from all participating subjects, and the study was conducted in accordance with the human experimental guidelines of our institution. Synovial cells were isolated from the synovial tissues by an enzymatic digestion as described previously [3]. Adherent synovial cells of at least four passages were examined for surface molecule expression with a flow cytometer (Epics XL; Beckman Coulter, Hialeah, FL, USA). Less than 1% of adherent synovial cells expressed CD2, CD3, CD20 and CD14, confirming that the adherent synovial cells used were FLS.

Adipocyte-like cell differentiation from FLS by troglitazone

Adipocyte-like cell differentiation from FLS was induced by stimulation with 10 μM troglitazone for 3 weeks, as described previously by our laboratory [3]. Briefly, FLS were cultured to confluence in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). The cells (3×10^5 per well in 12-well culture plate; Figs 1 and 4) were cultured for another 3 weeks in the same media containing 10 μM troglitazone. The culture media were changed every 3 days for 3 weeks. After cultivation, adipogenesis was confirmed by intracellular lipid deposition by staining with Oil Red O, as described previously [3]. In some experiments, FLS were cultured in the presence of TNF-α (200 IU/ml), IL-1β (20 IU/ml) or IFN-γ (500 IU/ml) for 3 weeks with troglitazone, and adipocyte-like cell differentiation from FLS was also examined.

Determination of IL-6, IL-8 and MMP-3 in culture supernatants from FLS and troglitazone-differentiated adipocyte-like cells

Production of IL-6, IL-8 and MMP-3 was determined in culture supernatants from FLS and troglitazone-differentiated adipocyte-like cells. Culture supernatants (5×10^6 in 100 mm dish; Fig. 2) were collected, and protein concentrations of IL-6, IL-8 and MMP-3 were examined by enzyme-linked immunosorbent assays (ELISA) (Fujirebio, Tokyo, Japan).

Expression of PPARγ in FLS

Expression of PPARγ was examined by Western blotting. Briefly, FLS (1.5×10^6 in 60 mm dish; Fig. 5) were cultured in the presence or absence of TNF-α (200 IU/ml), IL-1β (20 IU/ml) or IFN-γ (500 IU/ml) for the indicated times. After cultivation, the cells were washed three times with phosphate-buffered saline and lysed by addition of lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% NP-40 and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF)]. Protein concentrations of cell extracts were determined using a protein assay kit (Bio-Rad, Melville, NY, USA). An identical amount of protein for each lysate (5 μg/well) was subjected to 12% SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a poly vinilidene difluoride (PVDF) filter, which was subsequently blocked for 1 h using 5% non-fat dried milk in TBS containing 0.5% Tween 20 (TBS-T). The filter was then washed with 1% non-fat dried milk in TBS-T, and incubated at room temperature for 1 h with anti-PPARγ antibody (0.4 μg/ml). The filter was washed with TBS-T and incubated with a 1:1000 dilution of sheep anti-rabbit IgG coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham, Amersham, UK) was used for detection.

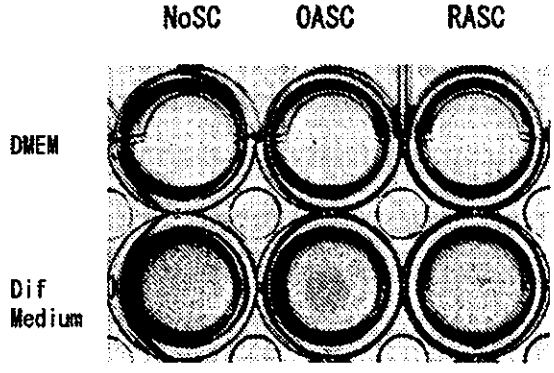

FIG. 1. Adipocyte-like cell differentiation of FLS in response to troglitazone. Three weeks of cultivation with troglitazone (10 μM) induced adipocyte-like differentiation of FLS derived from normal synovial tissues (NoSC), OA (OASC) and RA (RASC). Intracellular lipid accumulation was observed by Oil Red O staining.

Determination of nuclear NF-κB and C/EBP activities by electrophoretic mobility shift assay (EMSA)

NF-κB and C/EBP nuclear activities were examined by EMSA using the Gel Shift Assay System (Promega, Madison, WI, USA) as described previously [3, 12]. Briefly, nuclear proteins from the cells (5×10^6 in a 100 mm dish; Figs 3 and 6) extracted from the conditioned cells (7.5 μg protein from each cell lysate) were mixed with ^{32}P-radiolabelled double-stranded oligonucleotide containing NF-κB binding sequence (5′-AGTTGAGGGGACTTTCCA GGC-3′) or C/EBP binding sequence (5′-TGCAGATTGCGCAA TCTGCA-3′) and mutant oligonucleotide of C/EBP (5′-TGCAGA GACTAGTCTCTGCA-3′), 0.25 mg/ml of poly(dI-dC) (Sigma, St Louis, MO, USA) in 10 mM Tris (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol and 4% glycerol. Reactions were incubated for 30 min at room temperature and analysed with 5% PAGE. Cold competition was performed by adding excess unlabelled oligonucleotide (data not shown).

Statistical analysis

Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test. A *P* value of less than 0.05 denoted the presence of a statistically significant difference.

Results

Low production of IL-6, IL-8 and MMP-3 by adipocyte-like cells

Oil Red O staining demonstrated marked lipid deposition in all synovial cell cultures isolated from RA, OA and traumatic joints without arthritis in response following their culture with the synthetic PPARγ ligand troglitazone for 3 weeks (Fig. 1). The differentiation potential of FLS into adipocyte-like cells was similar among RA, OA and traumatic patients.

We next examined the functional changes in FLS during adipocyte-like cell differentiation based on our previous study that showed diminished IL-6 production by FLS during adipocyte-like differentiation [3]. Cytokines and proteases are crucial mediators for joint destruction in patients with RA [2, 13]. In addition to IL-6, protein concentrations of IL-8 and MMP-3 in culture supernatants of troglitazone-differentiated adipocyte-like cells were reduced compared with FLS. Interestingly, the production of IL-6, IL-8 and MMP-3 was restored 8 days after the

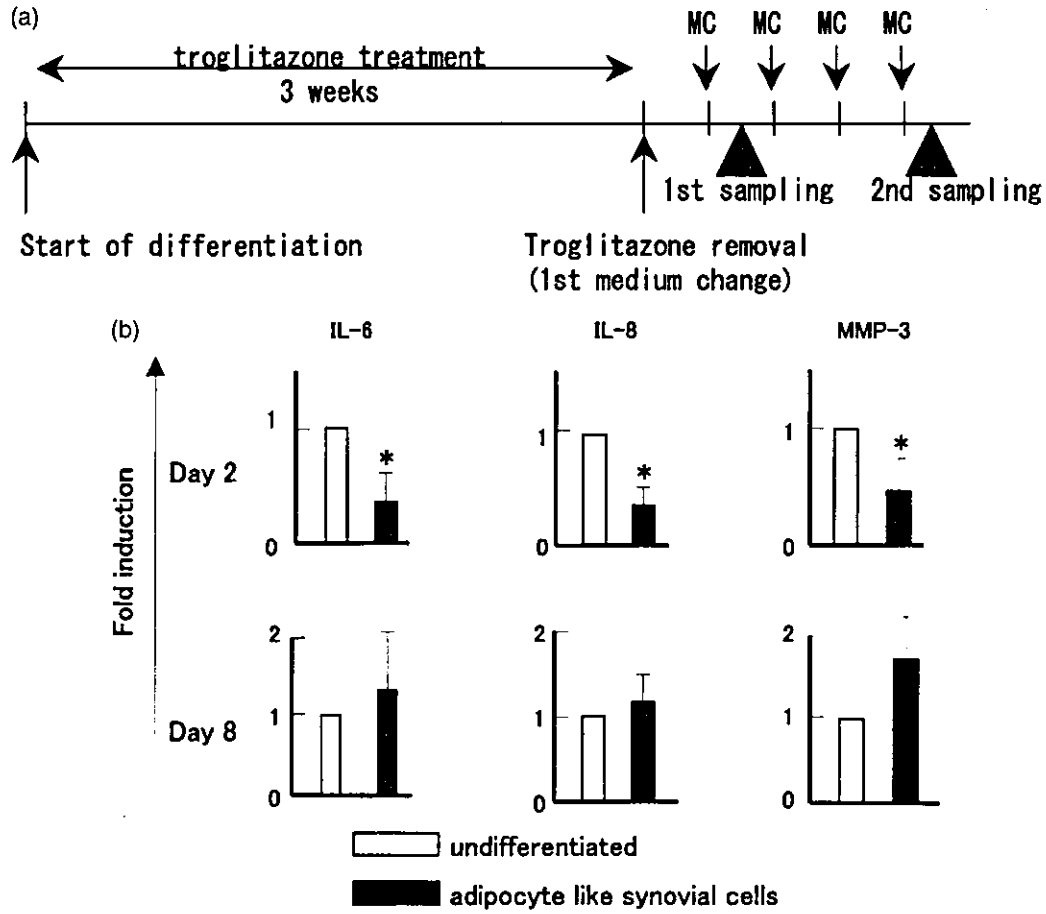

FIG. 2. Low IL-6, IL-8 and MMP-3 production after adipocyte-like cell differentiation. (a) Protocol for sample collection for ELISA. Troglitazone was removed from the culture medium after 3 weeks of induction of adipocyte-like differentiation of FLS. Troglitazone-free medium was changed every 48 h (MC). Culture medium was collected twice (arrowheads; the first sampling was 24 h after the second medium change and the second sampling was 24 h after the fifth change). (b) Protein concentrations of IL-6, IL-8 and MMP-3 in undifferentiated FLS were calculated as 1.0 in each experiment. Data represent the mean ± S.D. of samples from seven RA patients. Note that the production of IL-6, IL-8 and MMP-3 was diminished after adipocyte-like cell differentiation but was restored 8 days after troglitazone withdrawal. $^*P < 0.01$.

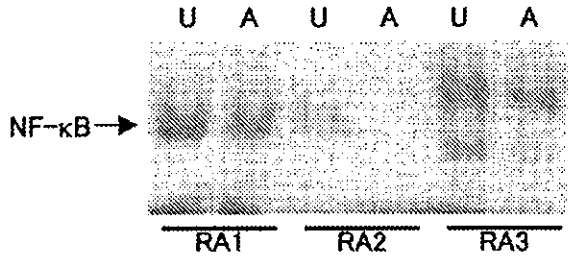

FIG. 3. Decrease in NF-κB DNA binding activity in FLS after adipocyte-like cell differentiation. Nuclear proteins were extracted from undifferentiated FLS and troglitazone-differentiated adipocyte-like cells, and NF-κB DNA binding activity was examined by EMSA. After 3 weeks of cultivation with troglitazone, adipocyte-like FLS were further incubated in the absence of troglitazone for 48 h to exclude a direct effect of troglitazone on NF-κB nuclear activity. Results shown are for three RA patients labelled as RA-1, RA-2 and RA-3. U, undifferentiated FLS, A, troglitazone-differentiated adipocyte-like cells.

withdrawal of troglitazone (Fig. 2). NF-κB is an indispensable transcription factor for IL-6, IL-8 and MMP-3 [14, 15], and therefore we studied NF-κB nuclear activity in FLS and troglitazone-differentiated adipocyte-like cells. NF-κB nuclear activity was not detected in either FLS or troglitazone-differentiated adipocyte-like cells (data not shown). Stimulation of FLS with TNF-α clearly induced NF-κB nuclear activity in FLS, which was diminished in troglitazone-differentiated adipocyte-like cells in response to TNF-α (Fig. 3).

