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Cytokines regulate fibroblast-like synovial cell differentiation to adipocyte-like cells

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Objectives. **Our recent work showed that fibroblast-like synovial cells (FLS) could differentiate into adipocyte-like cells *in vitro* in response to stimulation with peroxisome proliferator-activated receptor γ (PPARγ) ligand. The aim of the present study was to determine the role of cytokines in the regulation of FLS differentiation to adipocyte-like cells.**
Methods. **FLS isolated from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) and from normal synovial tissues were incubated with the synthetic PPARγ ligand troglitazone to induce adipocyte-like differentiation of the cells.**
Results. **Production of interleukin (IL)-6, IL-8 and matrix metalloproteinase-3 was reduced in adipocyte-like cells compared with FLS. DNA binding activity of nuclear factor κB (NF-κB) was clearly inhibited in adipocyte-like cells. Cultivation of FLS with interferon γ (IFN-γ), tumour necrosis factor-α (TNF-α) or IL-1β inhibited the expression of PPARγ as well as CCAAT/enhancer binding protein (C/EBP) nuclear activity, and thus suppressed adipocyte-like cell differentiation *in vitro*.**
Conclusion. **Our results indicate the importance of PPARγ and C/EBP in adipocyte-like cell differentiation of FLS and that the process is influenced by inflammatory cytokines, and suggest that the proinflammatory character of FLS in patients with RA is diminished during adipocyte-like cell differentiation.**

KEY WORDS: Adipogenesis, Cytokines, NF-κB, PPARγ, CCAAT/enhancer binding protein.

Hyperplasia of synovial tissues is the pathologic hallmark of rheumatoid arthritis (RA). Proliferating synovial cells produce a number of cytokines and proteases, leading to joint destruction [1, 2]. However, the mechanisms that alter the functions of synovial cells are not yet fully understood.

Our recent work has demonstrated the expression of peroxisome proliferator-activated receptor γ (PPARγ) in cultured fibroblast-like synovial cells (FLS) and revealed a notable characteristic of FLS: these cells can differentiate *in vitro* into adipocyte-like cells in response to stimulation with the PPARγ ligand troglitazone [3]. Since the gene expression pattern of FLS is similar to that of mesenchymal stem cells [4], FLS, acting as mesenchymal stem cells, might therefore exist as a progenitor cell population in the joints that can differentiate into multiple cell lineages during skeletal tissue regeneration or repair.

PPARγ is a nuclear transcriptional factor known to be essential for adipocyte differentiation from murine 3T3-L1 preadipocytes, in conjunction with another class of nuclear factor, CCAAT/enhancer binding protein (C/EBP) [5–8]. On the other hand, other investigators have demonstrated that PPARγ can inhibit cytokine synthesis [9] and that PPARγ can stimulate apoptotic cell death, including that of FLS [9, 10]. These results indicate that PPARγ is a multifunctional factor and that PPARγ stimulation could be beneficial for the treatment of inflammatory diseases, including RA. In fact, Kawahito *et al.* [10] have shown that administration of PPARγ ligand suppresses adjuvant arthritis in rats by induction of apoptosis.

The present study was designed to determine the importance of PPARγ and C/EBP in the differentiation of FLS and the role of inflammatory cytokines in this process. We first showed that adipocyte-like cell differentiation induced by PPARγ ligand was associated with suppression of nuclear factor κB (NF-κB) activity in FLS, with consequent reduction of production of interleukin (IL) 6, IL-8 and matrix metalloproteinase-3 (MMP-3). We next focused on cytokine regulation in PPARγ-mediated adipogenesis and showed that FLS differentiation into adipocyte-like cells in response to PPARγ ligand was suppressed in the presence of interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) or IL-1β, by inhibiting the expression of PPARγ and C/EBP nuclear activity in FLS. Our data demonstrated cross-talk between PPARγ and cytokine signalling pathways in FLS, and may flag a new therapeutic application of PPARγ signalling in RA.

Materials and methods

Reagents

Troglitazone, a synthetic PPARγ ligand, was kindly provided by Sankyo (Tokyo, Japan). Rabbit polyclonal antibody against human PPARγ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human IFN-γ was kindly provided by Shionogi (Osaka, Japan). Human recombinant TNF-α and IL-1β were purchased from R & D Systems (Minneapolis, MN, USA).

Isolation of fibroblast-like synovial cells

We obtained synovial tissue specimens from patients with RA ($n = 12$) who met the American College of Rheumatology criteria for the disease [11], patients with osteoarthritis (OA, $n = 5$) and traumatic patients without arthritis ($n = 4$) at the time of orthopaedic surgery in the National Ureshino Hospital between April 2000 and November 2002. Informed consent was obtained

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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.

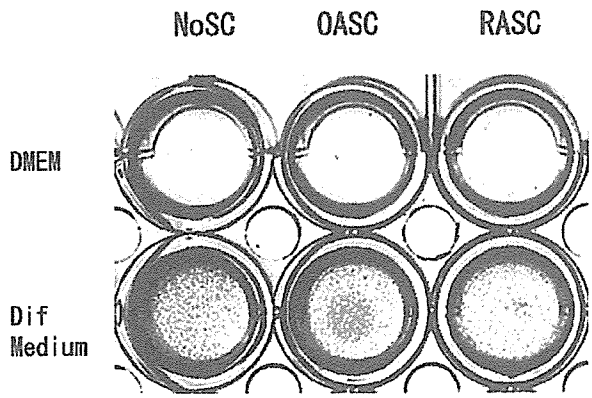

FIG. 1. Adipocyte-like cell differentiation of FLS in response to troglitazone. Three weeks of cultivation with troglitazone (10 μM) induced adipocyte-like differentiation of FLS derived from normal synovial tissues (NoSC), OA (OASC) and RA (RASC). Intracellular lipid accumulation was observed by Oil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

