032, also have been studied in clinical trials, showing good tolerability (11).

The primary known role for CDKI is suppression of CDKs required for cell cycle progression. However, our group as well as other investigators have shown that $p16^{INK4a}$ and $p21^{Cip1}$ also have immunomodulatory effects (7, 18, 19). Notably, overexpression of $p21^{Cip1}$ in RA FLSs reduced production of inflammatory cytokines and tissue-degrading enzymes involved in rheumatoid pathology. This is at least partly a result of inhibition of JNK by $p21^{Cip1}$ (18). Thus, immunomodulation by CDKI, which potentially contribute to antiarthritic effects of CDKI gene transfer, would not necessarily depend on inhibition of CDK activity, raising a question as to whether inhibition of CDK per se can control arthritis. Of special interest here is inhibition of CDK4/6, which are shared targets of $p16^{INK4a}$ and $p21^{Cip1}$.

In the present study we evaluated how systemic administration of two smCDK inhibitors affects animal models of RA. One was alvocidib, while the other was a newly synthesized CDK4/6-selective inhibitor. Systemic administration of either compound inhibited arthritis without obvious immunosuppression or side effects. The results argue that alvocidib exerted its effects primarily by CDK4/6 inhibition.

Materials and Methods

Reagents

Alvocidib was provided by Aventis Pharmaceuticals. A stock solution of alvocidib prepared in DMSO was diluted to working concentrations before each experiment. In addition, a CDK4/6-selective inhibitor compound A (*N*-{5-[2-(cyclohexyloxy)-6-methylpyrimidin-4-yl]-1,3-thiazol-2-yl}-5-[(4-methylpiperazin-1-yl)methyl]pyrazin-2-amine), which was characterized as compound 4 in the previous report (20), was synthesized at Merck-Banyu. It was dissolved in DMSO for in vitro analyses and in 5% glucose containing 10 mM citrate buffer (pH 4) for treatment of mice. Human and mouse IL-1β and TNF-α were purchased from WAKO.

Cells

FLSs were prepared from synovial tissues of patients with RA who underwent total joint replacement surgery or synovectomy at Tokyo Medical and Dental University Hospital (Tokyo, Japan) or National Shimoshizu Hospital (Chiba, Japan). RA was diagnosed according to the criteria of the American College of Rheumatology (21). Written consent forms concerning experimental use of resected tissues were completed by patients before surgery. All procedures in the present studies were approved by the ethics committees of RIKEN and Tokyo Medical and Dental University. Mouse fibroblast-like synoviocytes (MFLSs) were prepared from synovial tissues from knee joints of CIA mice as previously described (22). Although FLSs were used from 7 to 11 passages, MFLSs were used from 6 to 11 passages. Both were cultured in DMEM (Sigma-Aldrich) supplemented with L-glutamine, penicillin, streptomycin, and 10% FBS (Sigma-Aldrich). Purity of the fibroblasts was assessed with flow cytometry (23). CD14 or HLA class II were not expressed by FLSs, suggesting that macrophages and dendritic cells were not present in FLSs.

Proliferation assays

From 2000 to 5000 cells/well were grown overnight in 96-well plates and stimulated with 10 ng/ml IL-1β and 10 ng/ml TNF-α. These cytokine concentrations were determined to be optimal in preliminary experiments. Alvocidib (1–300 nM), compound A (0.01–10 μM), or DMSO alone was placed in wells in the presence of 10% FBS. After 24–48 h, BrdU was added and culture was continued for 20 h. Then incorporation of BrdU was quantified by ELISA with a BrdU cell proliferation ELISA kit (Exalpha Biologicals).

Cell cycle analysis

Cells were stimulated for 24 h in the presence or absence of smCDK inhibitors, washed with PBS, and resuspended in 0.15% Triton X-100/PBS before staining with propidium iodide (50 μg/ml) and examination for DNA content with a FACSCaliber flow cytometer (BD Biosciences).

Animal models of arthritis

CIA was induced in 7-wk-old male DBA/1 mice purchased from Japan Charles River Breeding Laboratories. Each mouse was immunized with 200 μg of bovine type II collagen (CII; Collagen Research Center) emulsified with CFA (Difco) by injection at the tail base. Immunization was repeated 21 days after primary immunization.

Serum-transfer arthritis was induced by transfer of serum from arthritic K/BxN mice, which spontaneously develop arthritis resembling RA. These mice were provided by Drs. C. Benoist and D. Mathis (Joslin Diabetes Center, Boston, MA) (24). Preliminary titration experiments showed that injection of 300 μl of serum into the peritoneal cavity transferred arthritis consistently in RAG2-null mutant ($RAG2^{-/-}$) and C57BL/6 wild-type mice. All procedures met institutional regulations for animal experiments.

Assessment of arthritis

Arthritis in each limb of arthritic mice was assessed clinically by visual scoring from 0 to 4: 0, no swelling; 1, detectable swelling in one joint; 2, non-severe swelling in two or more joints; 3, severe swelling in two or more joints; and 4, severe swelling in two or more joints including digital swelling. Maximal score for an individual animal was 16. Joint swelling was quantified by measuring hindpaw thickness and the ankle width within a micrometer (Ozaki Manufacturing). Arthritis scores were analyzed statistically with unpaired Student's *t* test.

In histological examination, hindpaws were obtained and fixed in 10% buffered formalin, decalcified in 10% EDTA, and embedded in paraffin. Sections (4 μm) were stained with H&E for histologic examination. Infiltration of inflammatory cells, transformation of synovial lining, cartilage destruction, and pannus formation were independently scored in a blind manner from 0 to 3 as previously described (25). Maximum histological score was 12. Histological scores were analyzed statistically with unpaired Student's *t* test. Radiographs of formalin-fixed hindpaws were obtained with a cabinet soft x-ray apparatus (CMB-2; Softex).

Measurement of serum levels of anti-CII Abs

Serum samples were collected at indicated time points and examined for anti-CII Ab concentrations by ELISA. Briefly, wells of 96-well plates were

coated overnight at 4°C with bovine CII in PBS, washed with PBS-0.05% Tween 20, and blocked with 2% BSA in PBS. Samples were incubated for 2 h at room temperature. After washing, HRP-conjugated rabbit anti-mouse IgG1, IgG2a, or IgG2b Ab (Zymed Laboratories) was added. Reaction product formed with 3,3',5,5'-tetramethylbenzidine was measured as OD at 450 nm. A standard mixture serum from arthritic mice was placed on each plate in serial dilution. Arbitrary units for anti-CII IgG1, IgG2a, and IgG2b Abs were determined with this standard curve.

Western blot analysis

Whole-cell lysates (15 μg of protein) were separated by SDS-PAGE on 7.5% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Anti-phospho RB (Ser^{807}/Ser^{811}) Ab (catalog no. 9308; Cell Signaling Technology) at 1/1000 dilution and anti-RB mAb (clone G3-245; BD Pharmingen) at 1 μg/ml were used as primary Abs. Bound Abs were visualized with peroxidase-conjugated secondary Ab (Amersham Biosciences) at 1/3000 dilution and ECL system (ECL; Amersham Biosciences).

Immunohistochemistry

Paraffin-fixed tissue sections were deparaffinized and incubated with anti-phospho RB (Ser^{807}/Ser^{811}) Ab. They were then incubated with biotinylated anti-rabbit IgG Ab and with ABC reagent (VectaStain Elite ABC kit; Vector Laboratories). Color was developed with diaminobenzidine (Kirkegaard & Perry Laboratories), whereas the sections were counterstained with hematoxylin. Diaminobenzidine-positive cells and stained nuclei were counted by observers in a blind manner at three independent sites per slide to calculate percentage of nuclei with phosphorylated RB gene product. The data were analyzed statistically with unpaired Student's *t* test.

Lymph node (LN) cell responses to CII

Single-cell suspensions derived from inguinal LNs (4×10^5 cells per well in a 96-well plate) were stimulated with denatured CII (0–100 μg/ml) in RPMI 1640 medium (Sigma-Aldrich) supplemented with L-glutamine, penicillin, streptomycin, 2-ME, and 10% FBS (Sigma-Aldrich). After 72 h, BrdU was added and culture was continued for 17 h to quantify incorporated BrdU.

Results

Alvocidib suppresses proliferation of synovial fibroblasts

FLSs from the inflamed joints of rheumatoid patients were isolated and cultured in the presence of alvocidib, a pan-CDKI. MFLSs from arthritic joints of CIA mice were isolated and treated in the same way. When alvocidib was present in culture medium, growth of FLS and MFLS was inhibited in a concentration-dependent manner (Fig. 1*A*). No cell death was visually apparent at concentrations below 300 nM in FLSs and 150 nM in MFLSs. We next analyzed the cell cycle in these fibroblasts after treatment with alvocidib. In either human or mouse cells, alvocidib increased cell populations at the G_0/G_1 phase of cell cycle without increasing the sub-G_1 population (Fig. 1*B*). Although alvocidib has been reported to induce apoptotic death in some cell types (12–14), this result was not seen in synovial fibroblasts.

Alvocidib treatment suppresses arthritis in CIA mice

Next, mice with experimentally induced arthritis were treated with alvocidib. In the first series of experiments, the drug was given i.p. to DBA/1 mice with CIA. Treatment was started on day 25 after the first immunization, when joint swelling became evident according to examination by our protocol. Repeat injection of 1 or 2.5 mg/kg of alvocidib for 10 consecutive days significantly suppressed severity of arthritis in a dose-dependent manner (Fig. 2*A*). Therapeutic effect was seen even when the treatment was started 3 days later, showing that alvocidib suppressed ongoing disease (Fig. 2*B*). A similar effect was observed when alvocidib was given only twice weekly, starting at about the time of clinical disease onset (day 24). The effect persisted for as long as 5 wk (Fig. 2*C*).