Inhibition of troglitazone-induced adipocyte-like cell differentiation by cytokines

As shown in Fig. 1, FLS differentiation into adipocyte-like cells was similar *in vitro* in RA, OA and normal synovial tissues, indicating that the synovial microenvironment *in vivo* may be important for FLS differentiation. We focused on whether cytokines affect adipocyte-like cell differentiation from FLS *in vitro*. Confluent FLS were cultured with 10 μM troglitazone in the presence of TNF-α, IL-1β or IFN-γ for 3 weeks, and the results

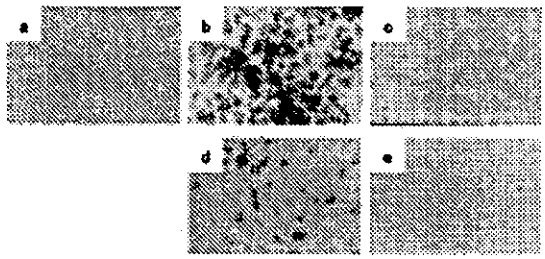

FIG. 4. Inhibition of adipocyte-like cell differentiation from FLS by TNF-α, IL-1β and IFN-γ. FLS were cultured with troglitazone for 3 weeks in the presence or absence of TNF-α (200 IU/ml), IL-1β (20 IU/ml) or IFN-γ (500 IU/ml). After cultivation, adipocyte-like cell differentiation was examined by Oil Red O staining. (a) FLS cultured in the absence of troglitazone and cytokines. (b) FLS cultured with troglitazone. (c) FLS cultured with troglitazone in the presence of TNF-α. (d) FLS cultured with troglitazone in the presence of IL-1β. (e) FLS cultured with troglitazone in the presence of IFN-γ. Results shown are representative data from five RA patients. Magnification, $\times$40.

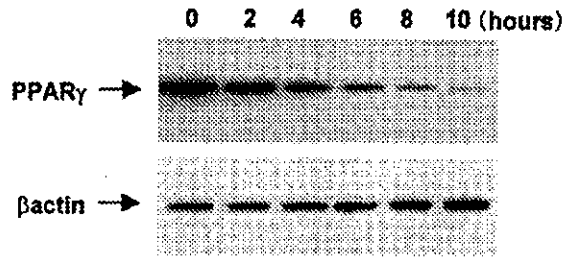

FIG. 5. Inhibition of PPARγ expression in FLS by IFN-γ. FLS isolated from RA patients were cultured with IFN-γ (500 IU/ml) for the indicated times and PPARγ expression was examined as described in the text. Results shown are representative data from four experiments. β-Actin was assayed as an internal control protein.

demonstrated that troglitazone-induced adipocyte-like cell differentiation from FLS was clearly inhibited by TNF-α, IL-1β and IFN-γ (Fig. 4). IFN-γ exhibited the highest inhibitory effect on adipocyte-like cell differentiation (Fig. 4). Troglitazone-induced adipocyte-like cell differentiation was additively inhibited in the presence of TNF-α plus IL-1β; however, the inhibition of which was less than that of IFN-γ alone (data not shown).

Inhibition of adipogenic transcription factors by cytokines

Expressions of PPARγ and C/EBP are crucial for adipogenesis. As we described previously [3], PPARγ was expressed in FLS, but its expression was clearly suppressed by IFN-γ (Fig. 5). We next examined C/EBP expression in FLS. Since constitutive expression of C/EBPβ was found in FLS by western blotting (data not shown), C/EBP nuclear activity was studied by EMSA. As shown in Fig. 6, DNA binding activity of C/EBP was determined in untreated FLS. C/EBP DNA binding activity of FLS was suppressed by stimulation with TNF-α, IL-1β or IFN-γ.

Discussion

Recent progress in research into the molecular basis of FLS has proved that FLS share some characteristics with malignant cells,

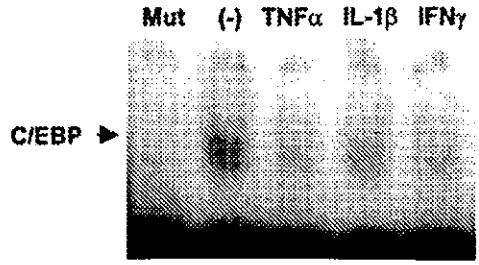

FIG. 6. Inhibition of C/EBP DNA binding activity in FLS by TNF-α, IL-1β and IFN-γ. FLS from RA patients were cultured in the presence or absence of TNF-α (200 IU/ml), IL-1β (20 IU/ml) or IFN-γ (500 IU/ml) for 12 h, nuclear proteins were extracted, and DNA binding activity of C/EBP was examined by EMSA. Mut, mutated oligo for negative control. Results shown are representative data from four experiments.

including mutations in p53 protein and lack of expression of the novel tumour suppressor gene *PTEN* (phosphatase and tensin homologous on chromosome ten) [16, 17]. In addition, FLS can differentiate into mesenchymal lineage cells, such as osteoblasts, chondrocytes and adipocytes [4], suggesting that FLS possess characteristics of immature or undifferentiated cells. We have recently found that IL-6 production from troglitazone-differentiated adipocyte-like cells is suppressed compared with FLS [3]. These results implicate that effective differentiation of synovial cells into adipocytes could lead to reduced cytokine production in RA. Thus, we conducted the present study to examine the role of cytokines in adipogenesis from FLS.

Adipogenesis is a cell differentiation process dependent upon the coordinated expression of two classes of transcriptional factors, PPARγ and C/EBP [5–8]. C/EBP family members are bZIP transcription factors that possess a leucine zipper and a basic DNA binding domain. Following hormonal stimulation in 3T3-L1 preadipocytes, C/EBP proteins, including C/EBPα, β and δ, are rapidly induced and function as indispensable nuclear factors for adipogenesis [7, 8]. PPARγ is expressed in FLS, and we showed here C/EBP nuclear activity in FLS, which may explain why FLS can differentiate into adipocyte-like cells. In addition to IL-6, endogenous production of IL-8 and MMP-3 in troglitazone-differentiated adipocyte-like cells was diminished. Furthermore, basal NF-κB nuclear activity was not detected in either FLS or troglitazone-differentiated adipocyte-like cells; however, TNF-α-induced NF-κB nuclear translocation was reduced in adipocyte-like cells compared with FLS. Since NF-κB is an indispensable transcription factor for IL-6, IL-8 and MMP-3 [14, 15], the small amount of active NF-κB that cannot be detected by EMSA might be suppressed during adipocyte-like cell differentiation. These transformations of FLS are very favourable for the regression of RA; however, the differentiation of the FLS into adipocyte-like cells may not be stable, because the productions of IL-6, IL-8 and MMP-3 were restored 8 days after the withdrawal of troglitazone.

We speculated that the inflammatory milieu in rheumatoid synovial tissues may contribute to the enrichment of immature FLS in the joints. Thus, we examined the effects of IFN-γ, TNF-α and IL-1β on troglitazone-mediated adipogenesis of FLS. Suzawa *et al.* [18] recently reported that troglitazone-induced adipocyte differentiation from mesenchymal stem cells can be inhibited by TNF-α and IL-1β via NF-κB-inducing kinase (NIK)-mediated NF-κB activation. Similar to results for mesenchymal stem cells, our results showed that both TNF-α and IL-1β suppressed troglitazone-mediated adipocyte-like cell differentiation from FLS. Interestingly, IFN-γ also inhibited the process, and its inhibitory effect was more prominent than that of TNF-α and IL-1β. These findings indicate that the Janus kinase (JAK)/signal

transducer and activator of transcription (STAT) cascade is another important inhibitory pathway for adipogenesis from FLS and our data are consistent with previous reports that stem cell self-renewal without differentiation is mediated through the JAK/STAT pathway in *Drosophila* spermatogenesis [19, 20]. Since TNF-α-induced NF-κB activation is inhibited in troglitazone-differentiated adipocyte-like cells, adipogenesis signals and cytokine signals apparently influence each other in FLS.

Pittenger *et al.* [21] showed that skin fibroblasts did not differentiate into mesenchymal lineage cells. However, FLS from RA, OA and normal synovial tissues can equally differentiate into adipocyte-like cells *in vitro*, suggesting that FLS are joint-specific pluripotent stem cells. Since the adipocyte-like cell differentiation potential of FLS did not differ among RA, OA and normal synovial tissues, the synovial microenvironment might be crucial in determining the mesenchymal lineage differentiation *in vivo*.

Our present data indicate the possibility that the inflammatory milieu affects the cell differentiation that results in cell proliferative disease in humans. Thus, we think it is possible to inhibit the activation of synovial cell in RA by artificial induction of adipogenesis of the cells.

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Osteoprotegerin (OPG) acts as an endogenous decoy receptor in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis of fibroblast-like synovial cells

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SUMMARY

We examined the role of osteoprotegerin (OPG) on tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in rheumatoid fibroblast-like synovial cells (FLS). OPG protein concentrations in synovial fluid from patients with rheumatoid arthritis (RA) correlated with those of interleukin (IL)-1β or IL-6. A similar correlation was present between IL-1β and IL-6 concentrations. Rheumatoid FLS *in vitro* expressed both death domain-containing receptors [death receptor 4 (DR4) and DR5] and decoy receptors [decoy receptor 1 (DcR1) and DcR2]. DR4 expression on FLS was weak compared with the expression of DR5, DcR1 and DcR2. Recombinant TRAIL (rTRAIL) rapidly induced apoptosis of FLS. DR5 as well as DR4 were functional with regard to TRAIL-mediated apoptosis induction in FLS; however, DR5 appeared be more efficient than DR4. In addition to soluble DR5 (sDR5) and sDR4, OPG administration significantly inhibited TRAIL-induced apoptogenic activity. OPG was identified in the culture supernatants of FLS, and its concentration increased significantly by the addition of IL-1β in a time-dependent manner. Neither IL-6 nor tumour necrosis factor (TNF)-α increased the production of OPG from FLS. TRAIL-induced apoptogenic activity towards FLS was reduced when rTRAIL was added without exchanging the culture media, and this was particularly noticeable in the IL-1β-stimulated FLS culture; however, the sensitivity of FLS to TRAIL-induced apoptosis itself was not changed by IL-1β. Interestingly, neutralization of endogenous OPG by adding anti-OPG monoclonal antibody (MoAb) to FLS culture restored TRAIL-mediated apoptosis. Our data demonstrate that OPG is an endogenous decoy receptor for TRAIL-induced apoptosis of FLS. In addition, IL-1β seems to promote the growth of rheumatoid synovial tissues through stimulation of OPG production, which interferes with TRAIL death signals in a competitive manner.

Keywords fibroblast-like synovial cells IL-1β OPG rheumatoid arthritis TRAIL

INTRODUCTION

The soluble receptor, osteoprotegerin (OPG), is a member of the tumour necrosis factor receptor (TNFR) superfamily and acts as a receptor antagonist. The decoy function of OPG towards receptor activator of nuclear factor κB ligand (RANKL) is well recognized; OPG binds RANKL, and thus prevents the interaction with, and stimulation of, RANK [1–3]. Hence, OPG inhibits osteoclast differentiation and survival as demonstrated both *in vivo* and *in vitro* [4]. OPG is also thought to be involved in inflammatory diseases based on results of experimental studies demonstrating stimulation of OPG production from endothelial cells by tumour necrosis factor (TNF)-α and interleukin (IL)-1β [5]. Furthermore, OPG has also been shown to exhibit mitogenic and/or anti-apoptotic properties for foreskin fibroblasts and/or endothelial cells [6,7].