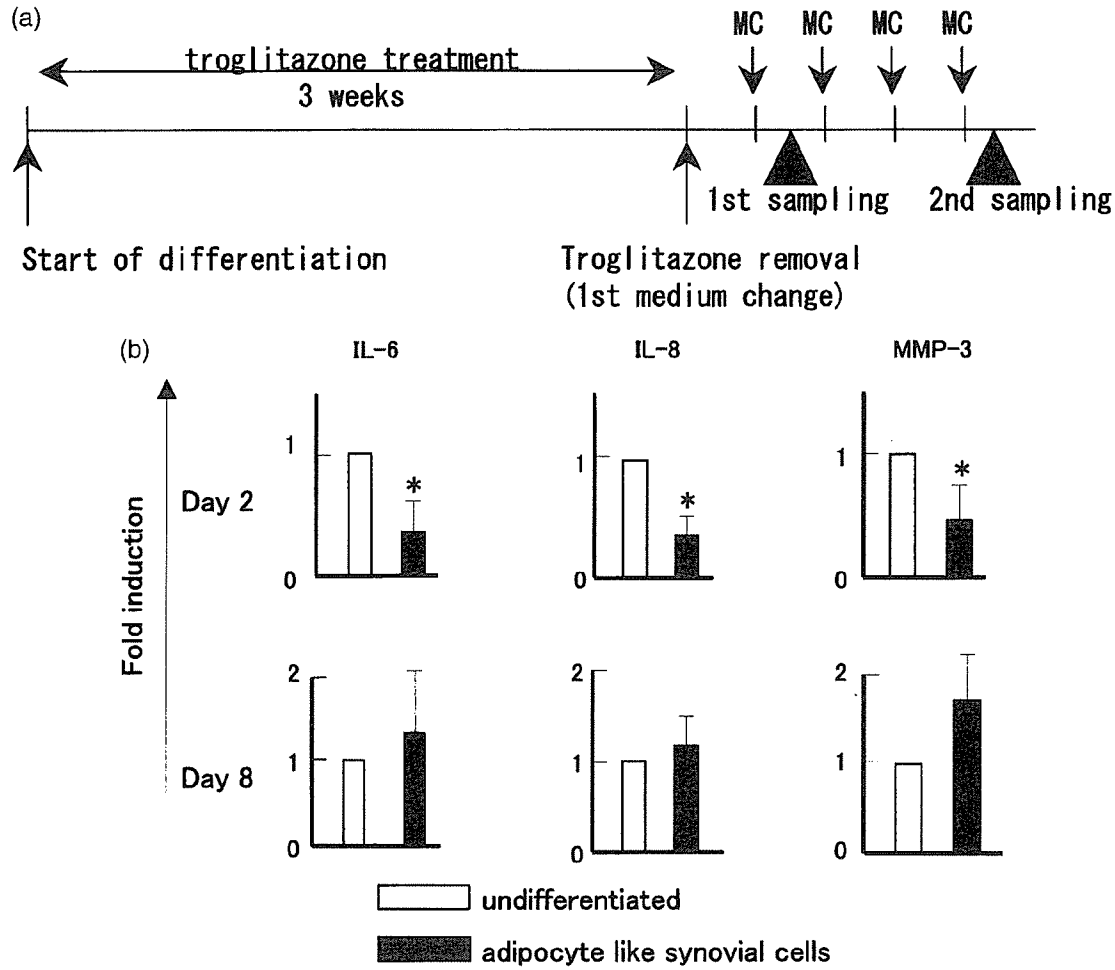

FIG. 2. Low IL-6, IL-8 and MMP-3 production after adipocyte-like cell differentiation. (a) Protocol for sample collection for ELISA. Troglitazone was removed from the culture medium after 3 weeks of induction of adipocyte-like differentiation of FLS. Troglitazone-free medium was changed every 48 h (MC). 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. $*P < 0.01$.

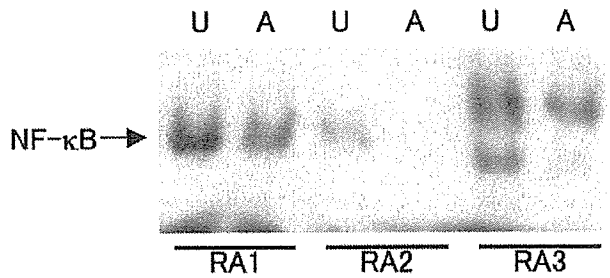

FIG. 3. Decrease in NF-κB DNA binding activity in FLS after adipocyte-like cell differentiation. Nuclear proteins were extracted from undifferentiated FLS and troglitazone-differentiated adipocyte-like cells, and NF-κB DNA binding activity was examined by EMSA. After 3 weeks of cultivation with troglitazone, 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

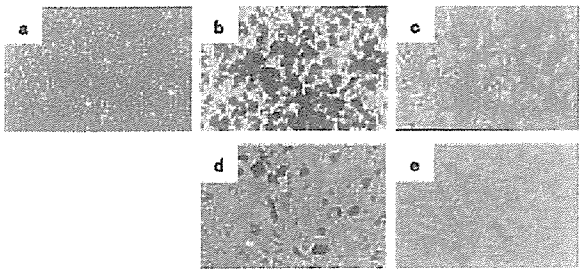

FIG. 4. Inhibition of adipocyte-like cell differentiation from FLS by TNF-α, IL-1β and IFN-γ. FLS were cultured with troglitazone for 3 weeks in the presence or absence of TNF-α (200 IU/ml), IL-1β (20 IU/ml) or IFN-γ (500 IU/ml). After cultivation, adipocyte-like cell differentiation was examined by 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, ×40.

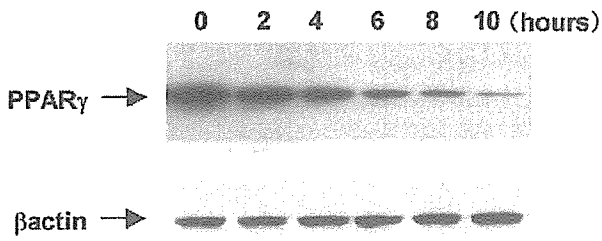

FIG. 5. Inhibition of PPARγ expression in FLS by IFN-γ. FLS isolated from RA patients were cultured with IFN-γ (500 IU/ml) for the indicated times and PPARγ expression was examined as described in the text. Results shown are representative data from four experiments. β-Actin was assayed as an internal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

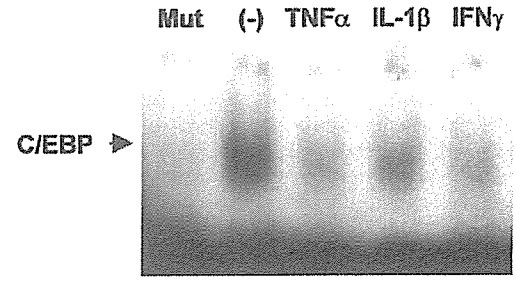

FIG. 6. Inhibition of C/EBP DNA binding activity in FLS by TNF-α, IL-1β and IFN-γ. FLS from RA patients were cultured in the presence or absence of TNF-α (200 IU/ml), IL-1β (20 IU/ml) or IFN-γ (500 IU/ml) for 12 h, nuclear proteins were extracted, and DNA binding activity of C/EBP was examined by EMSA. 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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Lipopolysaccharide signaling induces serum amyloid A (SAA) synthesis in human hepatocytes in vitro

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Abstract To investigate the role of lipopolysaccharide (LPS) in hepatocyte activation, we examined the expression of Toll-like receptor 4 (TLR4), the putative receptor for LPS in human hepatocytes. TLR4 mRNA and protein expression was confirmed in human hepatocytes. Stimulation of human hepatocytes with LPS results in rapid degradation of IkappaB-α and mitogen activated protein kinase activation. Human hepatocytes stimulated by LPS produced serum amyloid A protein. Our data suggest that human hepatocytes utilize components of TLR4 signal transduction pathways in response to LPS and these direct LPS-mediated effects on hepatocytes may contribute to liver inflammation and injury.

Keywords: Hepatocyte; Lipopolysaccharide; Toll-like receptor; Serum amyloid A protein

1. Introduction

Bacterial lipopolysaccharide (LPS), an essential component of the outer membrane of gram-negative bacteria, provokes a generalized proinflammatory response in the infected host that leads to septic shock and multiple organ failure [1]. LPS also causes liver injury [2]. The liver is an immunocompetent organ that plays a key role in the innate immune responses to pathogens [3]. The liver produces both inflammatory mediators and acute-phase reactants and functions to remove pathogens and microbial products from the blood [4]. Although hepatocytes have been reported to respond to LPS, the mechanisms by which LPS stimulates human hepatocytes remain uncertain. It has been suggested that Kupffer cells, as well as other macrophage populations, are responsive to LPS and produce TNF-α and IL-1 that activate hepatocytes [5]. Recently, Toll-like receptors (TLRs), several mammalian *Toll* homologues, have been identified and shown to play important roles in the recognition of various bacterial components [6]. In humans, the TLR family consists of 10 members, all of which are involved in the recognition of pathogen-associated molecular patterns [7]. Among these, Toll-like receptor 4 (TLR4) has been shown to be critical for LPS and endotoxin signaling [8]. TLR receptors have been identified on monocytes, macrophages, Kupffer cells and dendritic cells, and LPS binds to Kupffer cells via TLR4 [9]. LPS activation of Kupffer cells leads to upregulation of inflammatory cytokines, which are involved in liver damage [10]. Therefore, the general view is that the Kupffer cell is the major target of LPS in the liver.