Histologic analysis of synovial tissues from DMSO-treated control mice showed hyperplastic pannus tissues massively infiltrated

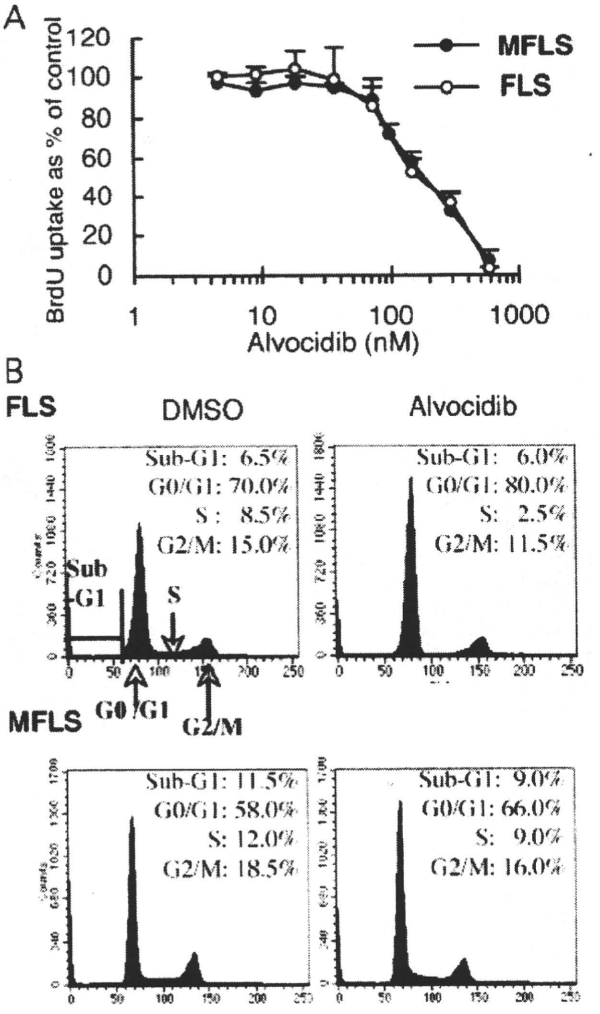

FIGURE 1. Alvocidib suppresses proliferation of synovial fibroblasts. *A*, Proliferation was assessed by BrdU incorporation. FLSs or MFLSs were stimulated with TNF-α and IL-1β for 24 h, and cultured for 24 h with indicated concentrations of alvocidib in the presence of TNF-α and IL-1β. The growth inhibitory effect as alvocidib concentrations increase is shown as declining BrdU incorporation relative to incorporation into cells incubated without alvocidib. DMSO, which was used to solubilize alvocidib, had no effects on incorporation at concentrations used in these experiments. Mean percentage is derived from three wells with error bars representing SD. Data are representative of three independent experiments. *B*, Cellular DNA content was assessed cytometrically as a means of cell cycle analysis. FLSs and MFLSs prestimulated as in *A* were cultured for 18 h with either 100 nM alvocidib or DMSO as a control before flow cytometry. Histograms of DNA content and percentage of population at each phase of the cell cycle are presented. Data shown are representative of three independent experiments. Two different cell lines of FLSs and MFLSs were tested for *A* and *B*. One line was tested twice in two independent experiments. The two lines responded quite similarly; calculated IC_{50} of alvocidib were 130 and 90 nM in one FLS sample and 75 nM in the other sample. Cells in the G_0/G_1 phase of cell cycle increased to 81 and 80% in one FLS sample and 72% in the other sample.

by mononuclear cells, cartilage destruction, and bone erosion, which are all characteristic of the pathology of RA disease. These features were suppressed in synovial tissues from alvocidib-treated mice. Drug effects were dose-dependent; joints from most mice treated with 2.5 mg/kg of alvocidib appeared normal (Fig. 2,

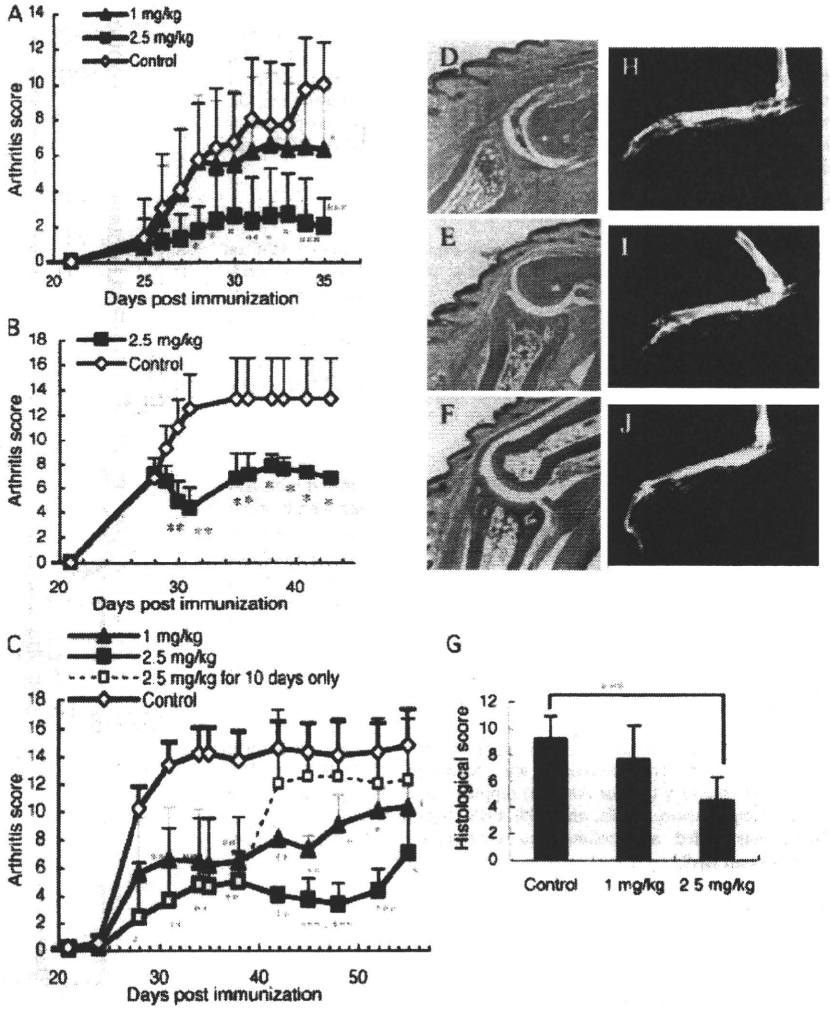

FIGURE 2. Alvocidib treatment suppresses arthritis in CIA mice. Mice with CIA were treated by i.p. injection of alvocidib at 1 mg/kg, or 2.5 mg/kg in saline containing 0.01% DMSO, or injection of only DMSO in saline, for 10 consecutive days (*A* and *B*) or twice weekly for 5 wk (*C*). Treatment was started 25 (*A*), 28 (*B*), or 24 (*C*) days after the initial immunization. Severity of arthritis was rated with an arthritis score. In other mice with CIA, twice-weekly treatment with 2.5 mg/kg of alvocidib was terminated after 10 days (*C*) (□). Mean score ± SD of seven mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *D–F*, H&E staining of joints. Original magnification, ×100. *G*, Histological score of the arthritis. Results are the mean ± SD from 10 paws. ***, $p < 0.001$. *H–J*, Specimen radiographs. Paws were examined at completion of the treatment for 10 consecutive days with DMSO in saline (control) (*D* and *H*) or 1 mg/kg alvocidib (*E* and *I*) and 2.5 mg/kg (*F* and *J*). Representative staining and radiograph are shown.

D–G). These results agreed well with specimen radiographic findings in the feet. DMSO-treated control mice displayed severe bone destruction, most characteristically as cystic changes and ankylosis of the articular bones. These changes were inhibited dose-dependently by alvocidib (Fig. 2, *H–J*).

Immune responses to CII are not impaired by alvocidib treatment

CIA is triggered by host immune responses to CII. Ab response is of central importance because B cell-deficient mice do not develop the disease, whereas transfer of mAbs against CII can induce full-blown arthritis (26). Immune responses require activation and proliferation of lymphocytes. Although peripheral lymphocyte counts were not affected by the dose of alvocidib used in the present study (data not shown), lymphocyte activation might be blocked by alvocidib. To assess immune suppression by alvocidib treatment, we determined serum concentrations of anti-CII Abs in the treated mice. When anti-CII-specific Abs of IgG1, IgG2a, and IgG2b subclasses were quantified separately with a specific ELISA, alvocidib-treated mice had serum IgG concentrations comparable to

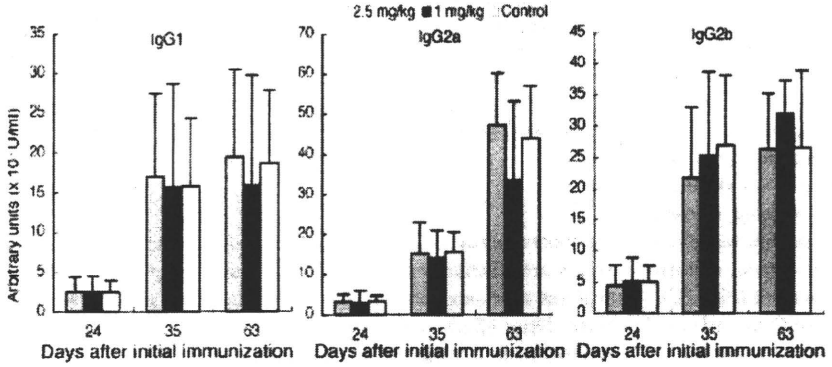

FIGURE 3. CII-specific IgG responses are maintained during alvocidib treatment. Serum concentrations of anti-CII Abs with IgG1, IgG2a, and IgG2b subclasses were determined by specific ELISA. Sera were collected 24, 35, and 63 days after the primary CII immunization in a series of experiments shown in Fig. 2*C*. Data are in arbitrary units for mean ± SD (n = 7 mice per group).