Marked hyperplasia of synovial tissues is a characteristic feature of rheumatoid arthritis (RA), which is mediated, at least in part, by impaired apoptosis of synovial cells *in situ* [8]. The anti-apoptotic feature of rheumatoid synovial cells *in situ* may not be intrinsic, but rather develop in the inflammatory rheumatoid microenvironment. In this regard, we have demonstrated that the sensitivity of fibroblast-like synovial cells (FLS) to apoptogenic

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stimuli is modulated by various inflammatory cytokines [9–11]. OPG acts also as a receptor antagonist for TNF-related apoptosis-inducing ligand (TRAIL) [12]. We have shown recently that FLS are sensitive toward TRAIL-mediated apoptosis [13]. TRAIL triggers the activation of caspase-8 in FLS, which induces mitochondrial perturbation [13]. As both mitochondrial perturbation and DNA fragmentation were completely inhibited by caspase-8 inhibitor, FLS are classed into type II cell death in response to TRAIL [13]. The regulatory mechanisms of inflammatory cytokines on OPG production may influence TRAIL-induced apoptosis of synovial cells, and thus defines a new functional role for OPG in the pathological process of RA.

In the present study, we demonstrate that IL-1β in rheumatoid synovial fluid is the major inflammatory cytokine responsible for stimulation of OPG synthesis from FLS. Furthermore, our data suggest that OPG produced by FLS is an endogenous receptor antagonist of TRAIL-induced apoptosis in FLS which may, in part, explain the growth-promoting activity of IL-1β for rheumatoid synovial tissues.

MATERIALS AND METHODS

Determination of OPG, IL-1β, IL-6 and TNF-α concentrations in synovial fluid from patients with RA

Synovial fluid samples were obtained from patients with RA, who fulfilled the criteria of the American Rheumatism Association for RA [14], admitted to the National Ureshino Hospital (28 synovial fluid samples from 28 RA patients). The experimental protocol was approved by the Hospital Human Ethics Review Committee and signed consent was obtained from each patient. The concentrations of OPG, IL-1β, IL-6 and TNF-α in the rheumatoid synovial fluid were examined by a sandwich enzyme-linked immunosorbent assay (ELISA) (OPG; Immunodiagnostik AC, Bensheim, Germany: IL-1β, IL-6 and TNF-α; Otsuka Pharmaceutical Co., Tokushima, Japan). In brief, the plates precoated with monoclonal antibody (MoAb) against OPG, IL-1β, IL-6 or TNF-α were incubated with the samples, incubated further with the secondary antibody, and colour was developed according to the protocol provided by the supplier.

Effect of inflammatory cytokines on OPG production from cultured FLS

FLS were isolated from 20 patients with RA at the time of orthopaedic surgery (total knee replacement) conducted at National Ureshino Hospital. Signed consent was also obtained from each patient. Briefly, the synovial tissues were trimmed of fat and minced with scissors, then added to a mixture of collagenase (Sigma Chemical Co., St Louis, MO, USA) and dispase (Godo Shusei Co., Tokyo). The tissue mixture was digested over a 45-min period during gentle stirring at 37°C, and the harvested cells were allowed to adhere to Petri dishes (Falcon 3003, Becton Dickinson Co., Oxnard, CA, USA). The adherent cells used in the present study at third to fifth passages were less than 1% reactive with various MoAbs, including CD3, CD68, CD20 and von-Willebrand factor, which define FLS. Isolated FLS were cultured in the presence or absence of recombinant IL-1β (rIL-1β, Otsuka Pharmaceutical Co., 20 IU/ml), rIL-6 plus r-soluble IL-6 receptor (sIL-6R) (R&D Systems Inc., Minneapolis, MN, USA, 100 ng/ml each) or rTNF-α (R&D Systems, 200 IU/ml) for indicated time intervals in RPMI-1640 containing 10% fetal calf serum (FCS) (1×10^5/35 mm dish). OPG protein concentration in the culture supernatants was examined by the sandwich ELISA as described above.

Expression of TRAIL receptors on cultured FLS

Expression of TRAIL receptors on cultured rheumatoid FLS was examined by flow cytometric analysis. Treated FLS were detached by addition of 0·265 mM EDTA, washed with phosphate buffered saline (PBS) and incubated with antihuman death receptor 4 (DR4; R&D Systems), antihuman DR5 (R&D Systems), antihuman decoy receptor 1 (DcR1; R&D Systems) or antihuman DcR2 (R&D Systems) at 4°C for 30 min. After incubation, the cells were washed with PBS, incubated further with phycoerythrin (PE)-conjugated antigout IgG (Sigma Chemical Co.), and the expression of DR4, DR5, DcR1 and DcR2 was examined by flowcytometer (Epics XL, Beckman Coulter, Hialeah, FL, USA).

Effect of OPG toward TRAIL-induced apoptosis of cultured FLS

We have shown recently that cultured FLS are committed to type II cell death in response to TRAIL [13], thus disruption of

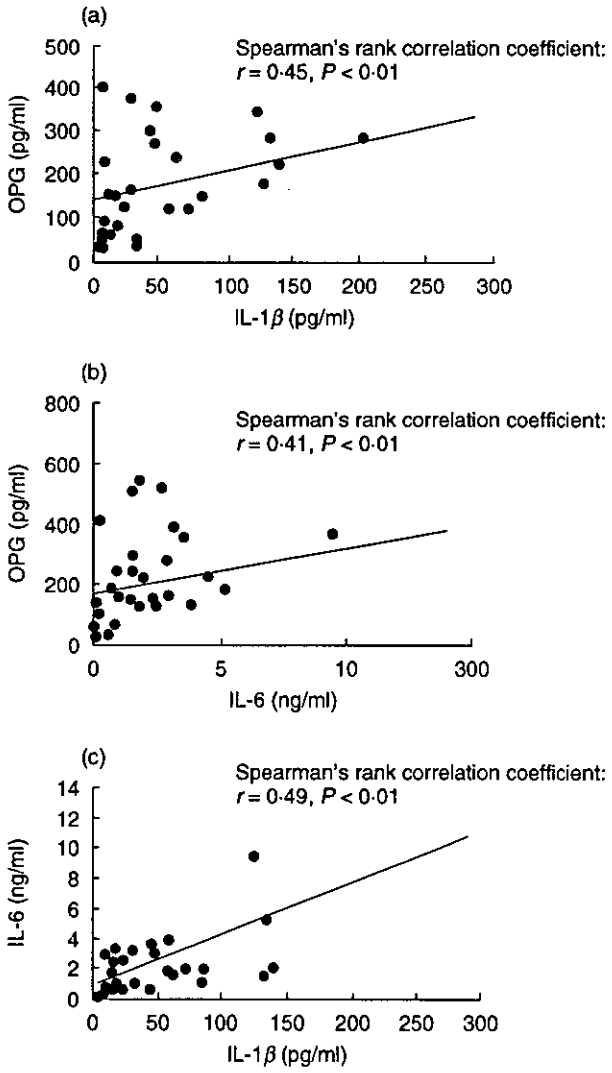

Fig. 1. Positive correlation of protein concentration between OPG and IL-1β (a), OPG and IL-6 (b) and IL-1β and IL-6 (c) found in synovial fluid samples (total 27 synovial fluids examined) of RA patients.

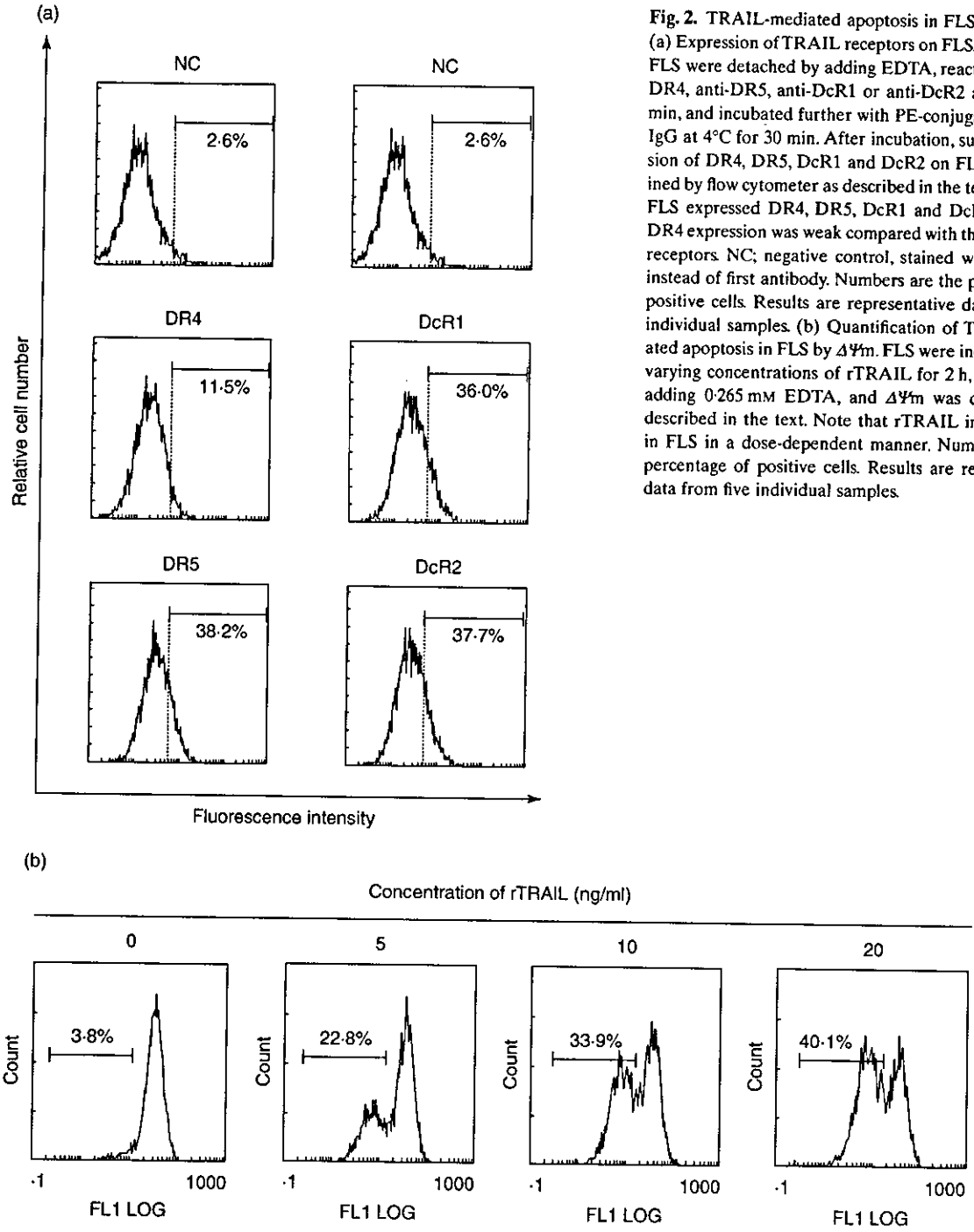

Fig. 2. TRAIL-mediated apoptosis in FLS by rTRAIL. (a) Expression of TRAIL receptors on FLS. Rheumatoid FLS were detached by adding EDTA, reacted with anti-DR4, anti-DR5, anti-DcR1 or anti-DcR2 at 4°C for 30 min, and incubated further with PE-conjugated antigout IgG at 4°C for 30 min. After incubation, surface expression of DR4, DR5, DcR1 and DcR2 on FLS was examined by flow cytometer as described in the text. Note that FLS expressed DR4, DR5, DcR1 and DcR2; however, DR4 expression was weak compared with the other three receptors. NC; negative control, stained with goat IgG instead of first antibody. Numbers are the percentage of positive cells. Results are representative data from five individual samples. (b) Quantification of TRAIL-mediated apoptosis in FLS by $\Delta\Psi m$. FLS were incubated with varying concentrations of rTRAIL for 2 h, detached by adding 0·265 mM EDTA, and $\Delta\Psi m$ was quantified as described in the text. Note that rTRAIL induced $\Delta\Psi m$ in FLS in a dose-dependent manner. Numbers are the percentage of positive cells. Results are representative data from five individual samples.