Serum amyloid A protein (SAA) is a major acute-phase protein (APP) produced in the liver after various insults such as infection and inflammation [11]. SAA is an apolipoprotein that belongs to class 1 APP in which IL-1 and IL-6 are the main inflammatory mediators involved in its transcriptional induction [12]. Multiple *cis*-acting elements, including C/EBP and NF-κB, have been found to be important for SAA genes [13]. LPS injection in rabbit results in the activation of C/EBP and NF-κB, which may be responsible for LPS-induced SAA induction [14]. NF-κB and NF-IL6 are involved in the cytokine-induced SAA gene expression [15]. SAF-1 is a zinc finger transcription factor that is essential for cytokine-induced SAA induction [16]. It was demonstrated that MAPK signaling pathway regulated the DNA-binding activity and transactivation potential of SAF-1 and phosphorylation of SAF-1 in response to cytokines was markedly inhibited by MAPK inhibitors [17]. More recently, it was demonstrated that LPS-stimulated SAA protein induction was significantly reduced in TLR4-deificient mice, suggesting the critical role of TLR4 in SSA induction [18]. In the present study, to determine whether hepatocytes respond directly to LPS and produce SAA, we investigated the presence of TLR4, a ligand of LPS, in human hepatocytes.

2. Materials and methods

2.1. Cells

Human primary hepatocytes were purchased from Cell Systems (Kirkland, WA). The cells were cultured in a basal medium composed of Ham's F-12 and Leibovitz L-15 (1:1) medium (Invitrogen, Carlsbad, CA), 0.2% (v/v) bovine serum albumin, 5 mM glucose (Wako Chemical Co Inc., Osaka, Japan), 10^{-8} M dexamethasone (Wako), and 10^{-8} M bovine insulin (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco, Grand Island, NY). These hepatocytes prepara-

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Abbreviations: ERK, extracellular signal-related kinase; IκB-α, IkappaB-α; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; SAA, serum amyloid A protein; TLR, Toll-like receptor

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tions were less than 0.1% reactive with the CD68 monoclonal antibodies (eBioscience, San Diego, CA), indicating that these cells were free of monocyte/macrophage.

2.2. Flow cytometry for determination of TLR4 expression

Adherent human hepatocytes were detached using 1 mM EDTA. Cell surface staining was performed using anti-human TLR4 phycoerythrin (eBioscience, San Diego, CA). Isotype-matched non-bonding control antibodies were used for comparison. Cells were analyzed using a EPCS XL (Coulter, Fullerton, CA). A total of 10 000 immunofluorescent events were acquired for each sample.

2.3. Immunoblot analysis

Whole cell lysates were prepared from LPS-stimulated hepatocytes using Triton lysis buffer containing protease and phosphatase inhibitor (1% Nonidet-P 40, 50 mM Tris, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM β-glycerophosphate, 1.0 mM sodium orthovanadate, 10 μg/mL aprotinin and 10 μg/mL leupeptin). One hundred micrograms of protein was electrophoresed on 10% SDS polyacrylamide gels. The fractionated proteins were transferred to nitrocellulose membranes and probed by anti-IkappaB-α (IκB-α), phospho-extracellular signal-related kinase (ERK)1/2, phospho-p38 and phospho-JNK1/2 antibodies (1:1000 dilution, Biosource, Camarillo, CA). Hepatocytes culture supernatants were also electrophoresed on 14% polyacrylamide gels. The fractionated proteins were transferred to a nitrocellulose membrane (pore-size: 0.2 μ, Bio-Rad, Hercules, CA) and probed with rabbit anti-SAA antibodies (1:2000 dilution) and developed using an enhanced chemiluminesence (ECL) system (Amersham, Arlington Heights, IL).

2.4. RNA preparation and RT-PCR assay

Total cellular RNA was extracted from hepatocytes using guanidium thiocyanate and phenol (RNAzol B, Cinna/Biotek Labs Int. Inc., Friendswood, TX). First-strand cDNA was synthesized by reverse transcription at 45 °C for 45 min in a 50 μl reaction mixture containing 1 μg of total RNA and MuLV reverse transcriptase (Invitrogen). After denaturing at 99 °C for 5 min followed by cooling at 5 °C, the cDNA was amplified using PCR. Two microliters of denatured cDNA was amplified in a 20 μl final volume containing 1 U *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD), 1 μM of each primer, *Taq* polymerase buffer, 1.5 mM $MgCl_2$ and 1.5 mM of each dNTP. PCR was performed in a thermal cycler (Perkin–Elmer–Cetus, Foster City, CA) using a program of 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final 10 min extension at 72 °C. The amplified products were subjected to electrophoresis on 2% agarose gel.

The specific primers used for TLR4 were
5′-TTGTATTCAAGGTCTGGCTGG-3′ (forward),
5′-GCAACCTTTGAAACTCAAGCC-3′ (reverse).
The predicted size of the fragment was 436 bp.
For *MyD88*:
5′-CCGCGCTGGCGGAGGAGATGGAC-3′ (forward),
5′-GCAGATGAAGGCATCGAAACGCTC-3′ (reverse).
The predicted size of the fragment was 356 bp.
For β-actin:
5′-GACGAGGCCCAGAGCAAGAGAG-3′ (forward),
5′-ACGTACATGGCTGGGGTGTTG-3′ (reverse).
The predicted size of the fragment was 236 bp.
For *SAA1*:
5′-CAGACAAATACTTCCATGCT-3′ (forward),
5′-ATTGTGTACCCTCTCCCCC-3′ (reverse).
The predicted size of the fragment was 303 bp.
For *SAA2*:
5′-CAGACAAATACTTCCATGCT-3′ (forward),
5′-ATTATATGCCATATCTCAGC-3′ (reverse).
The predicted size of the fragment was 328 bp.

3. Results

3.1. Expression of TLR4 in human hepatocytes

We first examined the mRNA expression of the LPS receptor molecule TLR4 and an intracellular adaptor protein for TLR4, *MyD88*, in human hepatocytes. Total RNA was extracted from hepatocytes and THP-1 cells, a monocyte cell line. The expression of TLR4 and *MyD88* genes was analyzed by reverse-transcription PCR. As shown in Fig. 1, TLR4 and *MyD88* mRNA were detected in human hepatocytes. THP-1 cells, which express TLR4 and *MyD88* mRNA, served as a positive control. To assess the protein expression of TLR4 in human hepatocytes, we undertook immunofluorescence staining for the presence of TLR4 in human hepatocytes using flow cytometry. As shown in Fig. 2, significant fractions of human hepatocytes constitutively expressed TLR4. To inves-

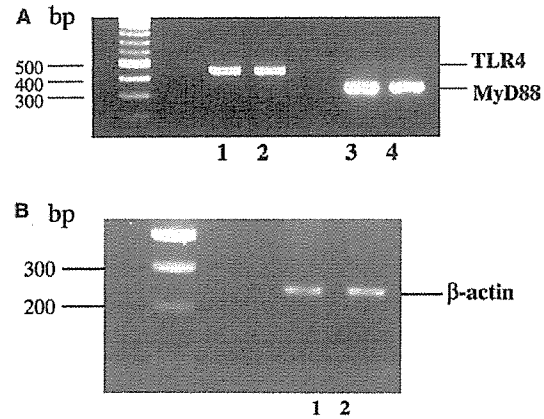

Fig. 1. Expression of TLR4 and *MyD88* mRNA in human hepatocytes. (A) Total RNA was obtained from THP-1 cells (lanes 1 and 3) and human hepatocytes and TLR4 and *MyD88* mRNA were analyzed by PCR following reverse transcription. (B) β-Actin expression in THP-1 cells (lane 1) and human hepatocytes (lane 2) were used as control.