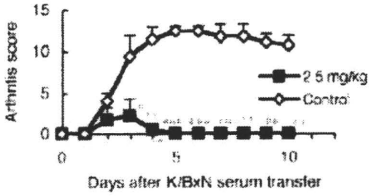

FIGURE 4. Alvocidib treatment suppresses arthritis in RAG2$^{-/-}$ mice induced by K/BxN serum transfer. Arthritis was induced in RAG2$^{-/-}$ mice by transfer of serum from arthritic K/BxN mice. Beginning on the day after serum transfer, mice were treated with 2.5 mg/kg of alvocidib for 10 consecutive days. Control mice were treated with saline containing DMSO. Severity of arthritis was assessed by an arthritis score. Mean score ± SD is shown (n = 5 mice per group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

those in control mice (Fig. 3). No difference was seen in relation to dose or time during the experiment. We then studied effect of terminating alvocidib treatment, following the clinical disease. In a group of CIA mice given 2.5 mg/kg of alvocidib twice weekly, treatment was terminated after 10 days. Arthritis became evident ~8 days later, and then progressed rapidly until the disease score approached that in untreated mice (Fig. 2*C*). The results showed that the underlying immune reaction against CII was not impaired during alvocidib treatment.

Alvocidib shows efficacy in treating a lymphocyte-independent arthritis

To substantiate that alvocidib can inhibit arthritis without suppressing lymphocyte function, we investigated its effect on a K/BxN serum-induced arthritis model induced in lymphocyte-deficient RAG2$^{-/-}$ mice. K/BxN mice spontaneously develop arthritis similar to RA in several aspects (24). Transfer of serum from these arthritic mice induces arthritis depending upon the genetic background of recipient mice (27). Pathology in the joints is characterized by acute edema in synovial tissues followed by proliferative and erosive arthritis that can be induced in lymphocyte-deficient mice (28). Making use of this model, we induced arthritis in RAG2$^{-/-}$ mice and began i.p. administration of 2.5 mg/kg of alvocidib on the day after serum transfer, continuing for 10 days. Treatment reduced edematous swelling of the joins in the early phase, and then abrogated proliferative arthritis (Fig. 4). Thus, suppression of immune responses is not required for the antiarthritic effect of alvocidib.

A newly synthesized CDK4/6-selective inhibitor suppresses synovial fibroblasts growth and CIA

Alvocidib was tolerated well in clinical trials except for a few occurrences of diarrhea when administered as a prolonged infusion; its toxicity profile was dose- and schedule-dependent (11). This agent has broad activity, inhibiting all CDKs and some other kinases (29). To study further whether cell cycle inhibition by CDK4/6 inhibition had a important role in ameliorating arthritis, a selective inhibitor, compound A, was synthesized (20). It is an aminothiazole CDKI that is structurally unrelated to alvocidib (Fig. 5*A*). It selectively inhibited CDK4 and CDK6, both of which have been shown to have indistinguishable activities in vitro (20). The IC_{50} for CDK4 and CDK6 was 9.2 and 7.8 nM, respectively, which were at least 100-fold less than concentrations for other CDKs.

As alvocidib did, compound A inhibited growth of FLSs and MFLSs in a concentration-dependent manner (Fig. 5*B*) and induced cell cycle arrest at the G_1 phase (Fig. 5*C*). It suppressed RB

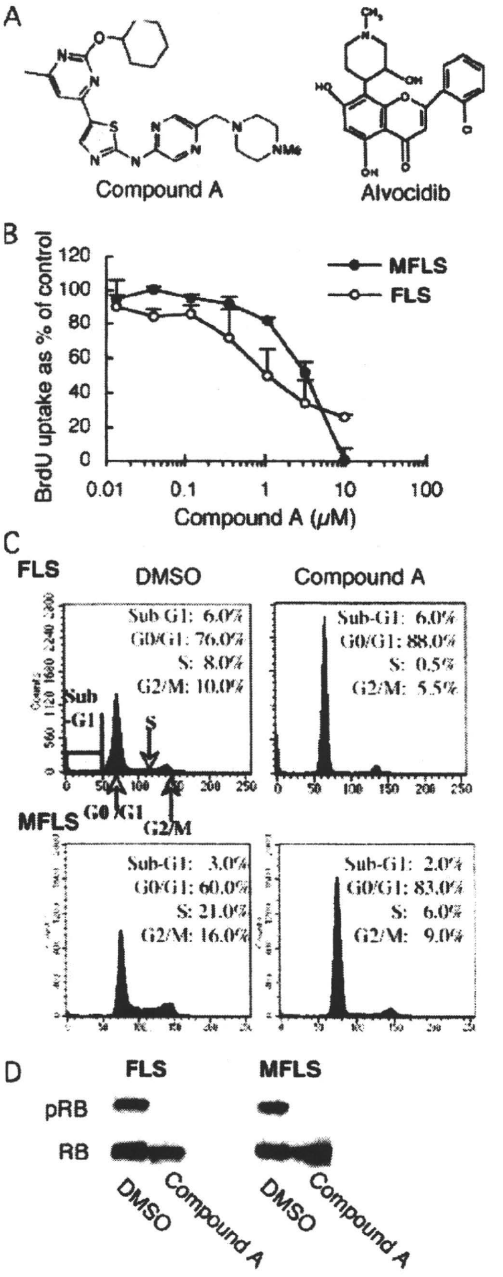

FIGURE 5. Compound A suppresses proliferation of synovial fibroblasts. *A*, Chemical structure of compound A and alvocidib. *B*, Proliferation was assessed as in Fig. 1. FLSs or MFLSs were stimulated with TNF-α and IL-1β for 24 h, and then cultured for 24 h with indicated concentrations of compound A in the presence of TNF-α and IL-1β. Shown are mean percentage ± SD of BrdU incorporation relative to uptake by control cells, derived from three wells. Data are representative of three independent experiments. *C*, DNA content of FLSs and MFLSs were analyzed as in Fig. 1. Instead of alvocidib, compound A (2.5 μM) was used. Histograms of DNA content and the percentage of population at each phase of the cell cycle are indicated. Data shown are representative of three independent experiments. Two different cell lines of FLSs and MFLSs were tested for *B* and *C* as was in Fig. 1. Again, they responded similarly; calculated IC_{50} of compound A were 1.0 and 0.6 μM in one FLSs sample and 1.8 μM in the other sample. Cells in the G_0/G_1 phase increased to 88 and 87% in one FLSs sample and 85% in the other sample. *D*, RB gene product phosphorylation of FLSs and MFLSs treated as in *C* were analyzed by Western blot using specific Ab to phospho-RB (pRB) or RB. The results are representatives of two samples.

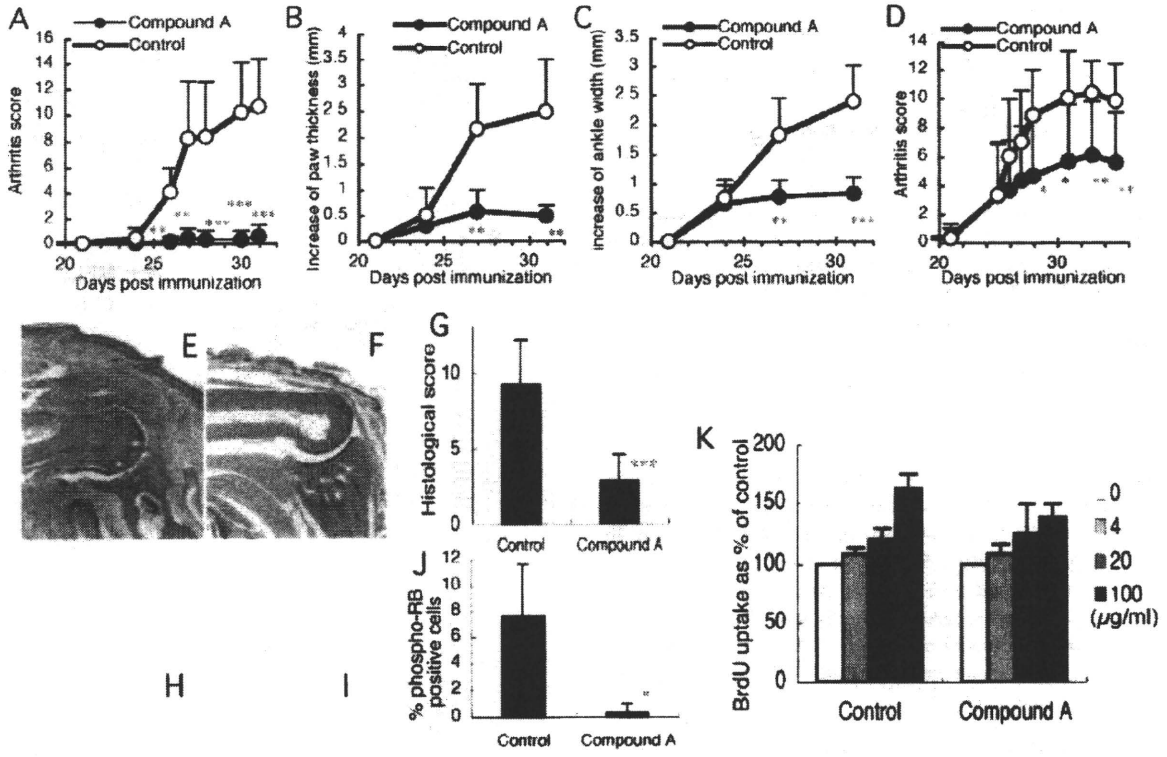

FIGURE 6. Treatment with compound A suppresses CIA in mice. *A–C*, Mice with CIA were treated with compound A orally (200 mg/kg) every 12 h for 7 days. Treatment was started when arthritis became evident, 24 days after the initial immunization. *D*, Other mice with CIA were treated i.p. with compound A (30 mg/kg for 5 days, repeated after a 1-day hiatus). Data are representative of two independent experiments. Control mice were treated with buffer alone. Severity of arthritis was assessed by an arthritis score (*A* and *D*), paw thickness (*B*), and ankle width (*C*). Data are the mean ± SD ($n = 8$ per group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *E* and *F*, H&E staining of joints at original magnification {times}100. Paws were examined at completion of the treatment with buffer alone (*E*) or compound A (*F*). Representative staining is shown. *G*, Histological abnormality of the mice treated as in *D* was scored. Results are mean ± SD from 12 paws. ***, $p < 0.001$. *H* and *I*, Immunohistological staining of joints. Original magnification at ×200. The paw samples in *E* and *F* were stained with phospho-RB Ab. Representative staining is shown. *J*, Phospho-RB positive cells were semiquantified as percentage of positive nuclei in total nuclei. Data are mean ± SD from 5 paws. *, $p < 0.05$. *K*, LN cells from the mice treated as in *D* were isolated at completion of the treatment. Their proliferative responses to different concentrations of CII were assessed by BrdU incorporation. Data were displayed as BrdU incorporation relative to that in cells cultured without CII. Data are mean ± SD from three mice.

phosphorylation at Ser^{807}/Ser^{811}, which are among residues specifically targeted by CDK4/6 in FLS and MFLS (Fig. 5*D*). Titration experiments showed that 0.25 μM compound A, which was below IC_{50} for other CDKs, started inhibiting the RB phosphorylation and also increasing cells at the G_0/G_1 phase; 83.0% in FLS and 72.0% in MFLS.