mitochondrial transmembrane potential ($\Delta\Psi m$) was used to quantify TRAIL-mediated FLS apoptosis in the present study. FLS were cultured in the presence or absence of various cytokines for indicated hours, washed, and incubated further with varying concentrations of rTRAIL (R&D Systems) with or without rOPG (R&D Systems), soluble DR4 (sDR4; R&D Systems) or sDR5 (R&D Systems) for an additional 2 h. After incubation, $\Delta\Psi m$ was examined as described recently [13]. In brief, treated FLS were detached by adding 0·265 mM EDTA, washed, and incubated further with a saturating amount of DiOC6 (3,3′-dihexyloxacarbocyamine iodide, Fluoreszenztechnologie, Grottenhofstr, Austria) at 37°C for 15 min. After incubation, the percentage of $\Delta\Psi m$ in FLS was quantified by flow cytometer (Epics XL, Beckman Coulter).In some experiments, rTRAIL was added to FLS culture without exchanging the culture media, and TRAIL-induced apoptosis in FLS was also examined by $\Delta\Psi m$. To neutralize endogenous OPG secreted from FLS in culture, anti-OPG MoAb (mouse IgG1; R&D Systems) was added, and TRAIL-mediated apoptosis in FLS was examined by $\Delta\Psi m$.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test or Spearman's rank correlation analysis. *P*-values <0·05 were selected as the level of significance.

RESULTS

Determination of OPG protein in synovial fluid of patients with RA

First, we examined whether OPG protein was present in the rheumatoid synovial fluid. As reported recently [15,16], OPG protein was detected in all samples examined, although the level varied from one sample to another (Fig. 1a). Furthermore, there was a positive correlation between the concentrations of OPG and IL-1β. In addition, a similar correlation was demonstrated between the protein concentration of OPG and IL-6, and between that of IL-1β and IL-6 (Fig. 1b,c). In contrast, TNF-α was detected in only a proportion of the samples, and the levels of this cytokine did not correlate with OPG concentration (data not shown).

TRAIL induces apoptosis in FLS through DR4 and DR5

As shown in Fig. 2a, rheumatoid FLS *in vitro* expressed DR4, DR5, DcR1 and DcR2. Expression of DR4 was not so obvious compared with DR5, DcR1 and DcR2. Figure 2B shows that rTRAIL induced apoptotic cell death in FLS in a dose-dependent manner. To clarify the functional role of DR4 and DR5 in TRAIL-mediated apoptosis in FLS, we performed the blocking experiments by the use of sDR4 and sDR5. sDR4 interferes with the interaction between TRAIL and DR4, and sDR5 interferes with that between TRAIL and DR5. As shown in Fig. 3, TRAIL-induced $\Delta\Psi$m in FLS was inhibited partially by sDR4 or sDR5, whereas it was inhibited completely by adding both sDR4 and sDR5. DR5 on FLS was suggested to be more functional to induce TRAIL-mediated apoptosis compared with DR4 (Fig. 3). In addition, OPG administration significantly suppressed TRAIL-mediated apoptosis in FLS (Fig. 3).

IL-1β-stimulated production of OPG by cultured FLS, which interferes with TRAIL-induced apoptogenic activity toward FLS

We examined the effects of inflammatory cytokines on OPG production by FLS. As shown in Fig. 4, OPG protein concentration increased time-dependently in the culture supernatants of FLS. Furthermore, such production was augmented by IL-1β at all time-points examined. Compared with IL-1β, the stimulatory effect of IL-6 (IL-6 + sIL-6 receptor) and TNF-α on OPG production from FLS was not found (data not shown). It was interesting to note that the rate of FLS apoptosis in response to TRAIL was decreased when rTRAIL was added without replacing the culture media (Fig. 5). Inhibition was more prominent in IL-1β-stimulated FLS compared with untreated FLS (Fig. 5), which was mainly restored by adding anti-OPG MoAb in culture media (Fig. 5). IL-1β treatment itself modulated neither TRAIL receptor expression (Fig. 6) nor TRAIL-mediated apoptosis (Fig. 5). These data suggest that OPG produced from FLS into the culture media is an endogenous receptor antagonist towards TRAIL-induced apoptosis of FLS.

DISCUSSION

Apoptosis occurs in a variety of physiological situations such as embryogenesis, and plays a crucial role in normal tissue homeostasis. However, a breakdown in the delicate balance between cell survival and apoptosis has been implicated in the pathogenesis of a number of rheumatic diseases, including RA [8]. The mechanisms responsible for synovial hyperplasia of RA patients may be explained by reduced synovial cell apoptosis, which cannot counteract the ongoing process of synovial cell proliferation.

TRAIL can interact potentially with five different receptors: two functional receptors DR4 and DR5, two decoy receptors DcR1 and DcR2, and a soluble decoy receptor OPG [12,17,18]. Other investigators have shown that DR5 is a sole death domain containing receptor for TRAIL on FLS [19]. We have shown here that DR5 was not sole but a prominent death domain-containing receptor for TRAIL. A previous study has shown that DR5 has the highest binding affinity towards TRAIL at 37°C [20];

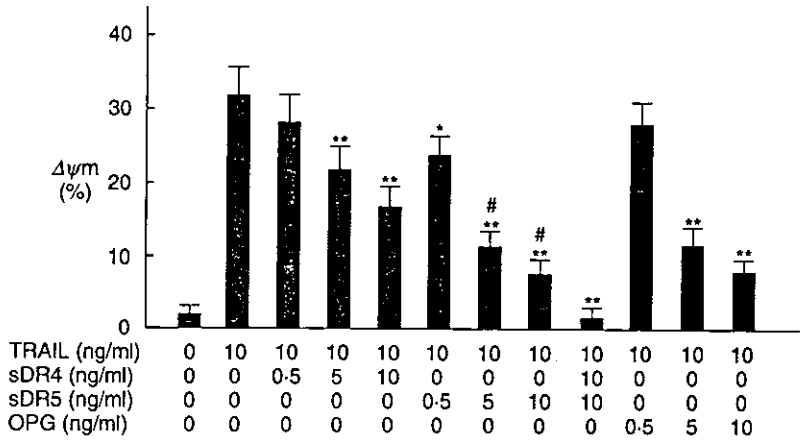

Fig. 3. Inhibition of TRAIL-mediated apoptosis in FLS by sDR4, sDR5 and OPG. FLS were cultured with 10 ng/ml of rTRAIL in the presence of varying concentrations of sDR4, SDR5 or OPG for 2 h. After incubation, apoptosis of FLS was quantified by $\Delta\Psi$m as described in the text. Note that TRAIL-mediated apoptosis in FLS was suppressed by sDR4, sDR5 and OPG; however, the inhibition was not complete. TRAIL-mediated apoptosis in FLS was inhibited completely by administration of both sDR4 and sDR5. *$P < 0·05$ *versus* FLS cultured with rTRAIL. **$P < 0·01$ *versus* FLS treated with rTRAIL. #$P < 0·05$ *versus* FLS treated with rTRAIL and sDR4. Data are the mean ± s.d. of four individual samples.

however, detailed experiments such as RNA interference for each TRAIL receptor are necessary to clarify the difference.

Recent studies reported the production of OPG in culture supernatants from rheumatoid FLS [16]. Thus, we focused on the regulatory role of inflammatory cytokines on OPG production by rheumatoid FLS. Our data suggest that OPG production in the rheumatoid microenviroment is, at least in part, positively regulated by IL-1β, which is consistent with recent observations by Ziokowska *et al.* [16]. The positive correlation between IL-6 and OPG protein concentration found in the rheumatoid synovial fluid may result from the inducible effect of IL-1β on the production of IL-6 [21], as the effect of IL-6 on OPG production by synovial cells was not found compared with IL-1β. A promising inhibitory role of OPG on osteoclastogenesis in murine adjuvant arthritis has been identified, which is achieved through decoy function of OPG for RANKL; however, treatment of the mice with OPG failed to improve the severity of synovial inflammation [22]. The present study has demonstrated that OPG is a functional inhibitor of TRAIL-induced apoptosis in FLS. Therefore, we speculate that the functional role of OPG on synovial cell growth is separate from its inhibitory action on osteoclastogenesis; the former is mediated through decoy function towards TRAIL while the latter through decoy function towards RANKL. Expression of TRAIL receptors on FLS was not affected by IL-1β treatment, and the sensitivity of TRAIL-mediated apoptosis was not suppressed directly by IL-1β; however, TRAIL-mediated apoptogenic activity towards FLS was inhibited significantly in IL-1β-stimulated FLS culture. The production of OPG from cultured FLS was augmented by IL-1β, thus IL-1β may inhibit primarily TRAIL-induced apoptosis of FLS at death receptor level by OPG in a competitive manner. This is consistent with data from the blocking experiment, that neutralization of OPG produced from FLS by anti-OPG MoAb mainly restored TRAIL-mediated apoptogenic activity towards FLS. Although OPG protein concentration in culture supernatants from IL-1β-treated synovial cells was comparable to that inhibiting TRAIL-induced FLS apoptosis (compare Figs 3 and 4), the concentration was clearly higher than that in the synovial fluid of RA patients (compare Fig. 1 with Figs 3 and 4), which could not suppress TRAIL-

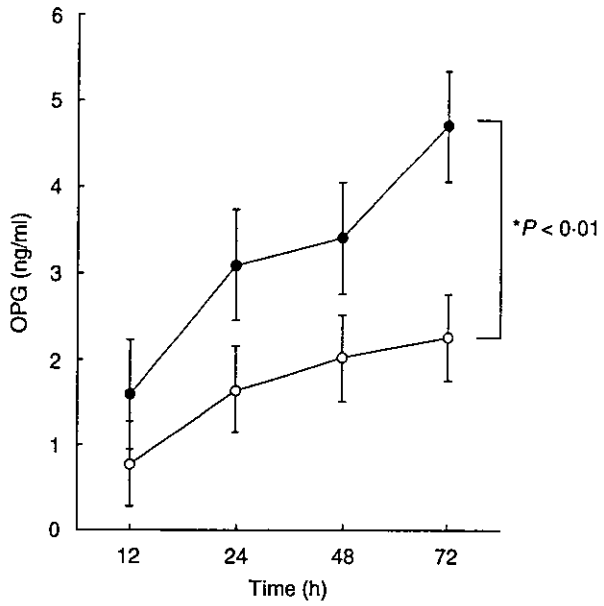

Fig. 4. IL-1β markedly stimulates OPG accumulation in culture supernatants of FLS. Rheumatoid FLS were cultured in the presence or absence of 20 IU/ml of IL-1β for indicated duration. After cultivation, OPG protein concentration in the culture supernatants was examined as described in the text. Open circles: unstimulated FLS, closed circles: IL-1β-treated FLS. *$P < 0.01$ *versus* unstimulated FLS. Data are mean ± s.d. of seven individual samples.