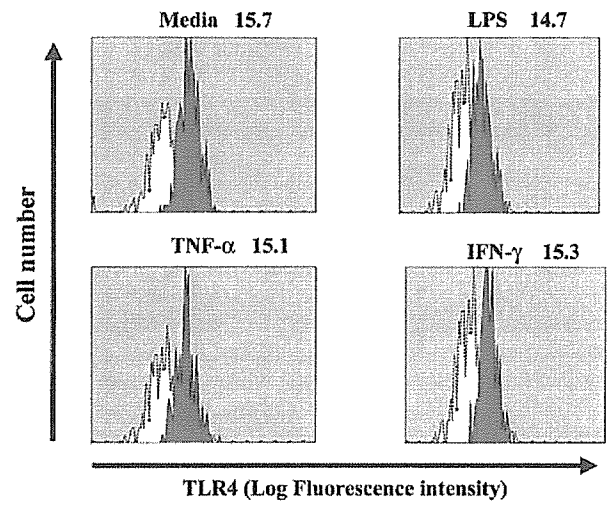

Fig. 2. Effects of cytokines on TLR4 expression on human hepatocytes. Human hepatocytes were treated with media, LPS (100 ng/ml), TNF-α (100 ng/ml) or IFN-γ(100 ng/ml) for 24 h. Expression of TLR4 on hepatocytes was measured by flowcytometer and represented as changes in fluorescence between TLR4 and the isotype control. Dotted line: isotype-matched control Ab. Solid line: TLR4 expression on hepatocytes. Values in upper right of graph represent mean fluorescence intensity of TRL4 expression. The data shown are representatives of at least three independent experiments.

tigate whether TLR4 expression could be regulated by inflammatory stimuli, we examined the effects of LPS, TNF-α and IFN-γ on TLR4 expression in hepatocytes. Human hepatocytes were stimulated with *E. coli* LPS (100 ng/ml), TNF-α (50 ng/ml) or IFN-γ (100 ng/ml) for 24 h, and the expression of TLR4 was analyzed by flow cytometry. TLR4 expression on hepatocytes was not modulated by these stimuli (Fig. 2).

3.2. LPS stimulates IκB-α degradation and MAPK activation

To determine whether LPS stimulation transmits a signal across the cell membrane in hepatocytes, we investigated the effects of LPS on the NF-κB and MAPK. Phosphorylation and subsequent degradation of IκB-α, an inhibitor of NF-κB, result in the activation of NF-κB [19]. To confirm LPS-induced NF-κB activation, we evaluated the IκB-α proteolysis. The protein levels of IκB-α in LPS-treated hepatocytes were measured by immunoblot analysis. LPS stimulation induced substantial IκB-α degradation in a time-dependent manner, suggesting the activation of NF-κB in hepatocytes (Fig. 3). LPS-mediated TLR4 signaling has also been found to trigger the activation of MAPKs. As shown in Fig. 4A, LPS had induced the phosphorylation of ERK1/2 at 15 min after stimulation. Similarly, LPS stimulation resulted in the phosphorylation of p38 (Fig. 4B) and JNK1/2 (Fig. 4C) in human hepatocytes. These results indicate that LPS activates MAPK signaling in human hepatocytes.

3.3. LPS induces SAA secretion from human hepatocytes

To investigate whether the TLR4 expressed on human hepatocytes is functional, the effects of LPS, a natural ligand for TLR4, on human hepatocytes were analyzed. We examined the secretion of SAA, a specific protein produced by hepatocytes. Human hepatocytes were incubated with varying doses of LPS in the presence or absence of 10% FCS for 24 h, and culture supernatants were removed and analyzed by anti-SAA immunoblot. Fig. 5A shows the secretion of SAA from LPS-stimulated human hepatocytes in a dose-dependent manner. LPS did not induce SAA production from human hepatocytes in the absence of serum (Fig. 5C). This result indicates that LPS-induced SAA production in hepatocytes was dependent on the presence of serum, a source of the LPS-binding protein (LBP) that is required for LPS to act through TLR4. Polymyxin B is an antibiotic that binds the lipid A motif of LPS and inactivates its biological function [20]. Preincubation with polymyxin B (100 IU/ml) before stimulation completely inhibited LPS-induced SAA production from human hepatocytes (Fig. 5B). To assess the functional role of TLR4, hepatocytes were incubated with anti-TLR4 monoclonal antibodies (HAT 125, eBioscience) for 6 h before stimulation with LPS. Hepatocytes preincubated with anti-TLR4 resulted in a reduced SAA synthesis, demonstrating that LPS utilizes TLR4 in human hepatocytes (Fig. 5D).

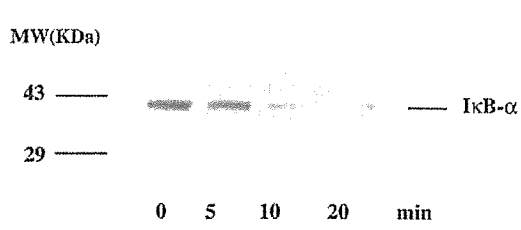

Fig. 3. LPS stimulated IκB-α degradation in hepatocytes. Human hepatocytes were stimulated with LPS (100 ng/ml) for the indicated times. Cells were lysed and cellular lysates were assessed by anti-IκB-α immunoblot analysis. The data shown are representatives of at least three independent experiments.

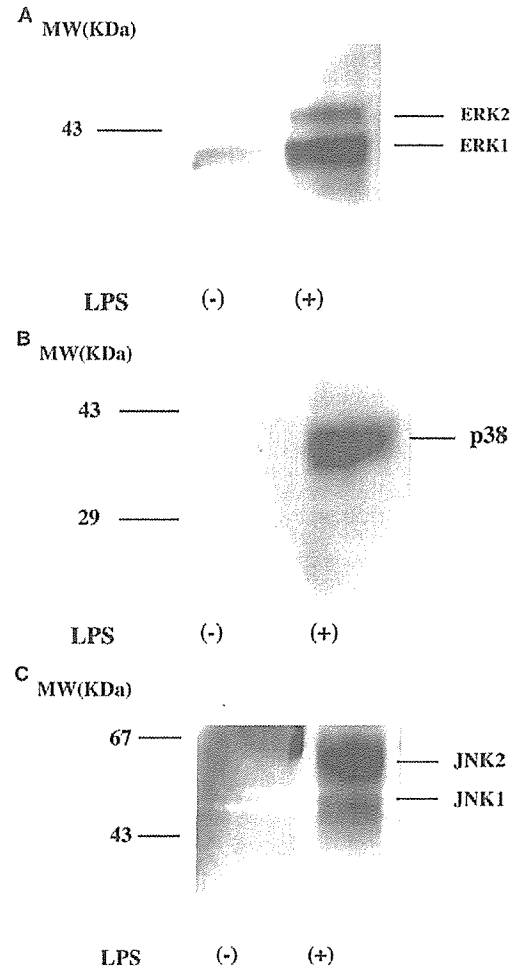

Fig. 4. LPS stimulation induces MAPK activation in hepatocytes. Quiescent human hepatocytes were stimulated with LPS (100 ng/ml) for 15 min. Cells were lysed and cellular lysates were analyzed by immunoblot using anti-phospho-specific ERK1/2 (A), p38 (B) and JNK1/2 (C) antibodies. The data shown are representatives of at least three independent experiments.

To confirm the LPS-stimulated induction of SAA at mRNA levels, we determined the levels of acute-phase SAA (*SAA1, SAA2*) in LPS-stimulated human hepatocytes using RT-PCR methods. Although the expression levels of *SAA1,2* mRNA in unstimulated hepatocytes were below the limit of detection, significant levels of *SAA1,2* mRNA expression were detected after 3 h of LPS stimulation (Fig. 6). In contrast, β-actin mRNA was constitutively expressed in hepatocytes and unchanged by LPS stimulation.