Compound A (200 mg/kg) was given orally twice daily for 7 days, beginning 24 days after the initial CII immunization. This treatment reduced arthritis score, ankle and paw swelling of the CIA mice (Fig. 6, *A–C*). The dose could be reduced to 30 mg/kg once daily i.p. injection without loss of therapeutic effects (Fig. 6*D*). This treatment was started after the onset of clinical arthritis. Five-day treatments repeated after a day hiatus suppressed ongoing arthritis. Histological analysis of the synovial tissues revealed that compound A, as well as alvocidib, suppressed characteristic features of pathology in the arthritis (Fig. 6, *E–G*). When nuclei with phosphorylated RB gene product in the synovial tissues were stained, their number was decreased in the compound A-treated mice (Fig. 6, *H* and *I*). Their ratio in the total nuclei was lower in the treated mice (Fig. 6*J*), showing that CDK4/6 was inhibited in vivo.

We found no abnormality by physical or behavioral observation in association with administration of compound A. At completion of the therapeutic experiment, inguinal LNs cells were isolated and stimulated with CII. No difference in proliferative response was observed between the compound A-treated and the nontreated mice (Fig. 6*G*). The preservation of T cell response was also observed in the alvocidib-treated mice (data not shown). Thus, inhibition of the lymphocytes did not play an important role in the therapeutic effects of the smCDK inhibitors.

Discussion

We have demonstrated that systemic administration of the two CDKIs exerted an antiarthritic effect without critical impairment of lymphocyte responses. Such an inhibitor proved effective in treating a lymphocyte-independent arthritis. Although alvocidib is known as a pan-CDKI, CDK4/6-selective inhibitor showed a comparable effect. This finding suggested that inhibition of CDK4/6 played an important role in the antiarthritic activity. It was reported that a CDK1/2/5 inhibitor, R-roscovitine, suppressed a passively induced arthritis model by promoting apoptosis of inflammatory cells (30). Alvocidib induces apoptotic cell death in various tumor cells (13, 14, 31, 32), likely by inhibition of other kinases than CDK (29). In this regards, TUNEL of the joint tissues treated with the two inhibitors did not show increase of apoptotic synovial cells (data not shown). This suggested that local induction of apoptosis in the joints was not responsible for the effect. Although we could not formally exclude effects of these agents on lymphocytes, the results argue that effects on nonlymphoid cells including synovial

fibroblasts and innate immune cells should account for the therapeutic activity.

A reported immunomodulatory action of endogenous CDKI does not depend upon CDK inhibition. This immune modulation could be at least partly responsible for therapeutic effects seen in CDKI gene therapy (18, 19). However, the present study demonstrated that inhibition of CDK activity alone is sufficient to ameliorate arthritis.

A major concern with systemic delivery of smCDK inhibitors is possible inhibition of normal cell turnover, which is essential for maintenance of organ homeostasis. Reassuringly, alvocidib, the prototype CDKI, was tolerated relatively well in clinical trials involving cancer (33). Diarrhea, the most frequent side effect in these trials, was not observed in any mice during our experiments. We suspect that proliferating cells involved in the arthritis were more sensitive than tumor cells because 2.5 mg/kg or less of alvocidib sufficed to treat arthritis, whereas at least 5 mg/kg was required to treat tumors (34, 35). Although alvocidib was injected i.v. for tumor treatment, oral administration of compound A was effective in treating arthritis. Although toxic effects might not be tolerated by patients without malignancies, route and dosage could be reconsidered in RA patients being treated in clinical trials. RA might respond to much lower doses than do tumors as is true in treatment with methotrexate.

Compound A is a more selective inhibitor acting primarily upon CDK4 and CDK6. Like alvocidib, it induced cell cycle arrest of synovial fibroblasts at the G_1 phase. The cell cycle arrest was accompanied by inhibition of RB phosphorylation. In selectivity testing of compound A against other serine/threonine or tyrosine kinases beyond those of the CDK family, only 6 of 45 kinases were inhibited >75% by compound A at 1 μM. No kinase among these six showed a IC_{50} comparable to those of CDK4 and CDK6. Partly because of structural irrelevance, alvocidib and compound A do not share the same kinases as off-target kinases. Accordingly, inhibition of cell cycling by CDK4/6 inhibition seemed important in suppressing arthritis. It has been proposed that MAPKs are potential therapeutic targets in RA (36). When their inhibitors showed efficacy, they suppressed Ab responses (37, 38). This was in contrast to the treatment with the CDKIs.

We assume that CDKIs might represent a new class of antirheumatic drugs. Based on the results of clinical trials of alvocidib as well as those of the present experiments, patients with RA easily should tolerate an antiarthritic dose of alvocidib because alvocidib seems to exert cytotoxic adverse effects primarily via inhibition of other kinases at antineoplastic doses (29). Alvocidib exhibited higher protein binding in human serum than in FBS, which led to development of an effective administration schedule (17). The optimal dose should be carefully determined for actual use in treating arthritic patients. In contrast, compound A needs to be modified to improve its pharmacokinetics. It was cleared quite rapidly in vivo, necessitating us using a relatively high dose as its present form.

The antiarthritic effect of these drugs did not require obvious suppression of the lymphocyte responses. Most of presently available antirheumatic treatments aim to inhibit harmful immune reactions. Combined use of conventional medication and smCDK inhibitors may well have a synergistic antiarthritic effect. As was revealed by our recent report and others (30, 39, 40), CDKI may have broader activity than anticipated. We found recently that both alvocidib and compound A suppressed matrix metalloproteinase-3 production by synovial fibroblasts and osteoclastogenesis of macrophages (data not shown). These effects may contribute to protection from joint damage.

The results of the present studies should encourage further development, clinical testing, and use of the smCDK inhibitors to treat human RA.

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Disclosures

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ORIGINAL ARTICLE

A1330V polymorphism of low-density lipoprotein receptor-related protein 5 gene and self-reported incident fractures in Japanese female patients with rheumatoid arthritis

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Abstract We attempted to determine whether the A1330V polymorphism of the *low-density lipoprotein receptor-related protein 5* (*LRP5*) gene is associated with a risk of self-reported incident fractures and hypercholesterolemia in Japanese patients with rheumatoid arthritis (RA). DNA samples, laboratory data, and clinical data were obtained from 563 female RA patients who participated in the Institute of Rheumatology Rheumatoid Arthritis (IORRA) observational cohort study. A1330V genotyping was performed using a custom TaqMan assay. Multiple logistic regression analyses showed that any incident fracture was significantly associated with older age ($P = 0.000000036$), high Japanese Health Assessment Questionnaire (J-HAQ) score ($P = 0.016$), and high daily prednisolone dose ($P = 0.031$), but not with the A1330V polymorphism, while serum total cholesterol levels ≥220 mg/100 mL were independently correlated with baseline older age ($P = 0.00011$), low J-HAQ score ($P = 0.0098$), high body mass index ($P = 0.024$), 1330VV genotype ($P = 0.027$), and high daily prednisolone dose ($P = 0.031$). Our results suggest that this *LPR5* polymorphism does not appear to be a clinically useful marker for the prediction of fracture risk in Japanese female RA patients, although it is associated with increased serum total cholesterol levels.

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Keywords Cohort study · Fracture · Low-density lipoprotein receptor-related protein 5 (LRP5) · Rheumatoid arthritis (RA) · Single-nucleotide polymorphism (SNP)

Introduction

Patients with rheumatoid arthritis (RA) have an increased risk of developing osteoporosis and osteopenia [1] as well as vertebral deformities [2] and hip fractures [3] compared to healthy controls. We and others have previously reported the clinical risk factors for incident fractures in RA patients [4–9]. While corticosteroid use [4–9], older age [4–7, 9], functional disability [5–8], and low bone mineral density (BMD) [4] have been identified as major risk factors, the identification of genetic risk factors is required to better predict which patients have an elevated fracture risk.

Limited data exist in the literature concerning the genetic risk factors for osteoporosis and fractures in RA patients. Gough et al. [10] have reported a positive association between a polymorphism of the vitamin D receptor and bone loss in patients with early RA, although Ho et al. [11] did not find any significant associations between this polymorphism and corticosteroid-related osteoporosis. The identification of individuals at high risk of fracture is important, as bisphosphonates [6, 12–14] and vitamin D3 [6, 15] have been documented to prevent osteoporosis and fractures in RA patients.

Low-density lipoprotein receptor-related protein 5 (LRP5) is a member of the low-density lipoprotein (LDL) receptor family [16]. Koay et al. [17] reported that the *LRP5* gene and the Wnt signaling pathway are key players in bone formation and the risk of osteoporosis, and that LRP5 signaling is essential for normal morphology,

developmental processes and bone health. A non-synonymous variant of *LRP5*, A1330V, has been identified in the Japanese population [18]. We and others previously reported positive associations between 1330V and low BMD in both Japanese women [19–21] and Caucasian men [22–25]. Recently, van Meurs et al. [26] reported significant associations between 1330V, low BMD, and fractures in Caucasian men and women in a large-scale, multicenter collaborative study. However, the association between the A1330V polymorphism and incident fractures has not been evaluated in the Japanese population.

LRP5 appears to be associated with dyslipidemia. Fujino et al. [27] showed that LRP5 is required for normal cholesterol metabolism, while Magoori et al. [28] demonstrated that the *LRP5* gene is involved in cholesterol metabolism, as atherosclerotic lesions develop in mice lacking both the apolipoprotein E and *LRP5* genes. Suwazono et al. [29, 30] reported that the 1330V allele contributes to hypercholesterolemia in the Japanese general population; however, their study did not evaluate specific risk factors in RA patients.