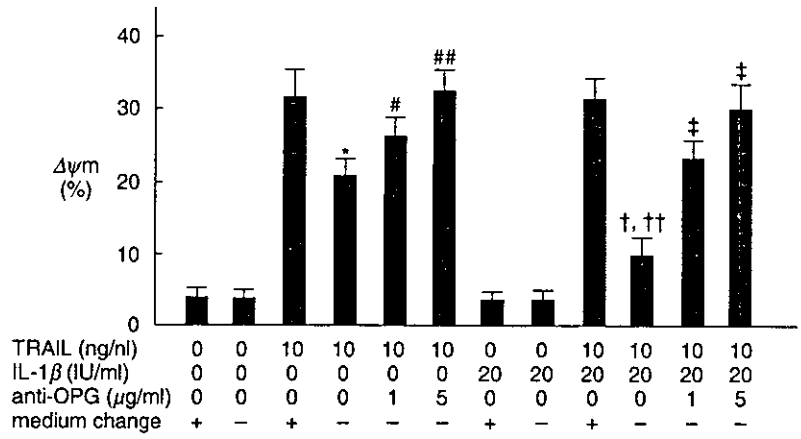

Fig. 5. OPG produced from FLS inhibits TRAIL-mediated apoptosis in FLS. FLS were cultured in the presence or absence of 20 IU/ml of IL-1β for 72 h with or without adding anti-OPG MoAb (1 μg/ml or 5 μg/ml). After cultivation, 10 ng/ml of rTRAIL was added to the culture with or without exchanging the culture media for an additional 2 h. After incubation, apoptosis of FLS was quantified by $\Delta\Psi$m as described in the text. Note that the sensitivity of FLS to TRAIL-mediated apoptosis was reduced when rTRAIL was added without exchanging the culture media, which was found significantly in IL-1β-treated FLS compared with unstimulated FLS. Furthermore, that inhibition was mainly restored by adding anti-OPG MoAb. Anti-OPG MoAb was mouse IgG1, thus 5 μg/ml of control mouse IgG1 (MBL) was used for negative control (0 μg/ml of anti-OPG MoAb means the addition of 5 μg/ml of control mouse IgG1). *$P < 0.01$ *versus* unstimulated FLS treated with rTRAIL with exchanging culture media. #$P < 0.05$. ##$P < 0.01$ *versus* unstimulated FLS treated with rTRAIL without exchanging culture media. †$P < 0.01$ *versus* IL-1β-stimulated FLS treated with rTRAIL with exchanging culture media. ††$P < 0.05$ *versus* unstimulated FLS treated with rTRAIL without exchanging culture media. ‡$P < 0.01$ *versus* IL-1β-stimulated FLS treated with rTRAIL without exchanging culture media. Data are the mean ± s.d. of four experiments.

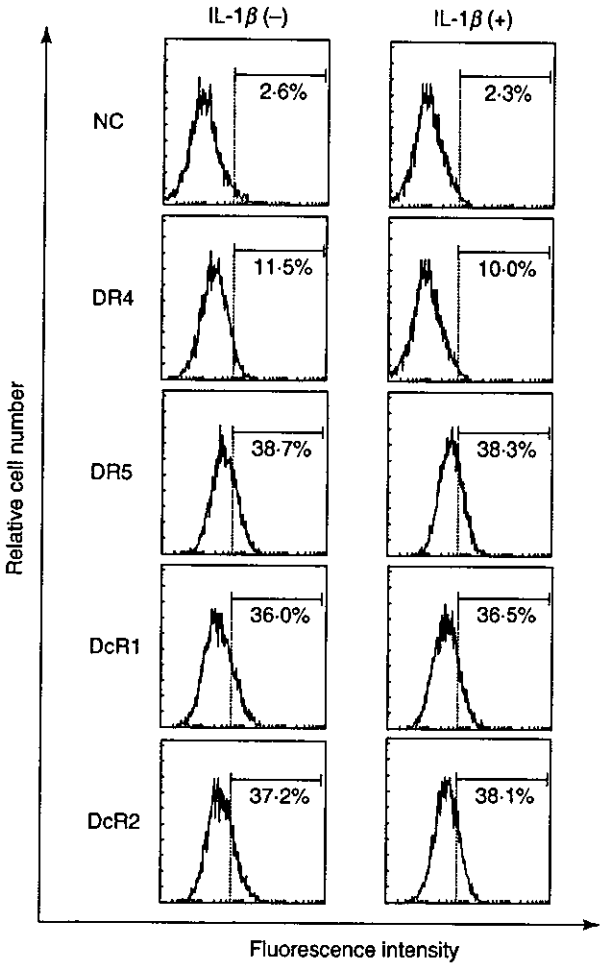

Fig. 6. IL-1β does not modulate the expression of DR4, DR5, DcR1 and DcR2 on FLS. Rheumatoid FLS, cultured in the presence or absence of rIL-1β (20 IU/ml) for 72 h, were detached by adding 0·265 mM EDTA and reacted with anti-DR4, anti-DR5, anti-DcR1 or anti-DcR2 at 4°C for 30 min. The cells were incubated further with PE-conjugated antigoat IgG at 4°C for 30 min, and surface expression of DR4, DR5, DcR1 and DcR2 on FLS was examined by flow cytometer as described in the text. Note that the expression of DR4, DR5, DcR1 and DcR2 on FLS was not changed by IL-1β treatment. NC: negative control, stained with goat IgG instead of first antibody. Numbers are the percentage of positive cells. Results are representative data from five individual samples.

induced apoptosis in FLS (see Figs 1 and 3). OPG protein concentration in rheumatoid synovial fluid reported by other groups [15,16,23] was much higher than the present data. Factors responsible for the difference are unclear; however, the variation in clinical situations of the subjects and MoAb used might result in the difference. Recent investigations have shown the elevation of OPG in rheumatoid synovial fluid, compared with serum consentration [15]; however, other investigators demonstrated a relatively low OPG protein concentration in the rheumatoid synovial fluid compared with synovial fluid from osteoarthritis patients [23]. Further investigation is necessary to clarify the functional expression of OPG in synovial tissue of RA patients.

TRAIL is thought to be an inhibitor of synovial cell hyperplasia as the blockage of TRAIL signalling by sDR5 in type II collagen-induced arthritis in mice exacerbates the proliferation of synovial cells [24]. Although we have not examined the expression of other soluble receptor antagonists such as sDR4 and sDR5 in the culture supernatants of FLS, our present study indicates that OPG produced by synovial cells acts as an endogenous decoy receptor of TRAIL-induced apoptosis, and part of the growth-promoting activity of IL-1β may be achieved by overproduction of OPG to suppress the biological function of TRAIL.

ACKNOWLEDGEMENT

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A novel mutation (T61I) in the gene encoding tumour necrosis factor receptor superfamily 1A (*TNFRSF1A*) in a Japanese patient with tumour necrosis factor receptor-associated periodic syndrome (TRAPS) associated with systemic lupus erythematosus

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Objective. To identify potential mutations in the tumour necrosis factor receptor superfamily 1A gene (*TNFRSF1A*) in a Japanese female patient with recurrent fever complicated by systemic lupus erythematosus (SLE), and in her family members.
Methods. DNA sequencing of exons 1–10 of the *TNFRSF1A* gene was performed to determine mutations that might be associated with the tumour necrosis factor receptor-associated periodic syndrome (TRAPS). Moreover, the *TNFRSF1A* gene was examined in Japanese patients with autoimmune diseases, including SLE, rheumatoid arthritis (RA), mixed connective tissue disease (MCTD) and Behçet's disease, and in healthy Japanese controls. Enzyme-amplified sensitivity immunoassay (EASIA) analysis was used to assess serum levels of TNF, the 55-kDa TNF receptor (TNFRSF1A) and the 75-kDa TNF receptor (TNFRSF1B). Membrane TNFRSF1A expression was analysed on the surface of peripheral blood mononuclear cells by flow cytometry.
Results. A novel mutation, a heterozygous C to T transition in exon 3 which substitutes an isoleucine for a threonine at position 61 (T61I) was detected in the *TNFRSF1A* gene derived from the genomic DNA of a Japanese female TRAPS patient. Two nieces and one nephew, all with a similar clinical phenotype, also possessed the same *TNFRSF1A* mutation. We further demonstrated the same mutation in five of 60 SLE patients (8.3%) and in five of 120 healthy individuals (4.2%), with no significant differences. Although high titres of serum TNF and soluble TNFRSF1B protein were observed in this patient, low titres of soluble TNFRSF1A protein were detected. However, a defect in TNFRSF1A shedding *in vitro* was not observed in monocytes derived from this patient.
Conclusion. This is the first report of a TRAPS patient associated with SLE with a novel *TNFRSF1A* mutation (T61I).

KEY WORDS: TRAPS, SLE, Mutation, TNF receptor, *TNFRSF1A*, Hereditary periodic fever syndrome.

Tumour necrosis factor (TNF) is a pleiotropic cytokine that plays a crucial role in a wide variety of proliferative responses, inflammatory effects and immune responses [1]. TNF has the ability to bind two distinct TNF receptors, TNFRSF1A (also known as p55 TNFR, TNFRI, CD120a) and TNFRSF1B (also known as p75 TNFR, TNFRII, CD120b) [2–5]. TNFRSF1A is known to initiate the majority of TNF's biological activities [6]. The binding of TNFRSF1A triggers the release of the inhibitory protein silencer of death domains (SODD) and forms a receptor-proximal complex containing the adapter proteins, such as TNF receptor-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), receptor-interacting protein (RIP) and Fas-associated death domain (FADD). Engagement of TNF receptors results in the activation of two major transcription factors, nuclear factor κB (NF-κB) and c-Jun. These transcription factors induce the expression of genes that mediate diverse biological processes [2–6].

Tumour necrosis factor receptor-associated periodic syndrome (TRAPS) is an autosomal dominant inherited disease causing recurrent episodes of fever, myalgia, rash, abdominal pain and conjunctivitis [7–13]. Impaired shedding of TNFRSF1A should cause excessive TNF signalling, leading to a wide range of biological responses [7, 12, 14, 15]. Any ethnicity may be affected in this disease; however, there have been few reports to date in patients from the Orient [7, 12, 14–22]. In this study we report a Japanese female SLE patient with recurrent high fever without disease activity. Gene analysis demonstrated a new *TNFRSF1A* mutation in this patient and in members of her family.