4. Discussion

Lipopolysaccharide (LPS) derived from gut bacteria has been implicated in this liver injury [21]. It has been suggested that LPS exerts an indirect effect on hepatocytes and that

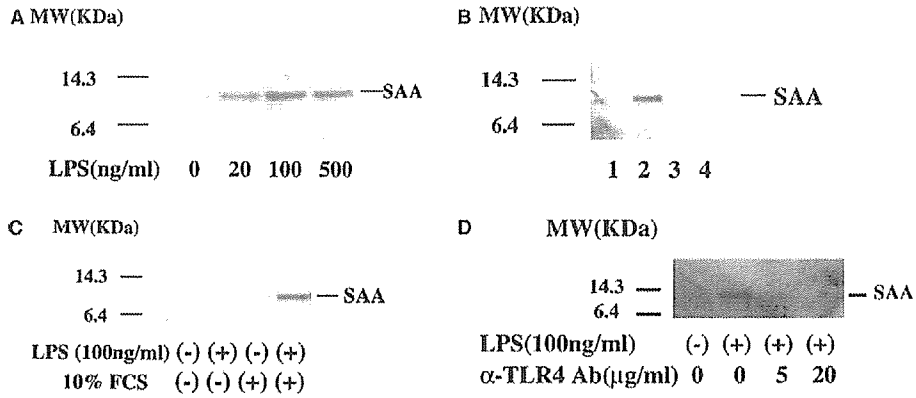

Fig. 5. (A) LPS induces SAA synthesis from human hepatocytes. Human hepatocytes were stimulated with the indicated concentrations of LPS for 24 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representatives of at least three independent experiments. (B) LPS-induced SAA production is inhibited by Polymyxin B. Human hepatocytes were stimulated by LPS (100 ng/ml) for 24 h with or without pretreatment of polymyxin B for 30 min. SAA production was measured by anti-SAA immunoblot using culture supernatants. Lane 1: untreated; lane 2: treated with LPS (100 ng/ml); lane 3: treated with Polymyxin B (2 IU/ml) plus LPS; lane 4: treated with Polymyxin B (10 IU/ml) plus LPS. The data shown are representative of at least two independent experiments. (C) LPS-induced SAA synthesis depends on the presence of serum. Human hepatocytes were stimulated with LPS (100 ng/ml) in the presence or absence of 10% FCS for 24 h. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representative of at least two independent experiments. (D) Effects of mAbs on TLR4 on the production of SAA by hepatocytes stimulated with LPS. Before the stimulation with LPS, human hepatocytes were pretreated with anti-TLR4 mAbs. SAA production was measured by anti-SAA immunoblot using culture supernatants. The data shown are representatives of at least two independent experiments.

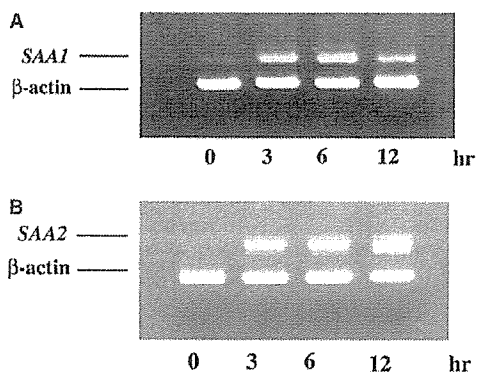

Fig. 6. LPS induces *SAA1,2* mRNA expression in human hepatocytes. Human hepatocytes were stimulated with LPS (100 ng/ml) for the indicated times. Expression on *SAA1* (A) and *SAA 2* (B) was analyzed by PCR following reverse transcription. β-Actin expression was used as control. The data shown are representatives of at least two independent experiments.

Kupffer cells are the major target of LPS [22]. However, several studies have raised the possibility that hepatocytes respond directly to LPS. Recently, it was demonstrated that murine hepatocytes express TLRs and respond to LPS through the TRL4 receptor pathway [23]. In this study, we demonstrated the mRNA expression of TLR4 and MyD88, a co-receptor for LPS, in human hepatocytes. Furthermore, hepatocytes expressed TLR4 on the cell surface at the protein level. These data indicate that human hepatocytes express LPS recognition molecules and suggest the possibility that hepatocytes respond to LPS directly.

Monocyte/macrophage exposed to LPS show the reduced surface TLR4 expression, which is thought to be one of the mechanisms of LPS tolerance [24]. In our data, TLR4 expression on hepatocytes was not modulated by LPS stimulation. Although the mechanism for the TLR4 downregulation in LPS-tolerant macrophages has not been clarified, these discrepancies in responses to LPS could be due to the differential cell types.

We then focused on the LPS signal transduction cascade in human hepatocytes. Upon binding of LPS to TLR4, MyD88, an adaptor protein, links TLR4 to IL-1R-associated protein kinase (IRAK) and IRAK is phosphorylated [25]. Phosphorylated IRAK dissociates from the receptor complex and activates tumor necrosis factor receptor-associated factor 6 (TRAF-6) [26]. Subsequently, TRAF-6 activates MAPKs and NF-κB to produce cytokines and chemokines [27]. To assess LPS signaling, we investigated whether LPS stimulation results in the activation of these kinases or transcription factors in human hepatocytes. MAPK are a group of serine/threonine protein kinases that participate in transmitting extracellular signals to the cell nucleus. LPS-induced MAPK activation was previously demonstrated in murine hepatocytes [23]. Consistent with these findings, our data indicate that LPS stimulation resulted in the activation of ERK1/2, p38 and c-Jun N-terminal kinase (JNK1/2). NF-κB is also shown to be activated by LPS stimulation. NF-κB is sequestered in an active form in the cytoplasm bound to inhibitor IκB-α. Extracellular stimuli induce IκB kinase (IKK), which degrades IκB [19]. After degradation of IκB, the NF-κB complex moves to the nucleus and activates NF-κB-dependent transcription [19]. The finding that LPS leads to the rapid degradation of IκB-α suggests that LPS stimulation induces IKK activation in human hepatocytes.

Hepatocytes support intra- as well as extrahepatic defense reactions by synthesis of APPs in inflammatory processes including endotoxemia. SAA, one of the APPs, is produced by hepatocytes in response to inflammatory cytokines such as TNF-α, IL-1β and IL-6 [11,28]. We evaluated the effects of LPS on SAA synthesis in hepatocytes. Our results clearly indicate that LPS directly induced SAA mRNA and protein expression in human hepatocytes. This LPS-induced SAA

synthesis was blocked by polymyxin B, a polypeptide antibiotic that inactivates the biological functions of LPS by removing FCS, a source of LPS-binding protein that is required for the interaction between LPS and TLR4 [29]. Recent reports indicated that NF-κB and SAA activating factor (SAF) are essential transcription factors for SAA gene expression [30] and that activation of SAF is mediated by MAPKs [17]. LPS-mediated NF-κB and MAPK activation coordinate the induction of SAA mRNA. SAA can induce chemotaxis of neutrophils [31] and matrix metalloproteinases (MMPs) induction [32], key processes involved in inflammation and tissue destructions. It is possible that human hepatocytes respond to LPS and participate in hepatic inflammation by producing SAA during LPS-induced liver injury.

In conclusion, our data suggest that human hepatocytes are capable of responding to a microbial product, LPS, directly without the mediation of Kupffer cells and macrophages. This LPS-mediated hepatocyte activation could be implicated in the pathogenesis of endotoxin-induced liver injury.

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MINIREVIEW

Nobuyuki Miyasaka · Tsutomu Takeuchi · Katsumi Eguchi

Official Japanese guidelines for the use of infliximab for rheumatoid arthritis

Abstract Differences in ethnic backgrounds as well as in medical and socioeconomic status often affect both the efficacy and adverse effects of medications. Recent data suggest an increased risk of opportunistic infections, especially tuberculosis (TB), among rheumatoid arthritis (RA) patients receiving infliximab, a chimeric monoclonal anti-tumor necrosis factor α (TNF-α) antibody. In this regard, the annual incidence of TB is approximately five times higher in Japan than in the United States. Furthermore, since Bacillus Calmette-Guérin vaccination is mandatory in childhood when the skin test for purified protein derivative (PPD) is negative, a high incidence of false-positive PPD skin tests is observed among the Japanese population. In addition, the upper limit of methotrexate dosage to be used for RA is lower in Japan. We have therefore established official guidelines for the proper use of infliximab in Japanese RA patients. In this review, an algorithm for the diagnosis and management of TB in RA is presented in an evidenced-based form.