Utilizing data from our prospective, observational study of RA in Japan (IORRA, Institute of Rheumatology Rheumatoid Arthritis) [31], we evaluated the associations of the *LRP5* A1330V polymorphisms with incident fracture and serum total cholesterol level in Japanese women with RA.

Patients and methods

Study cohort

The Tokyo Women's Medical University genome ethics committee approved the present study, and each individual signed an informed consent form after receiving a verbal explanation of the study. Participants were part of a large-scale Japanese RA cohort project, IORRA [31], which began in 2000. Details regarding the study's purpose and methodology have been previously reported [5, 6, 31, 32]. Study details were explained to each patient by 1 of 49 rheumatologists during their clinic visits. Informed consent was received from each patient. Each participant was asked to complete the questionnaire at home and mail it back in a pre-addressed, stamped envelope within 2 weeks.

Participant selection

Female RA patients who participated in IORRA from 2000 to 2005 and whose DNA samples were available were included in the present study. All of the patients had been diagnosed with RA according to the 1987 classification criteria for RA developed by the American College of Rheumatology [33]. Participants were followed for 6 to 54 months (October 2000 to March 2005). Patients were classified into three groups according to their self-reported incident fracture status compared to baseline: no new fractures, any new nonvertebral fracture, and new vertebral fracture.

Baseline assessments

Baseline demographics, clinical variables, and medication history obtained at study entry were as follows: age (years), height (cm), weight (kg), body mass index (BMI, kg/m^2), RA disease duration (years), rheumatoid factor (RF) level (IU/ml, Rose–Waaler test), RF positivity (≥35 IU/mL), serum C-reactive protein (CRP) level (mg/100 mL), serum total cholesterol level (mg/100 mL), Japanese Health Assessment Questionnaire (J-HAQ) scores [32], Disease Activity Score-28 (DAS28) [34], corticosteroid use, and daily prednisolone dose (mg/day), as described previously [5, 6]. Hypercholesterolemia was defined as serum cholesterol ≥5.7 mmol/L (220 mg/100 mL) based on the guidelines of the Japan Arteriosclerosis Society [35, 36].

Fracture assessments

Incident nonvertebral and vertebral fractures were enumerated from self-reports, as documented in the questionnaire. Participants were asked about fractures of the ankle, arm, clavicle, elbow, foot, hand, hip, knee, leg, nose, pelvis, rib, shoulder, thoracic spine, lumbar spine, and wrist, every 6 months from October 2000 to March 2005. They were then asked to state whether the fracture was due to a fall, accident, sports injury, or spontaneous event. Only the first fracture event reported by each patient was included in this study [5, 6, 37]. Asymptomatic vertebral fractures were not included because thoracic and lumbar spine radiographs for spinal morphometry were not routinely obtained. We did not verify self-reported fractures by radiology reports or medical records as in our previous studies [5, 6], as we were required to analyze patient DNA and clinical data without identification according to the guidelines of the genome ethics committee at our institute.

Genotyping

The *LRP5* A1330V (rs3736228) polymorphism was genotyped using the TaqMan Assay (Applied Biosystems) according to the manufacturer's protocol.

Statistical analysis

The Chi-squared test was used to compare the observed numbers of each genotype with those expected for a population in Hardy–Weinberg equilibrium. Statistical

significance of the differences between groups was determined using the Kruskal–Wallis test, Mann–Whitney *U* test (continuous variables), and Chi-square for independence test (counts) as appropriate. Pearson's correlation coefficient test was used to measure the correlation between DAS28 and J-HAQ scores. In the multivariate analyses, logistic regression was used to evaluate the effect of the A1330V genotype on self-reported incident fracture and hypercholesterolemia using the following confounding factors as independent variables: baseline age, BMI, J-HAQ score [32], and daily prednisolone dose. To detect the influence of the 1330VV genotype on self-reported incident fracture, serum total cholesterol level ≥220 mg/100 mL was included in the logistic model. We did not include the DAS28 score [34] into logistic regression analyses due to missing data (Table 1). $P < 0.05$ was considered significant. We did not include smoking or alcohol intake [38] into the analyses, as we have previously reported that they are not risk factors for fracture in Japanese women with RA [6]. All statistical analyses were conducted using R statistics software (Available at: http://www.r-project.org/).

Results

Table 1 shows baseline participant characteristics according to A1330V genotype. The observed polymorphism genotype frequencies were in Hardy–Weinberg equilibrium. Among the three A1330V genotypes, no significant differences in patient baseline characteristics were identified, except for weight ($P = 0.030$), DAS28 score ($P = 0.01$), and daily prednisolone dose ($P = 0.042$) (Table 1).

During the follow-up period from October 2000 to March 2005, 122 (21.7%) of the participants reported any type of fracture. Of these patients, 95 (77.9%) and 24 (19.6%) reported nonvertebral fractures and vertebral fractures, respectively; three did not report their fracture site and were thus excluded from this analysis. Among the patients who reported nonvertebral fractures ($n = 95$), the most frequent fractures were rib ($n = 20$, 21.1%) and toe ($n = 11$, 11.6%) fractures, followed by wrist ($n = 10$, 10.5%), ankle ($n = 8$, 9.5%), finger ($n = 7$, 7.4%), hip ($n = 7$, 7.4%), collar bone ($n = 6$, 6.3%), elbow ($n = 5$, 5.3%), knee ($n = 5$, 5.3%), shoulder ($n = 5$, 5.3%), pelvis ($n = 4$, 4.2%), arm ($n = 3$, 3.2%), foot ($n = 3$, 3.2%), and leg ($n = 1$, 1.1%) fractures. Among the patients who reported vertebral fractures ($n = 24$), vertebral fracture sites included the cervical spine ($n = 1$, 4.2%), thoracic spine ($n = 9$, 37.5%), lumbar spine ($n = 13$, 54.2%), and unknown ($n = 1$, 4.2%). Self-reported fractures occurred due to falls ($n = 61$, 51.3%), spontaneously ($n = 42$, 35.3%), accidentally ($n = 8$, 6.7%), due to sports injury ($n = 3$, 2.5%), and due to unknown causes ($n = 5$, 4.2%).

Table 1 Baseline characteristics of 563 Japanese female patients with rheumatoid arthritis

	LRP5 A1330V genotype			Data missing	*P*
	AA	AV	VV		
Number of patients (%)	304 (54.0%)	215 (38.2%)	44 (7.8%)	–	–
Age (years)	56.0 (49.0–62.3)	57.0 (50.0–64.5)	57.5 (50.0–68.0)	0	0.26†
Height (cm)	155 (152–159)	155 (151–158)	157 (153–160)	5 (0.9%)	0.071†
Weight (kg)	51.0 (46.0–56.0)	49.0 (45–54.3)	52.0 (48.5–57.5)	10 (1.8%)	0.030†
Body mass index (kg/m^2)	21.2 (19.4–23.3)	20.4 (19.0–22.7)	21.4 (20.1–22.3)	10 (1.8%)	0.13†
RA disease duration (years)	8.0 (4.0–13.0)	8.0 (3.0–14.0)	10.0 (5.8–16.3)	2 (0.4%)	0.30†
Rheumatoid factor positive[a] (%)	241 (81.7%)	160 (76.9%)	35 (81.4%)	16 (2.8%)	0.16‡
Rheumatoid factor, IU/mL	64 (23–126)	47 (17–109)	64 (29–116)	16 (2.8%)	0.34†
C-reactive protein (mg/100 mL)	0.7 (0.2–1.9)	0.6 (0.2–1.6)	0.4 (0.2–1.5)	9 (1.6%)	0.51†
Serum total cholesterol level					
mg/100 mL	197.5 (173.0–221.3)	204.0 (178.0–222.0)	214.0 (193.5–236.0)	8 (1.4%)	0.092†
≥220 mg/100 mL	80 (26.7%)	60 (28.4%)	19 (44.2%)	8 (1.4%)	0.059‡
J-HAQ score	0.6 (0.1–1.3)	0.6 (0.1–1.1)	0.5 (0.1–1.0)	0	0.70†
Disease activity score 28	4.2 (3.3–5.0)	3.9 (3.1–4.8)	4.2 (3.6–5.3)	66 (11.7%)	0.010†
Corticosteroid use	163 (53.6%)	97 (45.1%)	17 (38.6%)	0	0.060‡
Daily prednisolone dose (mg/day)	1.2 (0–5.0)	0 (0–4.0)	0 (0–3.3)	0	0.042†

Values are expressed as a median (25th–75th percentile) or number (%)

LRP5 low-density lipoprotein receptor-related protein 5, *RA* rheumatoid arthritis, *J-HAQ* Japanese Health Assessment Questionnaire

†, ‡ *P* values were calculated with Kruskal–Wallis test (†) or Chi-square for independence test (‡)

[a] Cut-off value = 35.0 IU/ml

Table 2 *LRP5* A1330V genotypes and self-reported incident fractures during the follow-up periods in 560 female Japanese patients with rheumatoid arthritis

Self-reported fracture	*LRP5* A1330V genotype			*P*
	AA	AV	VV	
Any fracture	67 (56.3%)	40 (33.6%)	12 (10.1%)	0.39[a]
Non-vertebral fracture	57 (60.0%)	27 (28.4%)	11 (11.6%)	0.088[a]
Vertebral fracture	10 (41.7%)	13 (54.2%)	1 (4.2%)	0.34[a]
No fracture	236 (53.5%)	173 (39.2%)	32 (7.3%)	–

Values represent numbers (%)

LRP5 low-density lipoprotein receptor-related protein 5

[a] *P* values were calculated with Chi-square for independence test versus no fracture group

The A1330V genotype distribution did not differ significantly between patients with self-reported any, nonvertebral, or vertebral fractures compared to that in patients without fractures (Table 2). The multiple logistic regression analysis indicated that the odds ratio for sustaining self-reported any incident fracture was 1.07 for baseline age ($P = 0.000000036$), 1.47 for each one-point increase in baseline J-HAQ score ($P = 0.016$), and 1.09 for baseline daily prednisolone dose ($P = 0.031$) after multiple adjustments (Table 3). The multiple logistic regression analysis also showed that the odds ratio for sustaining a self-reported incident nonvertebral fracture was 1.04 for baseline age ($P = 0.00034$) and 1.40 for each 1-point increase in baseline J-HAQ score ($P = 0.047$) after multiple adjustments (Table 3). Although not to a statistically significant degree ($P = 0.052$), baseline daily prednisolone dose appeared to be associated with self-reported incident nonvertebral fracture risk after multiple adjustments (Table 3).