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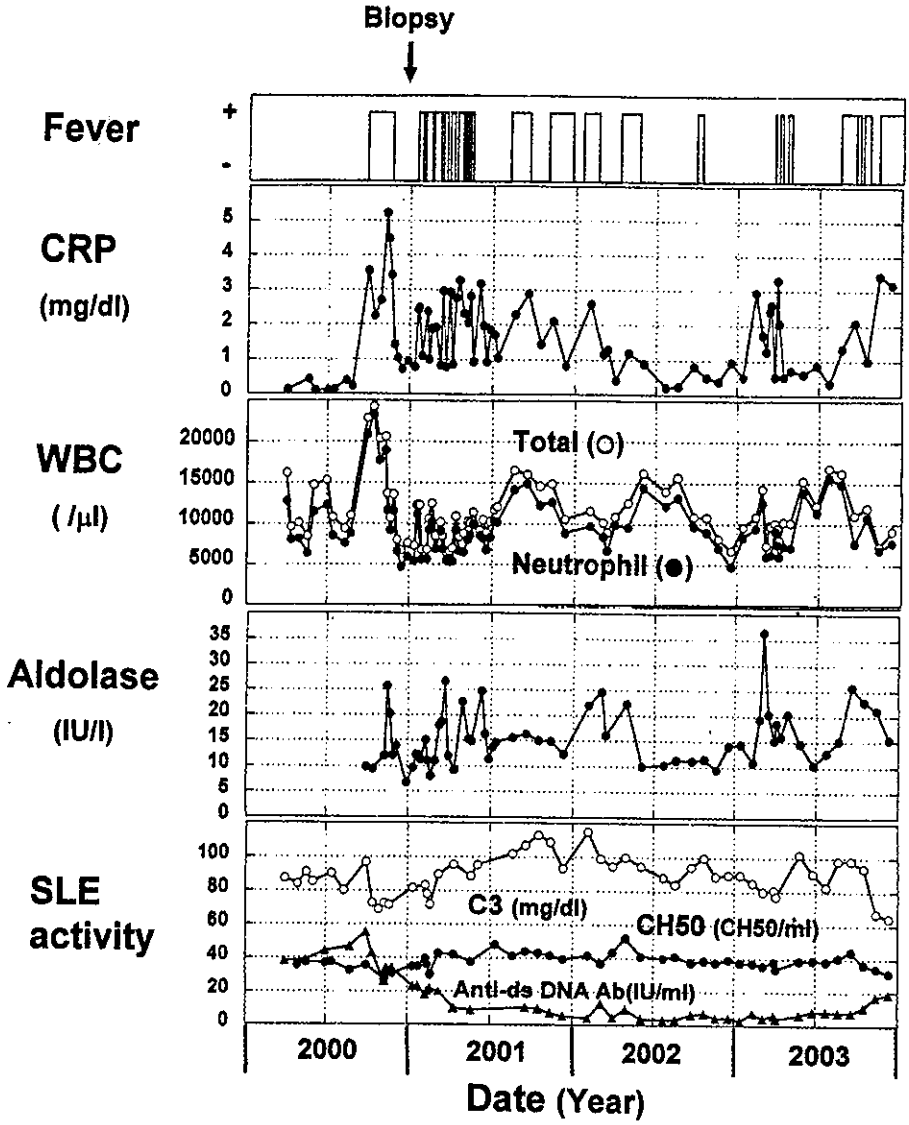

FIG. 1. Clinical course of the patient. Clinical symptom (fever) and clinical data were recorded after the onset of recurrent fever. Fever+ means fever over 38°C once a day. Clinical data include serum levels of CRP and aldolase, white blood cell count, number of neutrophils and SLE disease activity, which was evaluated by serum complement (CH50 and C3) and titres of anti-dsDNA antibodies. Normal range of CRP, WBC count, neutrophil count, aldolase, CH50, C3 and anti-dsDNA antibodies are <0.17 mg/dl, 3500–9000/μl, 1225–5850/μl, 2–7 IU/l, 20–60 CH50/ml, 65–135 mg/dl and <12 IU/ml, respectively.

Patients and methods

A Japanese family with recurrent fever

A 27-yr-old Japanese female patient was diagnosed with SLE at the age of 21 yr based on butterfly rash, fever, arthralgia, proteinuria, anaemia, antinuclear antibodies, a high titre of anti-double-stranded DNA (anti-ds DNA) antibodies (142.0 IU/ml), and low serum level of complement (CH50, 14.9 CH50/ml; C3, 33.0 mg/dl). After 3 yr of steroid therapy, recurrent fever appeared at the end of September, 2000 (Fig. 1). At this time, inflammatory signs were markedly elevated (high serum level of CRP and leucocytosis). In contrast, signs related to SLE, including butterfly rash, proteinuria, anaemia, high titres of anti-ds DNA antibodies and low levels of complement, were not observed in this period. The patient had undergone extensive diagnostic evaluation for infections, malignant and rheumatic aetiologies of her symptoms, with no positive findings. This led us to consider another aetiology for her illness. The patient also developed myalgia, arthralgia, a 'Still's rash' on the upper and lower extremities, and conjunctivitis during episodes of high fever. However, neither abdominal pain nor chest pain was present. Further examination demonstrated fasciitis in both thighs, detected by Ga scintigram, magnetic resonance imaging (MRI) (Fig. 2) [14, 23] and muscle and fascial biopsy (Fig. 3). CD68-positive cells were mainly infiltrated in fascia (data not shown), leading to the diagnosis of monocytic fasciitis [9, 24, 25]. We observed this patient carefully during more than 3 yr, and recurrent fever occurred repeatedly. Serum CRP and aldolase but not creatine phosphokinase (CPK) were elevated during periods of high fever (Fig. 1), suggesting that TRAPS symptoms resulted from the propagation of the fascial inflammation. Her family history demonstrated that two nieces (no. 11; 18 yr old; no. 17, 4 yr old) and 1 nephew (no. 14, 6 yr old) were also referred for recurrent fever (fever over 38°C lasting more than 7 days) with skin eruption (nos 11 and 17), arthralgia (no. 17) or abdominal pain (nos 11, 14 and 17), establishing the diagnosis of

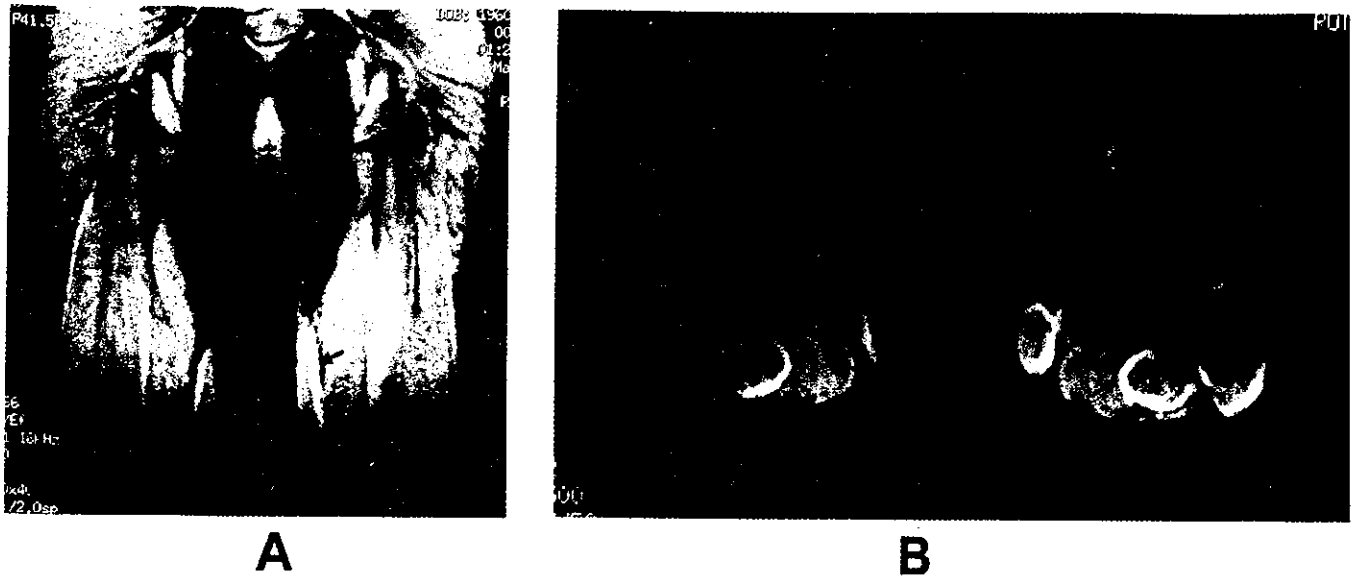

FIG. 2. Magnetic resonance imaging of the thigh. Sagittal (A) and coronal (B) views of the middle thighs of a patient with the T61I mutation using STIR magnetic resonance imaging,, demonstrating high-intensity signals in fasciâ (arrow).

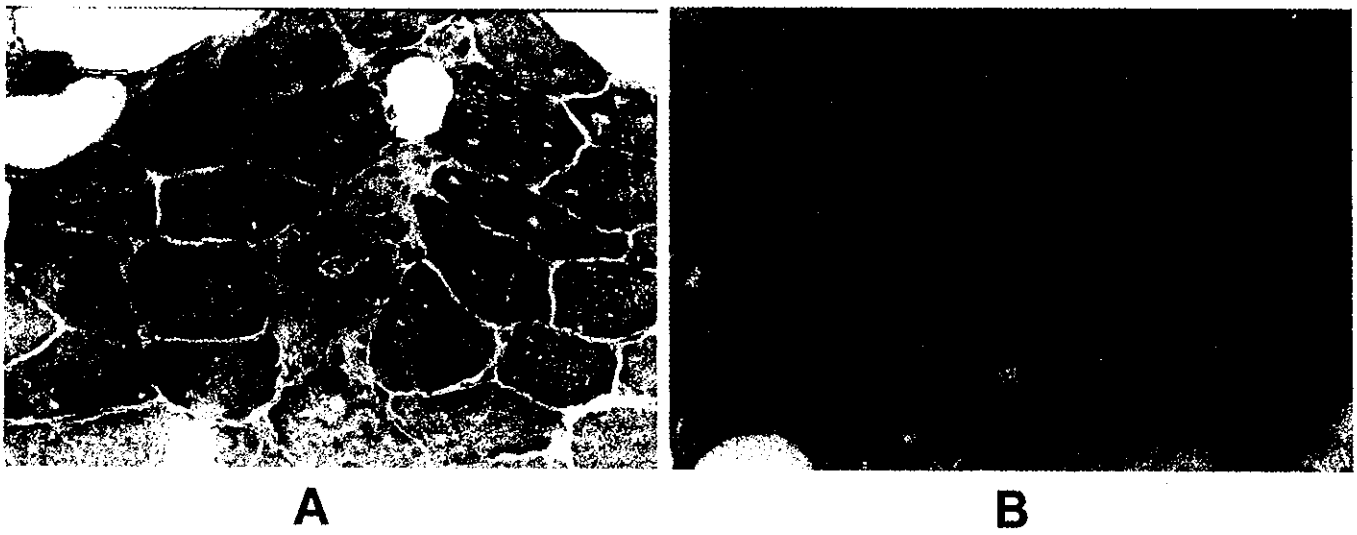

FIG. 3. Pathological findings in monocytic fasciitis. Cross-section of the frozen skeletal muscle biopsy samples from the right thigh, showing small numbers of infiltrated cells around endothelium, with well-preserved, intact myofibres and endomysium (A, H&E stained; magnification ×200). In contrast, the adjacent fascia was markedly inflamed with cells (B, H&E staining; magnification ×200).

TRAPS according to Hull's criteria [12]. They did not fulfil the criteria for SLE. Although the patient was from Nagasaki, her parents were not present in Nagasaki at the time of the atomic bomb detonation.

Japanese patients with autoimmune disease

We studied 154 patients diagnosed as having SLE (57 females, three males), RA (54 females, eight males), MCTD (16 females), and Behçet's disease (10 females, six males), as well as 120 healthy controls. All of the patients and controls were Japanese, and the patients were followed at our out-patient rheumatology facility at Nagasaki University Hospital.

Genomic DNA analysis

This study was approved by the ethics review board of Nagasaki University, and all participants gave their informed consent.