Key words Guideline · Infliximab · Rheumatoid arthritis (RA) · Tuberculosis (TB) · Tumor necrosis factor α (TNF-α)

Introduction

Rheumatoid arthritis is a chronic inflammatory disease of unknown etiology. Synovial proliferation results in the destruction of both cartilage and bone, and tumor necrosis factor α (TNF-α) is thought to play a central role in this process. Infliximab is a human-murine chimeric anti-TNF-α monoclonal antibody with high affinity and specificity.[1] It forms stable complexes with the monomeric and trimeric forms of soluble TNF-α and with the transmembrane forms of TNF-α. Not only neutralizing soluble TNF-α, but also the cytolysis of macrophages and monocytes by binding to transmembrane TNF-α in vitro, might be relevant in its potent anti-inflammatory and immunosuppressive actions. Infliximab, when used with methotrexate (MTX) in rheumatoid arthritis (RA) patients, induces not only significant improvement in the signs and symptoms of RA but also substantial inhibition of progressive joint damage.[2,3] Infliximab was approved for RA in the United States in 1999.

In spite of its dramatic efficacy against RA, it has been noted that opportunistic infections, especially tuberculosis (TB), can occur among patients treated with infliximab. According to the report by Keane et al.,[4] the background rate of TB in RA patients is 6.2 cases per 100000 per year, whereas the estimated rate of TB among RA patients receiving infliximab therapy was 24.4 cases per 100000 in the United States. Although this report was confirmed by Wolfe et al.,[5] no cases of TB have occurred in persons with recent purified protein derivative (PPD) skin tests or prophylaxis. These findings reemphasize the importance of TNF-α in host immune response to *Mycobacterium tuberculosis*. In addition, infliximab administration is accompanied with severe acute infusion reactions in approximately 0.5% of patients because of the chimeric structure of the molecule. Given the differences in the medicosocial status of Japan and the high incidence of infusion reactions, the Ministry of Health, Labor, and Welfare of Japan has decided to conduct a special post-marketing survey of the initial 5000 patients treated with infliximab in Japan.

The potential problems of using infliximab in Japanese RA patients are as follows: (1) TB is approximately five times more prevalent in Japan than in the United States, (2) Bacillus Calmette-Guérin (BCG) vaccination given in childhood yields false-positive tests among Japanese so that

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the PPD skin test may not be suitable for critically screening TB, (3) over 4% of TB patients are resistant to isoniazid (INH), (4) prophylactic use of INH can cause liver dysfunction in at least 10% of TB patients, and (5) infliximab can mask the clinical features of TB. These problems are discussed below.

Current situation of TB in Japan and the world

According to a report issued by the World Health Organization (WHO), there were 8.8 million new cases of TB in 2002, 3.9 million of which were smear-positive.[6] The global incidence of TB is growing at a rate of approximately 1.1% per year, and the number of cases at 2.4% per year.

In Japan, the annual incidence of new TB infection in 2002 was 25.8 per 100000, or 4.6-fold higher than in the United States.[7] Although the incidence has been decreasing year by year, the proportion of the elderly population is increasing. Among 32828 newly registered TB patients in 2002, patients over 60 years of age comprise 58.4% and those over 70 years of age 41.5%, whereas the respective figures in 1996 are 53.2% and 31.7%. Fifty-three percent are positive for *M. tuberculosis* either in smear or culture. Pulmonary TB accounts for 81 and extrapulmonary for 19. There is a regional bias in the annual incidence of TB with percentages of patient increasing in urban areas: 74.4 per 100000 in Osaka city and 36.6 in Tokyo special district, but only 12.5 in Nagano Prefecture. In addition, 4.4% of isolated *M. tuberculosis* in culture is resistant to INH.

Purified protein derivative skin test

In Japan, 0.1 cc of 3 tuberculin units of standardized PPD obtained from Aoyama B strain is injected intradermally into the volar surface of the forearm. Erythema, but not induration, is measured at 48 h, and the diameter is recorded in millimeters. The cut-off criterion for positivity is 10 mm in diameter. The size of induration, dual erythema, and presence of blisters and necrosis, if any, are also recorded. Those with diameters of more than 10 mm, but not with induration or dual erythema, are evaluated to be 1+; those with induration, 2+; and those with induration, dual erythema, blisters and necrosis, 3+. The reason why erythema but not induration is measured is mainly due to the feasibility of measuring its diameter on the skin in the Japanese population. The PPD skin test has been performed in Japan at the time of entry to elementary school (6 years of age) and to junior high school (12 years of age) but this was abolished in 2003. Two-step testing is performed only in settings where tight TB screening is necessary, such as the employment of medical professionals. The PPD skin test is known to be influenced not only by exposure to TB but also by repeated PPD skin testing, which creates a booster phenomenon, and by other *Mycobacterium* infections such as *Mycobacterium avium* complex.

Bacillus Calmette-Guérin vaccination

Bacillus Calmette-Guérin vaccination has been given to those who show a negative PPD skin test in childhood. That is, the PPD skin test has been mainly used in Japan to evaluate whether BCG vaccination is necessary in childhood. Those who are negative in the PPD skin test even after BCG vaccination are subjected to revaccination. However, revaccination has not been used since 2003. Furthermore, the Japanese Government recommended that BCG vaccination should be performed to all infants by 1.5 years of age from 2004 without doing a PPD skin test. Although BCG vaccination might substantially contribute to decreasing the incidence of TB in Japan, it creates a false-positive reaction on the PPD skin test and makes it difficult to discriminate actual positivity due to TB from false positivity.

Difference in MTX dosage

The maximal dosage of MTX approved for use in Japan is 8 mg/week. This dosage is based on a clinical trial conducted in Japan to determine the optimal dose, in which the efficacy of the 6 and 9 mg/week groups was comparable, and was significantly better than that of the 2 mg/week group.[8] Increased liver enzyme was observed at more than 9 mg/week, although folic acid was not given during the trial. However, in the clinical setting, dosages of MTX in excess of 8 mg/week are sometimes used if the physician deems it necessary. In such cases, informed consent is obtained from the patient.

Pneumocystis carinii pneumonia in MTX-treated RA patients

Pneumocystis carinii pneumonia (PCP) is a serious and potentially fatal infection often encountered in immunosuppressed patients such as those with acquired immunodeficiency syndrome (AIDS), cancer including hematological malignancies, and organ transplantation.[9] Patients with connective tissue diseases (CTD) are also at risk for PCP.[10] *Pneumocystis carinii* pneumonia affects 2.6%–4.3% of CTD patients with immunosuppressive treatments including corticosteroids.[11,12] Risk factors include the administration of high-dose corticosteroids or immunosuppressants, and low peripheral blood lymphocytes (PBL).[12] We have found that patients who developed PCP were significantly more intensively treated with corticosteroids and/or immunosuppressive agents and were more immunosuppressed than those who did not.[13] In our series of 124 patients who received more than 30 mg/day of prednisolone, nine patients in the non-prophylaxis group ($n = 82$) developed PCP, whereas none in the prophylaxis group receiving one tablet of TMP/SMX (containing 80 mg of trimethoprim and 400 mg of sulfamethoxazole) ($n = 42$) developed PCP. *Pneumocystis carinii* pneumonia was diagnosed when the clinical and ra-

diographic presentation was strongly suggestive of PCP and when microbiologic confirmation of *P. carinii* in respiratory samples was made or there was a response to treatment only with agents active against *P. carinii*. All the patients diagnosed with PCP had the following four features strongly suggestive of PCP: (1) clinical manifestation including pyrexia, dry cough, and dyspnea, (2) hypoxemia (PaO_2 < 80 torr) and/or increased A-aDO_2 (>15 mmHg),[14] (3) diffuse alveolar infiltrates or interstitial infiltrates on chest X-ray as well as on computed tomography of the thorax, and (4) increase of serum β-D-glucan level.[15] We also found that PBL counts 4 weeks after the institution of PSL in the patients who developed PCP were significantly lower than those in the other patients (476 ± 350 vs 1229 ± 1.019, mean ± SD, $P < 0.004$). Since we occasionally but infrequently experience PCP in MTX-treated RA patients and have experienced a case of PCP during the clinical trial of infliximab in Japan, we are extremely cautious regarding the possibility of PCP developing as a complication during treatment with infliximab in combination with MTX in Japanese RA patients.