Baseline serum total cholesterol level was significantly higher in patients with the VV genotype compared to those with the AA genotype ($P = 0.031$) and compared to patients with the AA and AV genotypes combined ($n = 519$, $P = 0.034$) (Table 1). Patients with the VV genotype also tended to have higher serum total cholesterol levels than those with the AV genotype ($P = 0.058$). The logistic regression analysis indicated that the odds ratios for serum cholesterol level ≥220 mg/100 mL were 1.04 for age ($P = 0.00011$), 0.68 for each 1-point increase in J-HAQ score ($P = 0.0098$), 1.08 for BMI ($P = 0.024$), 2.10 for the VV genotype ($P = 0.027$), and 1.09 for daily prednisolone dose ($P = 0.031$) after multiple adjustments (Table 4).

Discussion

In the present study, the *LRP5* A1330V polymorphism was not significantly associated with self-reported incident fractures in Japanese female RA patients (Table 3). Prednisolone use was significantly lower in the *LRP5* 1330VV group, and body weight was relatively high in this group (Table 1). These background characteristics may have contributed to increased BMD in this group and consequently led to a decrease in bone fracture occurrence although the association of genotype with fracture was analyzed in a multiple way including these factors. We and others have previously reported positive associations between the A1330V polymorphism and low BMD in both Japanese women [19–21] and Caucasian men [22–25]. Recently, van Meurs et al. [26] reported significant associations of the 1330V allele with low BMD and all fractures in both men and women in a prospective, multicenter, collaborative study of 37,354 Caucasians. In the present study, we did not evaluate BMD, which is the most sensitive screening method for osteoporosis and the best parameter for predicting fracture risk [39], because we did not collect BMD data. A large study that evaluates BMD is thus required to determine whether there is a significant association between the 1330V allele and osteoporosis in Japanese RA patients. Many clinical risk factors for incident fracture have been identified in patients with RA [4–9], although limited data exist in the literature concerning genetic risk factors for osteoporosis in RA patients. Any associations between vitamin D receptor polymorphisms and BMD in RA patients are also inconclusive [10, 11]. Thus, clinical factors, rather than genetic factors, may primarily influence the risk of incident fractures in RA patients, although further study is required to confirm this.

Our results also confirm the previously reported significant associations identified between incident fractures and older age [4–6] as well as functional disability [5, 6, 8] in Japanese RA patients (Table 3). Our study also showed that baseline daily prednisolone dose is a significant risk factor for self-reported incident fracture in Japanese women with RA (Table 3). Although we previously reported that baseline daily prednisolone dose is an independent risk factor for clinical vertebral fracture in

Table 3 Multiple logistic regression analysis results on self-reported any and nonvertebral fracture in Japanese female patients with rheumatoid arthritis

Independent variables	Any fracture		Nonvertebral fracture	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (years)	1.07 (1.04, 1.09)	0.000000036	1.04 (1.02, 1.07)	0.00034
J-HAQ score	1.47 (1.07, 2.00)	0.016	1.40 (1.00, 1.94)	0.047
Prednisolone dose (mg/day)	1.09 (1.01, 1.18)	0.031	1.09 (1.00, 1.18)	0.052
LRP5 1330VV genotype	1.40 (0.65, 3.01)	0.39	1.81 (0.84, 3.89)	0.13
Body mass index (kg/m^2)	0.98 (0.91, 1.05)	0.50	0.99 (0.92, 1.07)	0.85
Serum total cholesterol level ≥220 mg/100 mL	1.06 (0.66, 1.72)	0.80	1.00 (0.60, 1.67)	1.00

OR odds ratio, *CI* confidence interval, *J-HAQ* Japanese Health Assessment Questionnaire, *LRP5* low-density lipoprotein receptor-related protein 5

Table 4 Multiple logistic regression analysis results for serum total cholesterol level ≥220 mg/100 mL in Japanese female patients with rheumatoid arthritis

Independent variables	OR (95% CI)	*P*
Age (years)	1.04 (1.02, 1.06)	0.00011
J-HAQ score	0.68 (0.50, 0.91)	0.0098
Body mass index (kg/m^2)	1.08 (1.01, 1.15)	0.024
LRP5 1330VV genotype	2.10 (1.09, 4.07)	0.027
Prednisolone dose (mg/day)	1.09 (1.01, 1.17)	0.031

OR odds ratio, *CI* confidence interval, *J-HAQ* Japanese Health Assessment Questionnaire, *LRP5* low-density lipoprotein receptor-related protein 5

Japanese men with RA [5], we did not evaluate baseline daily prednisolone dose in our previous study in women [6]. Nampei et al. [8] reported that daily glucocorticoid dose is significantly associated with both any and vertebral fracture in Japanese RA patients. Thus, we verified this association in Japanese women with RA from our IORRA database. We and others reported that history of orthopedic surgery for RA [6], history of total knee replacement [5, 9], history of prior fracture [6, 9], low serum C-reactive protein levels [5, 6], and lack of concomitant use of bisphosphonates or active vitamin D3 [6] were potential risk factors for fractures in patients with RA; however, we did not evaluate these risk factors in the current study since our preliminary analysis indicated no significant association between these factors and incident fracture or due to missing data (data not shown).

Of all the parameters tested in our study, *LRP5* 1330VV genotype is a significant risk factor for hyperlipidemia (serum cholesterol level ≥220 mg/100 mL) (Table 4). Suwazono et al. [30] reported that A1330V was a genetic risk factor for hyperlipidemia in 936 male and 662 female Japanese workers [29] in a factory. Our results confirmed that this is also true in Japanese female RA patients, suggesting that the A1330V polymorphism is an important genetic risk factor for hyperlipidemia in both the Japanese general population and RA patients, although further studies are required to confirm this.

In our study, low J-HAQ disability scores were significantly associated with hyperlipidemia (Table 4), while high J-HAQ disability scores were significantly associated with the risk of self-reported any and nonvertebral fractures (Table 3). Boers et al. [40] reported that total cholesterol levels correlate inversely with disease activity in RA patients. Since J-HAQ scores were significantly correlated with DAS28 scores in the present study (correlation coefficient, 0.52; $P = 1.2 \times 10^{-35}$) (data not shown), our results are consistent with those of Boers et al. [40].

We observed a significant association between baseline daily prednisolone dose and hyperlipidemia (Table 4). These results are consistent with those of Okamoto et al. [41] who previously reported that serum cholesterol levels are significantly associated with corticosteroid dose in RA patients in the IORRA database. Our results also indicate that baseline older age and high BMI are independent predictors of hyperlipidemia in Japanese female RA patients (Table 4), which is consistent with the observation that serum total cholesterol level increases tend to accompany aging [42] and obesity [43].

Despite the advantages of a prospective, observational cohort study performed at a single institution, our study has some limitations. First, we did not evaluate the effects of statins on serum cholesterol because we did not collect these data at baseline. However, any effect of statin use is expected to be weak, as only a small number of patients (6.7%) in the IORRA database were undergoing statin treatment [41]. Second, fractures in our study were self-reported, although we observed a 69% agreement rate between self-reports and medical records in our previous study in women using the IORRA database [6]. Self-reporting of fractures has proven to be fairly reliable by several groups [44, 45], and this method has been used in many RA fracture studies [5, 6, 46, 47] and other recent

large studies [26, 37, 48]. Third, we did not perform spinal morphometry, which is the best method for detecting spinal fractures [49]. As over one-third of vertebral fractures are asymptomatic in RA patients [50], our study did not address the value of risk factors for non-clinical vertebral fractures. Fourth, we did not evaluate family history of hip fracture [38] because family hip fracture history data were not collected at baseline. Finally, we may not have sufficiently addressed clinical implications of fractures and fracture risk in our study.

In conclusion, we have evaluated the associations of the *LRP5* A1330V polymorphism with self-reported incident fractures and serum cholesterol levels in Japanese women with RA in a prospective, observational cohort study at a single institution. Our results suggest that the *LPR5* A1330V polymorphism does not appear to be a clinically useful marker for the prediction of fracture risk in Japanese female RA patients, although it is associated with increased serum total cholesterol levels.

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The Association of a Nonsynonymous Single-Nucleotide Polymorphism in *TNFAIP3* With Systemic Lupus Erythematosus and Rheumatoid Arthritis in the Japanese Population

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Objective. **Genome-wide association (GWA) studies in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in Caucasian populations have independently identified risk variants in and near the tumor necrosis factor α (TNFα)–induced protein 3 gene (*TNFAIP3*), which is crucial for the regulation of TNF-mediated signaling and Toll-like receptor signaling. The aim of this study was to assess the role of *TNFAIP3* in the development of SLE and RA in Japanese subjects.**

Methods. **We selected 2 single-nucleotide polymorphisms (SNPs) from previous GWA studies. Rs2230926 is a nonsynonymous SNP in *TNFAIP3* and is associated with SLE, while rs10499194 is an intergenic SNP associated with RA. We then performed 2 independent sets of SLE case–control comparisons (717 patients and 1,362 control subjects) and 3 sets of RA case–control comparisons (3,446 patients and 2,344 control subjects) using Japanese subjects. We genotyped SNPs using TaqMan assays.**

Results. **We observed a significant association between rs2230926 and an increased risk of SLE and RA in the Japanese population (for SLE, odds ratio [OR] 1.92, 95% confidence interval [95% CI] 1.53–2.41, $P = 1.9 \times 10^{-8}$; for RA, OR 1.35, 95% CI 1.18–1.56, $P = 2.6 \times 10^{-5}$). The intergenic SNP rs10499194 was also associated with SLE and RA, while the risk allele for RA in Caucasians was protective against the diseases in our population.**

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Conclusion. **We demonstrated a significant association between the nonsynonymous variant in *TNFAIP3* and the risk for SLE and RA in the Japanese population. *TNFAIP3*, similar to *STAT4* and *IRF5*, may be a common genetic risk factor for SLE and RA that is shared between the Caucasian and Japanese populations.**

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) represent multigenic diseases and are considered to be caused by interactions between susceptibility genes and environmental factors that result in an abnormal immune response. In fact, familial and linkage studies have provided strong evidence for the role of multiple genetic factors in the development of SLE and RA (1). In addition, association-based approaches in candidate loci using single-nucleotide polymorphisms (SNPs) have also identified several genes that contribute to these diseases. More recently, genome-wide association (GWA) studies in SLE and RA have revealed many susceptibility genes and pathways that contribute to disease development (2).