Genomic DNA was prepared from peripheral blood. Polymerase chain reaction (PCR) amplification of each of the 10 *TNFRSF1A*-coding exons was performed using oligonucleotide primer pairs specific for exons 1–10 of the *TNFRSF1A* gene [14]. The sequences of the primers were as follows: 1A 5′-CAGCACTGCCGCTGCCACAC-3′, 1B 5′-AGGTGCCTCGC CCACCAGCC-3′, 2A 5′-CCTCTCTTGATGGTGTCTCC-3′, 2B 5′-GCAGCACCCCAGACCTGAGG-3′, 3A 5′-GTGTTCT CACCCGCAGCCTA-3′, 3B 5′-CCCACACACCACTCAAGA CC-3′, 4A 5′-GAGGATGCAGGACTCATACCC-3′, 4B 5′-GG

AAGTGCCACCGCATGGG-3′, 5A 5′-TCACTTCCTCTGTC CTGTGG-3′, 5B 5′-TTGCCCAGCTAATGGTTCCC-3′, 6–7A 5′-CCAATGGTAGGGCCTCTGTT-3′, 6–7B 5′-CACCCATGT CCTCCCTCACC-3′ (exon 6, intron 6 and exon 7 were amplified as a single product), 8A 5′-AGCTGAGTCCAGGGTGCCAG-3′, 8B 5′-GAAAGTGAAGGATGATTCCAGG-3′, 9A 5′-TTCGCT GCCGCACTAATCTGC-3′, 9B 5′-CCCAGCCTCCTCGTCTC CAG-3′, 10A 5′-TGGGGTTGCCGCCCGAGGCT-3′ and 10B 5′-CATCTCGCAGGACGGTCCTTAG-3′. The PCR conditions were as follows: genomic DNA (50–100 ng), PCR buffer containing 10 mM Tris–HCl (pH 9.2), 50 mM KCl, and 1 mM $MgCl_2$, 10% DMSO, 0.01% gelatine, 200 mM deoxy-NTPs, 1 mM of each primer, 0.25 U AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA, USA). The cycling conditions were 5 min at 94°C followed by 32 cycles consisting of 45 s at 94°C, 30 s at 55°C and 45 s at 72°C. The PCR products were purified using a Microcon-100 filter (Amicon, Beverly, MA, USA) before both strands were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, PE Applied Biosystems). The reactions were analysed on an ABI Prism 3300 DNA Sequencer, as previously described [26].

Restriction analysis of the T61I mutation

Exon 3 of *TNFRSF1A* was PCR amplified in each member of the family, using primers 3A and 3B. The PCR products were digested by *Hph*I (MBI Fermentas, Lithuania) restriction enzyme at 37°C for 1 h and fragments were separated in 2.5% agarose gels stained with ethidium bromide. The PCR products with the T61I mutation produce two discrete fragments (163 and 51 b.p.), whereas those with no T61I mutation produce three fragments (107, 56 and 51 b.p.).

HLA-A, B, C typing and HLA-DRB1, DQB1 typing

In our TRAPS patient, HLA typing for antigens of class I (HLA-A, B and C) was performed with the lymphocyte microcytotoxicity test [27]. DNA typing of the HLA class II gene (HLA-DRB1 and DQB1) was performed with the PCR-SSO (sequence-specific oligonucleotide) method [28, 29].

Measurement of soluble TNF receptors and TNF

Soluble TNFRSF1A (p55), soluble TNFRSF1B (p75) and TNF were measured in the serum using a solid-phase enzyme-amplified sensitivity immunoassay (EASIA) performed in microtitre plates according to the manufacturer's instructions (Biosource, Nivelles, Belgium), as previously described [30]. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies directed against distinct epitopes of soluble TNFRSF1A, soluble TNFRSF1B or TNF are employed, resulting in highly sensitive detection. We examined 33 serum samples from our TRAPS patient, and serum from a 16-yr-old male with SLE (before and after therapy), a 19-yr-old female with virus-associated haemophagocytic syndrome (VAHS) (before and after therapy), and 13 normal individuals. Each sample was examined in duplicate and data were calculated as the mean.

Flow cytometry histograms of TNFRSF1A expression in monocytes after PMA and Ionomycin stimulation

Peripheral lymphocytes derived from a TRAPS patient and an SLE patient were purified by centrifugation over Ficoll–Hypaque. The lymphocytes were incubated with 10% fetal calf serum (FCS) RPMI in 5% CO_2 at 37°C for 15 min, then phorbol 12-myristate 13-acetate (PMA) (final concentration 20 ng/ml; Sigma, St Louis, MO, USA) and ionomycin (final concentration 500 ng/ml; Sigma) were added to the media. After incubation for 10 or 60 min, lymphocytes were harvested and washed once with cold washing buffer [phosphate-buffered saline (PBS) containing 2% FCS and 0.1% azide sodium]. The dual immunofluorescence analysis method has been described in detail elsewhere [30]. Briefly, cell pellets were incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-TNFRSF1A monoclonal antibody (mAb) (R&D, Minneapolis, MN, USA) and PC5-coupled anti-CD14 mAb (Beckman Coulter, Hialeah, FL, USA) or a FITC-conjugated anti-mouse IgG1 mAb (R&D) and PC5-coupled anti-CD14 mAb for 45 min at 4°C. After incubation, the cells were washed once with cold washing buffer. The dual-immunofluorescence experiments were analysed with a flow cytometer (Epics XL; Beckman Coulter).

Immunohistochemical analysis of tissues

Biopsy samples were fixed in 4% paraformaldehyde in PBS and were immersed successively in 10, 15 and 20% sucrose. Tissues were then frozen in liquid nitrogen and stored at −80°C until use. Tissue sections (4 mm thick) were cut and mounted on glass slides precoated with aminopropyltriethoxysilane. Dried cryostat sections of snap-frozen tissues were fixed with a mixture of methanol and acetone (vol/vol, 1/1) for 10 min on ice. Endogenous peroxide activity was inhibited by immersing the section in 3% H_2O_2. After blocking with 10% mouse IgG for 10 min, sections were incubated with anti-CD68 mAbs (Dako, Carpinteria, CA, USA), anti-CD3 mAbs (Becton Dickinson, Mountain View, CA, USA) or anti-CD19 mAbs (Becton Dickinson) in PBS containing 1% fetal bovine serum (FBS) for 60 min in a humidified chamber at room temperature. These sections were washed with PBS containing 0.25% Brij and then stained using the streptavidin–biotin method (Histofine staining kit, Nichirei, Tokyo, Japan) [31]. Haematoxylin and eosin (H&E) staining was performed using dried cryostat sections of snap-frozen tissues after fixation.

Statistical analysis

Data were analysed with Fisher's exact probability test. $P < 0.05$ was considered statistically significant.

Results

Mutation analysis of the TNFRSF1A gene and pedigree of a TRAPS family

Genomic DNA analysis identified a single mutation, a heterozygous C to T transition in exon 3, which substitutes an isoleucine for a threonine at position 61 (T61I) (Fig. 4A). No other mutations were observed in exons 1–10 of the *TNFRSF1A* gene using DNA sequencing analysis. The T61I mutation destroys an *Hph*I restriction site determined by DNA strider 1.2. Two nieces and one nephew, all with a similar clinical phenotype, also possessed the same *TNFRSF1A* mutation, which was monitored by *Hph*I (data not shown). Another five family members have the same mutation in the absence of TRAPS symptoms (Fig. 4B). This mutation was also confirmed by DNA sequencing analysis. As this patient (no. 8) also met diagnostic criteria for SLE, we examined whether the T61I polymorphisms was commonly associated with SLE. Surprisingly, this mutation was detected in another five SLE Japanese patients out of 60 analysed (8.3%), whereas no change of T61I was seen in other patients with RA, MCTD or Behçet's disease. As the same mutation was also found in five healthy Japanese individuals out of 120 (4.2%), we could not detect significant differences ($P = 0.305$) between SLE and healthy controls in the percentage of T61I mutation.

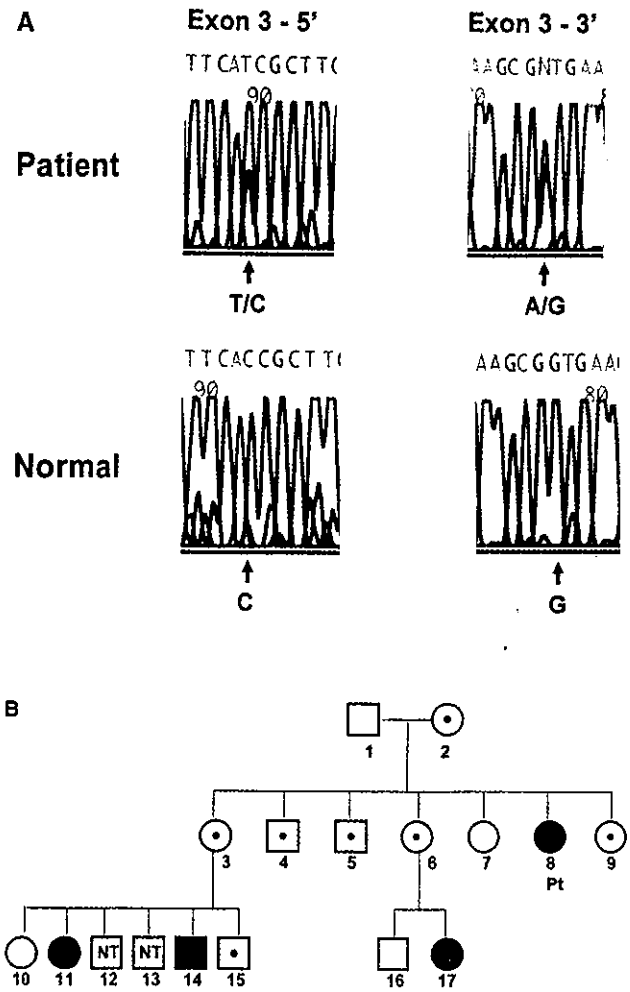

FIG. 4. Detection of *TNFRSF1A* mutation in the family. (A) Sequence analysis of exon 3 in the patient and a normal individual. The arrow points to the heterozygous C → T transition, resulting in a threonine-to-isoleucine substitution in the upper panel. (B) Pedigree of the family. The T61I mutation (square or circle with a black point) was confirmed by DNA sequencing analysis. The genomic DNA from two family members (nos 12 and 13) could not be provided (NT). Shaded symbols indicate the existence of TRAPS symptoms (nos 8, 11, 14 and 17).

HLA typing

For the haplotype analysis, our TRAPS patient possessed the following MHC class I antigens: HLA A24, A31, B39, B60, CW3 and CW7. For MHC class II antigens, DRB1*1501, DRB1*1101, DQB1*0602 and DQB1*0301 were detected. As HLA-B39, DRB1*1501, DRB5*0101 and DQB1*0602 have been reported to be susceptibility genes for SLE in Japanese patients [32, 33], this patient possessed three of four susceptibility genes.

Determination of soluble TNFR and TNF levels

To examine whether the T61I mutation in *TNFRSF1A* alters the shedding of soluble TNFRSF1A, we measured the serum level of soluble TNFRSF1A in this patient compared with the serum level of TNF and soluble TNFRSF1B using EASIA. We stored 33 samples of her sera during a 3 yr period and measured TNF, soluble TNFRSF1A and soluble TNFRSF1B in each sample. Moreover, we evaluated these proteins in sera from patients with active SLE and severe VAHS before and after therapy. Figure 5 shows that high titres of TNF were persistently detected in her sera during our clinical observations, and these data were correlated with high titres of TNFRSF1B. However, the titre of soluble TNFRSF1A was very low compared with sera from each SLE or VAHS patient whose disease was active, or was similar to normal controls (shaded area).