Treatment guidelines for using infliximab (Fig. 1)

We have therefore created guidelines for the use of infliximab to safely treat Japanese RA patients according to our data described above. The guidelines were initially established by the Study Group of Rheumatoid Arthritis, Ministry of Health, Labor, and Welfare, Japan (principal investigators; Nobuyuki Miyasaka, MD, Tsutomu Takeuchi, MD, and Katsumi Eguchi, MD), and were later officially approved by the Japan College of Rheumatology.

Inclusion criteria

Inclusion criteria are active RA with at least six swollen and tender joints with concomitant usage of methotrexate of over 6 mg/week. Patients must have either C-reactive protein >2.0 mg/dl or erythrocyte sedimentation rate >28 mm/h. Patients also are required to have more than 4000/mm^3 of white blood cells and more than 1000/mm^3 of peripheral blood lymphocytes in addition to negative β-D-glucan in sera, to avoid possible opportunistic infections including TB and *P. carinii*.

Exclusion criteria

Patients having concurrent infection or histories of serious infection for the last 6 months are excluded from the study. Patients who have chest X-ray findings indicative of old TB (pleural thickening, fibrotic scarring shadows, calcified shadows of more than 5 mm in diameter), a history of TB, extrapulmonary TB and PCP, congestive heart failure, malignancy, and demyelinating disease are also excluded from this study. However, if the physician thinks that the

A. Inclusion criteria

1. Disease activity
   Active RA patients with concomitant usage of methotrexate (MTX) of over 6 mg/week for more than three months who fulfill following conditions.
   1) tender joints ≧ 6
   2) swollen joints ≧ 6
   3) ESR ≧ 28 mm/hr or CRP ≧ 2.0 mg/dl
2. Laboratory data
   1) WBC ≧ 4,000/mm^3
   2) peripheral blood lymphocytes ≧ 1,000/mm^3
   3) serum β-D glucan: negative

B. Exclusion criteria

1. Ongoing infection
2. Past history of serious infection for the last six months
3. Abnormal shadows in chest radiographs suggestive of old pulmonary tuberculosis (TB) or tuberculous pleuritis (fibrotic scarring shadows, calcified shadows of more than 5 mm in diameter, pleural thickening)
4. History of pulmonary and extrapulmonary TB*
5. History of *Pneumocysits carinii* pneumonia
6. Congestive heart failure
7. Malignancy
8. Demyelinating disease

* If the physician thinks that the advantage of infliximab treatment outweighs its safety in patients with latent TB infection, prophylactic treatment of isoniazid (INH, 0.3 mg/day) is recommended.

Fig. 1. Treatment guidelines for using infliximab to rheumatoid arthritis patients in Japan

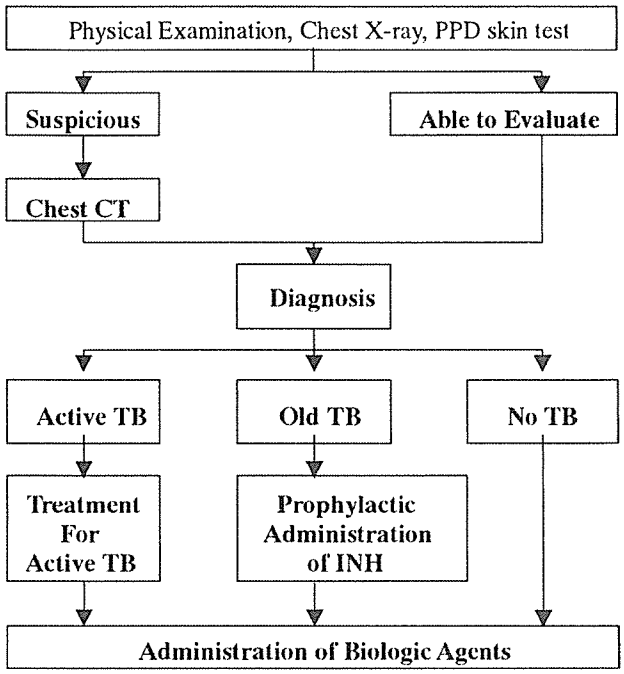

Fig. 2. Algorithm for the diagnosis and management of tuberculosis in patients with rheumatoid arthritis on biologic agents

advantage of infliximab treatment outweighs its safety in patients with latent TB infection, prophylactic treatment of INH (0.3 mg/day) is recommended.

Screening of TB (Fig. 2)

Intensive history taking and physical examination

Patients are asked whether they have had a history of TB or exposure to TB patients. They are screened for symptoms consistent with active TB, such as cough, sputum, or fever. It should be emphasized that signs and symptoms of TB can be masked by the use of infliximab, and therefore an extensive physical examination should be performed.

Purified protein derivative skin test

The PPD skin test is mandatory for all patients enrolled in this study and is performed before the initial infusion. If it is strongly positive (>2+), continuous use of INH (0.3 g/day) for 9 months, beginning one month prior to the initial infusion, is recommended.

Chest radiography and chest computed tomography (CT)

Chest radiography is also mandatory for all patients before the initiation of infliximab therapy. If the chest radiograph shows any findings suggestive of old TB, chest CT is recommended. Prophylactic use of isoniazid (INH) should be initiated in patients showing chest radiographs indicative of old pulmonary TB or tuberculous pleuritis. In the case of active TB, patients should be treated with a full course of anti-TB drugs for a sufficient period of time before starting infliximab. Physicians are simultaneously required to be capable of reading chest radiographs, evaluating them on the same day of clinical examination, and properly treating opportunistic infections including TB.

Treatment protocol

Intravenous infusion of infliximab (3 mg/kg) at weeks 0, 2, and 6 was performed, followed by subsequent infusions every 8 weeks. Intravenous infusion is initiated at a slow rate (15 ml/h) for the first 15 min and increased to 50 ml/h over a period of 2 h. The signs and symptoms of the patient are monitored throughout the infusion. Vital signs are closely monitored every 10 min during infusion with an automated manometer.

Management of infusion reactions

Physicians need to prepare for serious infusion reactions that may possibly occur during infliximab administration.[16] When mild to moderate infusion reactions occur, it is recommended to slow the infusion rate to 10 ml/h or stop infusion, if necessary. Further, p.o. or i.v. diphenhydramine (25–50 mg) and p.o. acetaminophen (650 mg) are administered. When wheezing is audible, i.v. hydrocortisone (100 mg) is given.

For severe acute reactions such as dyspnea with wheezing, significant discomfort, severe urticaria, or hypotension below 40 points of systolic blood pressure, s.c. epinephrine ([1:1000] 0.1–0.5 ml) is given and can be repeated every 5 min for three doses followed by i.v. methylprednisolone (50–100 mg) if necessary. The airway should be maintained with oxygen inhalation, and the patient should be transferred to the emergency room in case of anaphylaxis.

Assessment of the guidelines

As of July 11, 2004, 3011 cases were enrolled in this study and all of their case report forms were collected. The details of the study will be published elsewhere (Takeuchi T et al. Postmarketing survey of infliximab in 3000 cases of Japanese rheumatoid arthritis patients, in preparation). Briefly, TB was seen in nine patients, six of whom were observed in the initial 1000 cases, followed by two patients in the next 1000 cases, and one patient in the remaining 1000. Six of the cases were pulmonary and two extrapulmonary. None of the patients was under prophylactic use of INH. However, in six cases the chest radiographs turned out to be abnormal when retrospectively assessed by pulmonologists, although initial readings by attending physicians were assessed to be normal. Furthermore, three of these patients were strongly positive for the PPD skin test, and one had a history of TB. False-negative PPD skin tests were seen in two patients who had abnormal findings suggestive of old TB on chest radiographs.