Familial and linkage studies have also shown familial aggregation of RA, SLE, and other immune-mediated diseases (1). In fact, several gene polymorphisms, including *PTPN22*, *STAT4*, and *IRF5* variants, have been shown to predispose to SLE and RA. Recent GWA studies in Caucasian populations have also identified the tumor necrosis factor α (TNFα)–induced protein 3 gene (*TNFAIP3*) as another common genetic risk factor for SLE and RA (3–6). *TNFAIP3*, also known as the A20 protein, is a negative regulator of the NF-κB signaling pathway that is essential in the pathogenesis of both SLE and RA (7). The association of *TNFAIP3* with diseases has been independently reported in SLE and RA, and it is of great interest that the peaks in association in the GWA studies are different between SLE and RA. In Caucasian populations, the significantly associated SNP markers for SLE, including the nonsynonymous SNP termed rs2230926, are located in the *TNFAIP3* region, while those for RA are located in the intergenic region between *TNFAIP3* and the oligodendrocyte transcription factor 3 gene (*OLIG3*). In addition to the difference in the diseases themselves, the association between *TNFAIP3* polymorphisms and these diseases in the Asian populations remains unclear (8).

In order to elucidate a genetic role for *TNFAIP3* in the development of SLE and RA in the Japanese population, we investigated 2 independent case–control cohorts of patients with SLE and 3 independent cohorts of patients with RA.

PATIENTS AND METHODS

Subjects. The subjects in the SLE study group comprised 2 cohorts of Japanese patients with SLE and unrelated control subjects. An SLE case–control cohort from the RIKEN (SLE cohort 1) consisted of 376 patients (mean age 43.2 years, 90.3% women) and 934 unrelated control subjects (mean age 52.6 years, 25.0% women). An SLE case–control cohort at Hokkaido University (SLE cohort 2) consisted of 341 patients (mean age 46.2 years, 88.3% women) and 428 unrelated control subjects (mean age 47.7 years, 28.7% female). All patients with SLE fulfilled the 1997 American College of Rheumatology (ACR) revised criteria for SLE (9).

The subjects in the RA component of the study comprised 3 cohorts of Japanese patients with RA and unrelated control subjects. The first cohort of patients with RA from BioBank Japan (RA cohort 1) consisted of 1,112 patients (mean age 60.5 years, 89.7% female, 69.7% positive for rheumatoid factor [RF]), and 934 unrelated control subjects. The second cohort from RIKEN (RA cohort 2) consisted of 830 patients (mean age 64.3 years, 83.7% women, 75.0% RF positive), and 658 unrelated control subjects (mean age 48.6 years, 57.4% women). The 934 unrelated control subjects in the first cohort of RA patients were the same as those used in SLE cohort 1. An RA case–control cohort from the Institute of Rheumatology Rheumatoid Arthritis (IORRA) cohort (RA cohort 3), which is a prospective observational cohort of patients with RA studied at Tokyo Women's Medical University, comprised 1,504 patients (mean age 59.3 years, 84% women, 88% RF positive), and 752 control subjects (mean age 38.4 years, 50% women). All patients with RA met the 1987 ACR (formerly, the American Rheumatism Association) revised criteria for a diagnosis of RA (10).

All subjects entered into this study were self-identified as Japanese and were recruited through several medical institutions located in Japan. DNA samples from the patients in the first cohort of RA patients in BioBank Japan were provided by the Leading Project for Personalized Medicine from the Ministry of Education, Culture, Sports, Science and Technology, Japan (11). All subjects provided informed consent prior to their participation in this study, and the study was preapproved by the ethics committee of each institution.

SNPs. For the selection of SNPs required to genotype in and near *TNFAIP3*, we reviewed previous GWA studies of SLE and RA (3–6). We then selected 2 SNPs, rs2230926 and rs10499194. SNP rs2230926 is a nonsynonymous variant in exon 3 of *TNFAIP3* and was strongly associated with SLE in the GWA study by Musone et al (5). Although the GWA study of SLE by Graham et al indicated that rs5029939, located in intron 2 of the gene, is most significantly associated with a predisposition to SLE (6), there is strong linkage disequilibrium (LD) (r^2 = 0.86) between these SNPs according to HapMap phase II data for Japanese and evidence that rs5029939 may be substituted by rs2230926 (Figure 1). Two previous GWA studies in RA revealed that rs10499194 and rs6920220, which are located between *TNFAIP3* and *OLIG3*, were significantly associated risk variants for RA (3,4). The HapMap data for Japanese individuals indicate that the minor allele frequency (MAF) of rs6920220 is 0.011, and that the MAF for control subjects in RA cohort 3 (IORRA) was <0.01. Results of a recent study in Korean populations also indicated

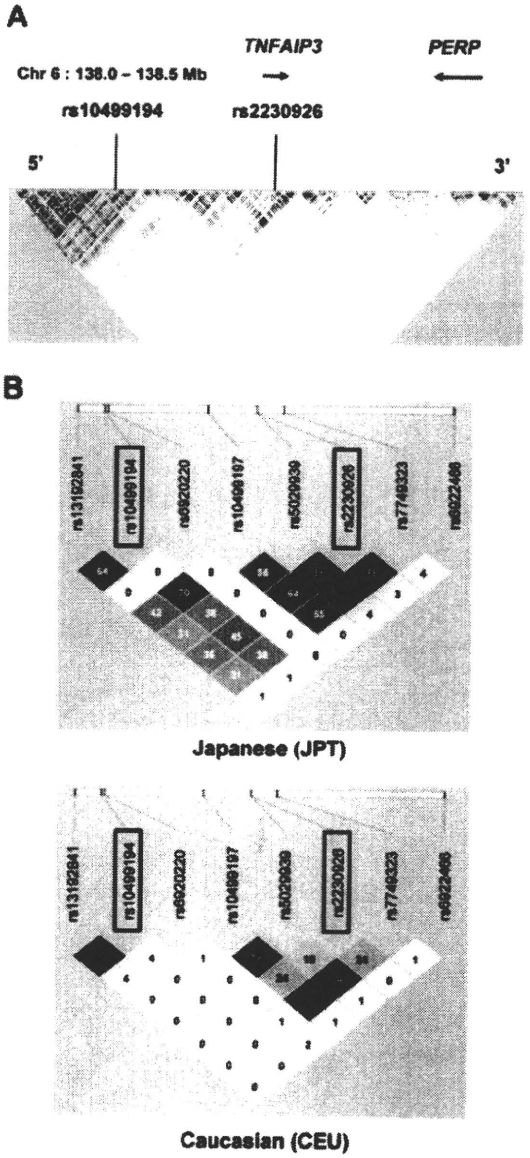

Figure 1. Pairwise linkage disequilibrium (LD) patterns for polymorphisms in the *TNFAIP3* region, according to HapMap phase II data. **A,** Pairwise LD pattern in the expanded *TNFAIP3* region derived from the HapMap data for Japanese patients, with r^2 values. *OLIG3* is located ~370 kbp away from *TNFAIP3* in the 5′ region and is not shown. **B,** Pairwise LD patterns for single-nucleotide polymorphisms (SNPs) in the *TNFAIP3* region that were significantly associated with systemic lupus erythematosus and rheumatoid arthritis in previous genome-wide association studies. The upper and lower panels were constructed using HapMap data for Japanese and Caucasian patients, respectively. The diagram shows pairwise LD values as quantified using the r^2 value. A stronger LD is depicted graphically by the densely shaded boxes. The boxed areas show the 2 SNPs genotyped.

that the variant was too rare (MAF <0.01) to be evaluated for associations (8).

Based on HapMap data for Japanese individuals, pairwise LD patterns for the SNPs in and near *TNFAIP3*, which were significantly associated with SLE and RA in the previous GWA studies, are presented in Figure 1 (for SLE, rs13192841, rs10499197, rs5029939, rs2230926, rs7749323, and rs6922466; for RA, rs10499194 and rs6920220).

Genotyping. We genotyped SNPs using TaqMan assays. For the selected SNPs, predesigned TaqMan SNP genotyping assays were used (probe ID: rs2230926, C.770116_10; rs10499194, C.1575581_10; Applied Biosystems, Foster City, CA). Fluorescence was detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Genotyping assessment was performed on >98% of the samples, for all of the polymorphisms genotyped. All of the SNPs were in Hardy-Weinberg equilibrium in control subjects, according to chi-square statistics ($P > 0.01$).

Case–control association tests. We first performed allele frequency comparisons of rs2230926 and rs10499194 in SLE cohort 1 and RA cohort 1. Then, further case–control association studies were conducted using SLE cohort 2 and RA cohorts 2 and 3, to validate the associations in the first cohorts. In the replication studies, we genotyped the SNPs with a P value less than 0.05 in either SLE cohort 1 or RA cohort 1 (the P value was determined after correction for conditional logistic analysis, as described below).

Measurement of autoantibodies. Sera from 1,104 patients in RA cohort 1 were available for the measurement of anti–cyclic citrullinated peptide (anti-CCP) antibodies and RF. Anti-CCP antibodies were measured using the Mesacup CCP test (Medical and Biological Laboratories, Woburn, MA), and RF was measured by enzyme-linked immunosorbent assay.

Statistical analysis. The case–control association of each SNP was tested with the Cochran-Armitage trend test. The genotype and allele frequencies for patients and control subjects were used to calculate the odds ratios (ORs) and 95% confidence interval (95% CIs) using Woolf's method. For the combined analysis, we used the Mantel-Haenszel test. We performed conditional logistic regression analysis to evaluate the effect of each polymorphism conditional on the remaining polymorphisms, using Statistica software (StatSoft, Tulsa, OK). We calculated pairwise LD indices between pairs of SNPs (the r^2 value), using HaploView software, version 4.0 (http://www.broad.mit.edu/haploview/ haploview). We calculated the population attributable risk (PAR) using the following formula: $PAR = f(OR - 1)/(1 + f[OR - 1])$, where f is the allele frequency in the control subjects. PAR is defined as the reduction in incidence that would be achieved if the population had been entirely unexposed. We calculated the statistical power of association using the R software program (http://www.r-project.org).