Expression of TNFRSF1A on monocytes from patients after PMA and ionomycin stimulation

As serum concentrations of the soluble form of TNFRSF1A are low, we assayed for defects in receptor shedding from monocytes. Peripheral lymphocytes derived from a TRAPS patient and a patient with SLE without the T61I mutation (control) were stimulated with PMA and ionomycin for 10 or 60 min, then stained with anti-TNFRSF1A mAb and anti-CD14 mAb. Figure 6 shows that normal shedding was observed in CD14-positive cells (monocytes) from both a TRAPS patient and a patient with SLE without T61I mutation, suggesting that abnormal shedding does not account for the low concentrations of the soluble form of the TNFRSF1A in sera from this TRAPS patient.

Discussion

A critical feature of this case was the presence of high fevers in the absence of SLE disease activity. During high fever (after at the end of September 2000), inflammatory disease signs were increased, despite normal levels of serum complement, leading us to consider another aetiology for her recurrent fever (rather than SLE flare). Periodic high fever appeared in association with monocytic fasciitis in both thighs, as determined by Ga scintigram and MRI (Fig. 2) [14, 23] and confirmed by immunohistological studies (CD68-positive cells were seen to infiltrate into fascia; data not shown) [9, 24, 25]. In spite of high titres of TNF and soluble TNFRSF1B, low levels of soluble TNFRSF1A were found in sera during our observation period (Fig. 5). A novel mutation (T61I) was detected in the *TNFRSF1A* gene derived from the genomic DNA of this patient. This is the first case of a TRAPS patient associated with SLE with novel *TNFRSF1A* mutation.

Many TRAPS families have been reported, and more than 20 different missense mutations, including novel T61I mutations, have been detected between exon 2 and exon 4 in the *TNFRSF1A* gene [10, 12], although a novel mutation (I199N) which locates in exon 6 was recently reported, and this portion might be involved in receptor shedding [34]. These portions of the receptor encode the cleavage sites in TNFRSF1A for the metalloprotease, TNF-α converting enzyme (TACE) [35–37]. TACE is known to be responsible for generating the soluble homotrimeric form of TNF and the shedding of TNFRSF1A [35, 37]. Although low titres of soluble TNFRSF1A were detected in our patient's serum, the function of TACE should be intact because TNF production was retained. Many investigators have reported a receptor shedding defect (i.e. H22Y, C30S, C33G, P46L, T50M and C52F mutations) in TRAPS patients [10–12]. Evidence for this includes lack of shedding of soluble TNFRSF1A, as detected by enzyme-linked immunosorbent assays (ELISA), and absence of alteration of TNFRSF1A expression on monocytes and neutrophils after PMA stimulation, as determined by flow cytometry [7, 14, 15]. In our case, although very low titres of soluble TNFRSF1A were detected in her sera during the observation period, the changes in titre of soluble TNFRSF1A correlated with the alterations of titre of TNF and soluble TNFRSF1B (Fig. 5), suggesting that TNFRSF1A was cleaved in response to TNF. This result was supported by experiments showing that normal shedding of TNFRSF1A was

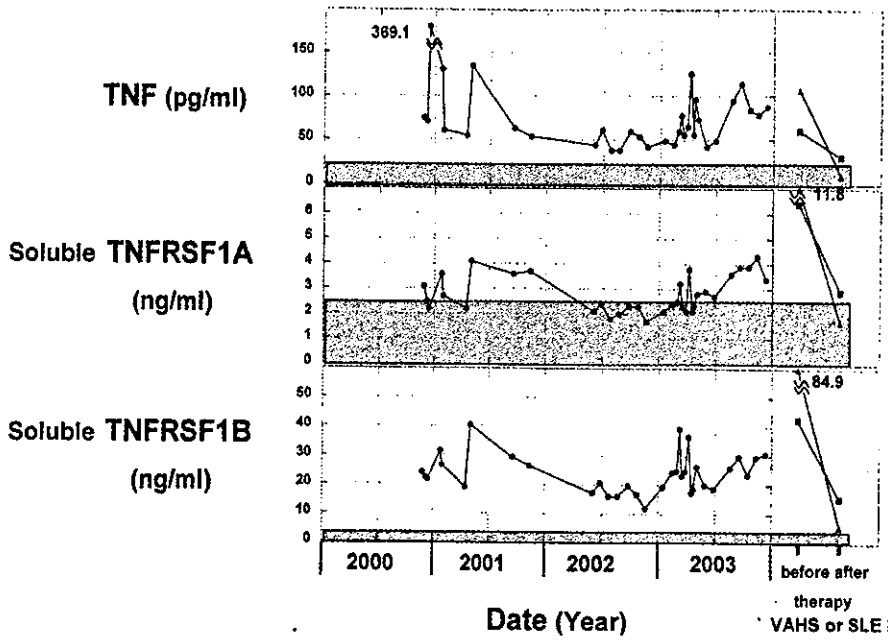

FIG. 5. Changes in TNF, soluble TNFRSF1A and soluble TNFRSF1B levels in sera from the TRAPS patient (closed circles). Each serum level in active SLE (closed squares) and severe VAHS (closed triangles) before and after therapy is shown on the right side of the graph. Average ± 2 S.D. in normal controls ($n = 13$; TNF, 10.5 ± 10.4 pg/ml; soluble TNFRSF1A, 1.2 ± 1.1 ng/ml; soluble TNFRSF1B, 3.0 ± 1.3 ng/ml) is indicated by the shaded area in each column.

observed in monocytes from this patient (Fig. 6). It has been reported that the changes of TNFRSF1A expression on cells in TRAPS patients harbouring the R92Q mutation are the same as the alteration of TNFRSF1A expression in normal individuals [10, 12]. This suggests that defective receptor shedding does not account for the entire pathophysiological mechanism underlying TRAPS. This family study and the high prevalence in the general population show that the T61I mutation has low penetrance, resembling the R92Q mutation [10, 12, 13, 21]. Moreover, a defect in TNFRSF1A shedding *in vitro* was not observed in monocytes derived from this patient (T61I), as also observed for patients with the R92Q mutation. Recent reports demonstrate that abnormal shedding accounts for a minority of TRAPS patients with the receptor defect, and there is a defect of receptor shedding from monocytes in some families with no *TNFRSF1A* mutations, indicating that the TRAPS clinical phenotype has a heterogeneous aetiology [13, 21]. After the appearance of TRAPS symptoms, serum levels of soluble TNFRAF1A remained low, whereas serum levels of TNF remained high in this patient (Fig. 5). TNFRSF1A is expressed on the cell surface but large amounts are found localized at the perinuclear–Golgi complex [38]. One possible reason for the low level of soluble TNFRSF1A is that the T61I mutation influences the transport of TNFRSF1A from the Golgi to the cell surface, leading to a low level of TNFRSF1A expression on cells, which correlates with the observed low level of soluble TNFRSF1A in serum after normal cleavage in response to TNF. Another possibility is that the T61I mutation interferes with TNFRSF1A protein folding and/or trimerization, resulting in low expression of TNFRSF1A. Further experiments comparing patients with the mutation with healthy individuals will be required to differentiate between these possibilities.

It is very important to know whether *TNFRSF1A* mutations contribute to the development of autoimmune diseases. Our patient was diagnosed with SLE, fulfilling six of the criteria from the revised ACR criteria. We speculate that monocytic fasciitis first appeared in this patient around September 2000; TRAPS symptoms might have arisen in response to TNF production, probably from fascia in both thighs. Hashimoto *et al.* reported SLE-associated MHC markers in Japanese patients with SLE,

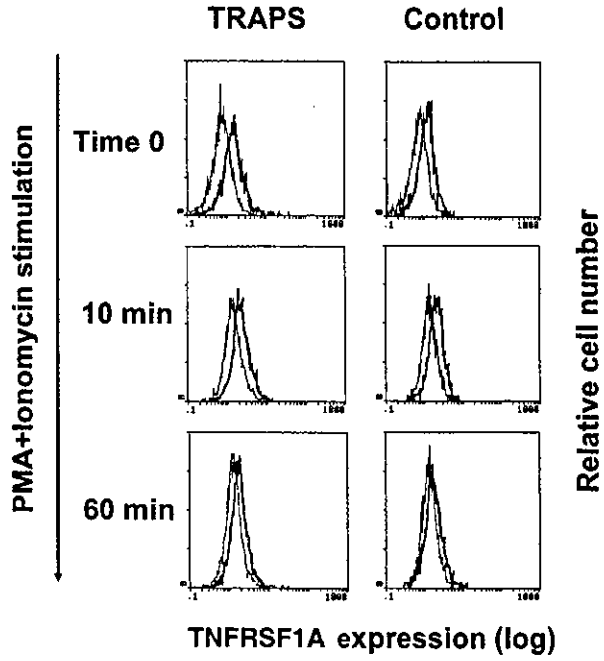

FIG. 6. Flow cytometry histograms of TNFRSF1A expression in monocytes after PMA and ionomycin stimulation. Peripheral lymphocytes derived from a TRAPS patient (TRAPS) and an SLE patient without the T61I mutation (Control) were stimulated with PMA (final concentration 20 ng/ml) and ionomycin (final concentration 500 ng/ml). After incubation for 10 or 60 min, lymphocytes were stained with an FITC-conjugated anti-TNFRSF1A mAb or an FITC-conjugated anti-mouse IgG1 mAb with PC5-coupled anti-CD14 mAb. The dual-immunofluorescence experiments were analysed with a flow cytometer. Monocytes (CD14-positive cells) were analysed for TNFRSF1A expression (thicker line in each panel). The negative control is depicted as a thinner line in each panel.

with significantly more SLE patients expressing HLA-B39, DRB1*1501, DRB5*0101 and DQB1*0602 than normal controls in Japan [32, 33]. As our TRAPS patient was found to possess three of four susceptibility genes for SLE, she is at higher risk of developing SLE. Aksentijevich *et al.* speculated that R92Q might have a broader influence on susceptibility to inflammation in early arthritis [10]. We reviewed the charts of five SLE patients with the T61I mutation, and demonstrated that four of the five patients suffered from severe complications which included lupus nephritis (WHO type V) with nephrotic syndrome, lupus nephritis (WHO type V) with haemophagocytic syndrome, autoimmune hepatitis with liver cirrhosis, and interstitial pneumonia with chronic thyroiditis. Interestingly, the T61I mutation exists in 8.3% of SLE patients, whereas T61I was not detected in patients with RA. Nevertheless, no significant differences were detected between SLE and healthy controls in the percentage of the T61I mutation. From these points of view, T61I could influence a generalized pro-inflammatory effect for autoimmune diseases by analogy to R92Q, rather than an additive effect to those provided by HLA-B39, DRB1*1501, DRB5*0101 and DQB1*0602 for susceptibility to SLE. Further investigation of a larger patient and control set for the T61I mutation is clearly needed for determining whether *TNFRSF1A* mutations contribute to the pathogenesis and development of autoimmune diseases.

Rheumatology	Key messages
	• This is the first report of a TRAPS patient associated with SLE with a novel *TNFRSF1A* mutation (T61I).

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The authors have declared no conflicts of interest.

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Anti-apoptogenic function of TGFß1 for human synovial cells: TGFß1 protects cultured synovial cells from mitochondrial perturbation induced by several apoptogenic stimuli

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