RESULTS

Our results revealed a significant association between rs2230926 and both SLE and RA when comparing allele frequency in the patients and control subjects in the first cohort (for SLE, OR 1.92, 95% CI

Table 1. Association study of rs2230926 and rs10499194 with SLE in Japanese subjects*

dbSNP number, major/minor allele	No. of patients	No. of controls	Minor allele frequency		OR (95% CI)	*P*
			Patients	Controls		
rs2230926, G/T						
SLE 1	376	934	0.113	0.062	1.92 (1.43–2.58)	1.2×10^{-5}
SLE 2	341	428	0.116	0.064	1.91 (1.33–2.73)	3.0×10^{-4}
Combined analysis†	717	1,362	0.114	0.063	1.92 (1.53–2.41)	1.9×10^{-8}
rs10499194, T/C						
SLE 1	376	933	0.084	0.061	1.42 (1.03–1.95)	0.030

* SLE = systemic lupus erythematosus; dbSNP = Database of Single-Nucleotide Polymorphisms; OR = odds ratio; 95% CI = 95% confidence interval.
† By the Mantel-Haenszel method.

1.43–2.58, $P = 1.2 \times 10^{-5}$; for RA, OR 1.52, 95% CI 1.20–1.92, $P = 5.6 \times 10^{-4}$) (Tables 1 and 2). We also observed an association between rs10499194 and SLE patients in cohort 1 (OR 1.42, 95% CI 1.03–1.95, $P = 0.030$) (Table 1). However, the T allele appeared to represent a susceptibility allele in the SLE and RA patients in cohort 1, whereas the C allele appeared to be a risk allele for RA in Caucasians (3). We speculated that this association could be secondary to the moderate LD between rs2230926 and rs10499194 ($r^2 = 0.14$) according to data on control subjects in SLE cohort 1, and we subsequently performed a conditional logistic regression analysis to evaluate the effects of each polymorphism conditional on the remaining polymorphisms. The results of this analysis indicated that rs10499194 did not retain the statistically significant association when conditionally evaluated on rs2230926 ($P = 0.73$), while rs2230926 retained the significant association when conditionally evaluated on rs10499194 ($P = 3.4 \times 10^{-4}$). We concluded that rs2230926 was primarily associated with SLE located at this locus, and therefore genotyped only rs2230926 for replication studies in SLE (3–6).

The results of a case–control association study in SLE cohort 2 confirmed the significant association between rs2230926 and the risk of SLE (OR 1.91, 95% CI 1.33–2.73, $P = 3.0 \times 10^{-4}$). A combined analysis also confirmed a significant association (OR 1.92, 95% CI 1.53–2.41, $P = 1.9 \times 10^{-8}$, PAR = 0.055). In RA cohort 2 a statistically significant association between rs2230926 and a predisposition for RA was also replicated; however, this was not replicated in RA cohort 3 (for cohort 2, OR 1.39, 95% CI 1.07–1.81, $P = 0.013$; for cohort 3, OR 1.19, 95% CI 0.94–1.50, $P = 0.15$) (Table 2). In RA cohort 3, the statistical power required to detect an association at rs2230926 was 0.54 at a significance level of $\alpha = 0.05$ when we presumed that the OR for RA was 1.4 (the combined OR for RA cohorts 1 and 2 was 1.46). It was possible that the statistical power for RA cohort 3 may have been insufficient. A combined analysis on these data suggested a significant association (OR 1.35, 95% CI 1.18–1.56, $P = 2.6 \times 10^{-5}$, PAR = 0.024).

We observed no significant association of rs10499194 in RA cohort 1, but the statistical power to detect the association in this study was insufficient (1 –

Table 2. Association study of rs2230926 and rs10499194 with RA in Japanese subjects*

dbSNP number, minor/major allele	No. of Patients	No. of controls	Minor allele frequency		OR (95% CI)	*P*
			Patients	Controls		
rs2230926, G/T						
RA cohort 1	1,112	934	0.091	0.062	1.52 (1.20–1.92)	5.6×10^{-4}
RA cohort 2	825	655	0.100	0.074	1.39 (1.07–1.81)	0.013
RA cohort 3	1,478	747	0.087	0.075	1.19 (0.94–1.50)	0.15
Combined analysis†	3,415	2,326	0.092	0.069	1.35 (1.18–1.56)	2.6×10^{-5}
rs10499194, T/C						0.26
RA cohort 1	1,112	933	0.069	0.061	1.15 (0.90–1.48)	0.26
RA cohort 2	827	650	0.072	0.048	1.52 (1.11–2.08)	0.0090
RA cohort 3	1,472	716	0.073	0.059	1.32 (1.02–1.73)	0.038
Combined analysis†	3,411	2,299	0.071	0.056	1.30 (1.11–1.53)	8.4×10^{-4}

* RA = rheumatoid arthritis; dsSNP = Database of Single-Nucleotide Polymorphisms; OR = odds ratio; 95% CI = 95% confidence interval.
† By the Mantel-Haenszel method.

$\beta = 0.31$) considering the previously reported OR of 0.75 and a significance level of $\alpha = 0.05$ (3). Therefore, we genotyped rs10499194 in RA cohorts 2 and 3 for confirmation. Unlike in RA cohort 1, a significant association of rs10499194 was observed in RA cohorts 2 and 3 (for cohort 2, OR 1.52, 95% CI 1.11–2.08, $P = 0.0090$; for cohort 3, OR 1.32, 95% CI 1.02–1.73, $P = 0.038$) (Table 2). However, the risk allele for Caucasian patients with RA was protective against RA in our population, just as was observed in SLE cohort 1. The combined analysis showed a significant association of rs10499194 with RA (OR 1.30, 95% CI 1.11–1.53, $P = 8.4 \times 10^{-4}$).

We stratified patients in RA cohorts 1 and 3 according to the presence of anti-CCP antibodies and RF and examined for the association between *TNFAIP3* polymorphisms (rs2230926 and rs10499194) and RA susceptibility (see Supplementary Table 1, available in the online version of this article at http://www3.interscience.wiley.com/journal/76509746/home). When the patients were stratified according to anti-CCP antibody status, the G allele of rs2230926 was found to confer increased risk for RA in anti-CCP antibody–positive patients relative to anti-CCP antibody–negative patients (for anti-CCP antibody–positive patients, OR 1.36, 95% CI 1.15–1.62, $P = 4.0 \times 10^{-4}$; for anti-CCP–negative patients, OR 1.16, 95% CI 0.83–1.61, $P = 0.39$ in the combined analysis). A similar trend was observed when patients were stratified according to RF status. A stratified analysis on rs10499194 also showed that the disease susceptibility allele in Japanese patients with RA (the T allele) conferred higher risk in autoantibody-positive patients than in autoantibody-negative patients.

DISCUSSION

In the current study, rs2230926, located in exon 3 of *TNFAIP3*, was shown to be significantly associated with a predisposition to both SLE and RA in 2 and 3 independent cohorts of subjects, respectively. Our results confirmed that *TNFAIP3* is one of the common genetic risk factors for both SLE and RA, similar to *STAT4* and *IRF5*, in the Japanese and Caucasian populations (2). In addition, recent studies in Caucasian patients with RA have demonstrated that the *TNFAIP3* variant conferred an increased risk of RA in anti-CCP antibody– and RF-positive patients compared with anti-CCP antibody– and RF-negative patients (12,13). Our analysis stratified according to the autoantibodies confirmed this observation in Japanese patients with RA.

TNFAIP3 encodes a cytoplasmic zinc finger protein that is also known as the A20 protein. The A20 protein is required for negative regulation of the NF-κB signaling pathway, which is mediated by innate immune receptors such as TNF receptors and Toll-like receptors, and it prevents overstimulation of the innate immune response (7,14). The disease-associated variant, rs2230926 (T/G), is a nonsynonymous variant that results in a phenylalanine-to-cysteine change at residue 127 of the A20 protein (5). The risk allele is known to be the G allele that encodes Cys. Musone et al have reported that Cys^{127} A20 protein was only modestly, but consistently, less effective at inhibiting TNF-induced NF-κB activity than the Phe^{127} protein (5). This result suggests that reduced negative regulatory activity of A20 protein may allow excessive immune activity, leading to enhanced autoreactivity.

GWA studies of SLE patients in Caucasian populations have suggested that several polymorphisms in the *TNFAIP3* region, including the nonsynonymous SNP rs2230926, are associated with a predisposition to the disease. The genetic significance of rs2230926 was evident in the Japanese patients with SLE or RA entered into our study, although its precise role in Caucasian patients with RA remains unclear. The intergenic SNP rs10499194 is one of the landmark polymorphisms identified in Caucasian patients with RA (3,15), although the significant association with RA could not be replicated in several Caucasian populations (3,12). Because rs10499194 is also associated with RA susceptibility and autoantibody status in our population, rs10499194 could be a landmark for disease causal variants in Japanese patients with RA. However, considering the inverted susceptibility allele of rs10499194 between Japanese patients (T allele) and Caucasian patients (C allele), this association of rs10499194 would appear to be secondary, as a result of LD between rs10499194 and the disease causal variants. This finding is further supported by the lack of independent association at rs10499194 in SLE when conditioned with the rs2230926 genotype, suggesting that the association observed in rs10499194 may be partially influenced by rs2230926.

Taking into account the biologic impact of rs2230926 demonstrated by Musone et al (5), rs2230926 seems likely to be an important candidate for a causal variant in *TNFAIP3* (5). However, additional polymorphisms that are located in the intergenic region of *OLIG3* and *TNFAIP3* as well as that of *TNFAIP3* and *PERP* may also independently exercise an effect on disease susceptibility, a hypothesis that was previously raised by Musone et al (5) and Graham et al (6). Further mapping of the *TNFAIP3* region in Asian and Caucasian