Pneumocystis carinii pneumonia was observed in six patients (0.2%), none of whom was undergoing prophylactic

use of TMP/SMX. Serious infusion reactions were observed in six patients (0.3%), but all were successfully treated.

Discussion

We have established official guidelines for the use of infliximab in combination with MTX to treat Japanese RA patients and have simultaneously developed an algorithm for the diagnosis and management of TB. Approval was given by the Japan College of Rheumatology in 2003. Nine cases of TB were observed in this study, and six of them occurred during the first 1000 cases. However, if the above guidelines had been strictly followed by the treating physicians, these TB cases might have been prevented. Strict enforcement of the guidelines after the experience of the initial 1000 cases prevented the subsequent occurrence of TB at the same frequency. In this respect, Spain is in the same situation as Japan, with the incidence of TB higher than in other west European countries and the United States. However, Spain overcame this situation by establishing official guidelines and recommendations, and succeeded in dramatically reducing new cases of TB among patients receiving infliximab treatment.[17]

RA patients taking corticosteroids or MTX are often anergic, but this study had only two cases of false-negative PPD skin tests. Even though a high incidence of false-positive PPD skin tests is expected owing to BCG vaccination in childhood, the PPD skin test was useful in screening latent TB, and chest radiography combined with chest CT was effective in detecting latent TB infection in the lung.

An increased risk of TB in patients with RA has been reported in Mexico[18] but not in the United States.[5] No epidemiological studies on the risk of TB in RA patients have been carried out in Japan; however, this type of study is essential. In any event, our study again demonstrated that TNF-α is essential in host immune response to *M. tuberculosis*.[19,20]

The guidelines should be revised in the near future by more closely analyzing the upcoming results of the post-marketing survey in Japan. Enrollment of the 5000 cases initially planned for the post-marketing survey will be completed by the end of 2004.

Finally, recognition of the potential risk of opportunistic infections in RA patients treated with infliximab in combination with MTX by medical professionals is strongly required, and close monitoring of these patients for the signs and symptoms of complicated diseases such as TB and PCP will enable physicians to safely treat RA patients with infliximab.

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REVIEW ARTICLE

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Guidelines for the proper use of etanercept in Japan

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Abstract Application of biological agents targeting inflammatory cytokines such as tumor necrosis factor-α (TNF-α) dramatically caused a paradigm shift in the treatment of rheumatoid arthritis (RA). Infliximab, a chimeric anti-TNF-α monoclonal antibody, has initially been introduced to Japan in 2003 and shown to be dramatically effective in alleviating arthritis refractory to conventional treatment. However, serious adverse events such as bacterial pneumonia, tuberculosis, and *Pneumocystis jiroveci* pneumonia were reported to be in relatively high incidence; i.e., 2%, 0.3%, and 0.4%, respectively, in a strict postmarketing surveillance of an initial 4000 cases in Japan. Etancercept, a recombinant chimeric protein consisting of p75 TNF-α receptor and human IgG, was subsequently introduced to Japan in March of 2005. We therefore drew up treatment guidelines for the use of etanercept to avoid potential serous adverse events, since only approximately 150 cases have been included in the clinical study of etanercept in Japan. The guidelines were initially designed by the principal investigators (N.M, T.T., K.E.) of rheumatoid arthritis study groups of the Ministry of Health, Labor and Welfare (MHLW), Japan, and finally approved by the board of directors of the Japan College of Rheumatology. The MHLW assigned a duty to the pharmaceutical companies to perform a complete postmarketing surveillance of an initial 3000 cases to explore any adverse events, and this was performed according to the treatment guidelines shown in this article.

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Introduction

In recent years, there has been a paradigm shift in the treatment of rheumatoid arthritis (RA). It is mostly attributed to the introduction of biological agents targeting inflammatory cytokines. Biological agents have approved and marketed for the treatment of RA in Europe and the United States include anti-tumor necrosis factor-α (TNF-α) antibodies such as infliximab (Remicade) adalimumab (Humira), soluble TNF-α receptor etanercept (Enbrel), and interleukin (IL)-1 receptor antagonist anakinra (Kineret). Among these, etanercept has drawn particular attention as a highly effective and safe biological product in the treatment of RA; it was approved in January 2005 in Japan.

Efficacy and adverse events of etanercept

Etanercept is a recombinant chimeric protein consisting of two molecules of p75 and the Fc portion of human IgG1 and is produced by introducing the fusion gene into Chinese hamster ovary cells (molecular weight, approximately 150kDa; total amino acid residues, 934). As compared with the natural-occurring soluble TNF-α receptor, etanercept showed 50-fold greater binding to TNF-α, 100- to 1000-fold greater biological activity, and 5- to 8-fold longer plasma half-life; therefore, treatment of RA with etanercept has been conducted.[1]

In a phase III study in the United States involving 234 patients with active RA who were resistant to disease-modifying antirheumatic drugs (DMARDs) including

methotrexate (MTX), treatment with etanercept 25 mg showed significantly greater efficacy than etanercept 10 mg or placebo.[1] Analysis of adverse events revealed that the incidence of injection-site reactions was significantly higher in the 25-mg dose group than other dose groups and that the active treatment groups had a higher incidence of infections, i.e., upper respiratory tract infections, than the placebo group.

In a double-blind study of concomitant MTX, 89 patients with active RA who had been treated with MTX for at least 6 months received either etanercept 25 mg or placebo twice a week subcutaneously in addition to MTX, resulting in improvement in a 20% American College of Rheumatology (ACR 20) response in 71% of patients receiving etanercept and an ACR 50 improvement in 39% of patients receiving etanercept. Moreover, there were no significant differences in incidence of adverse events such as infections between the two groups.[2]

On the basis of these results, etanercept was approved as a treatment for RA by the Federal Drug Administration (FDA) in the United States in November 1998. More than 6 years after its approval in the United States, the drug was approved in January 2005 and has been marketed since the end of March 2005 in Japan.

The most significant benefit of etanercept is to inhibit the progression of joint destruction. Its efficacy has been demonstrated to be far superior to that of MTX, which is known to have the potent effect of slowing the progression of joint destruction.[3] The remarkable effectiveness of etanercept has been shown particularly in the recently reported TEMPO (Trial of Etanercept and Methotrexate with Radiographic Patient Outcomes).[4] In this trial, 686 patients with RA who were resistant to one or more DMARDs other than MTX received one of the following three treatments for 2 years: MTX alone, etanercept alone, or etanercept plus MTX. The primary efficacy endpoint was the numeric index of the ACR response (ACR-N) area under the curve (ACR-AUC) over the first 24 weeks. The primary endpoint did not differ significantly between the MTX monotherapy group and etanercept monotherapy group; however, the etanercept plus MTX group had significantly higher ACR-AUC values than the two monotherapy groups. The proportion of patients achieving ACR 50, a clinically meaningful efficacy, at 2 years was 71% in the etanercept plus MTX group as compared with 42% in the MTX monotherapy group and 54% in the etanercept group, indicating greater efficacy of the combination of etanercept and MTX. Moreover, the change in total Sharp Score at 1 year of treatment was −0.54 in the etanercept plus MTX group as compared with +2.8 in the MTX monotherapy group and +0.52 in the etanercept group, suggesting the possibility that the combination may inhibit the progression of joint destruction and even heal the condition.

Frequently observed adverse events include injection-site reactions, which are characterized by erythema with pruritus, swelling, or pain at the injection sites. Most reactions disappear with only topical treatments such as antihistamines.

The most careful attention should be given to monitoring for infections. Among the more than 1100 patients receiving long-term treatment for at least 6 months, 50 experienced serious infections such as pyelonephritis, bronchitis, septic arthritis, and abscess formation, which were caused by various types of organisms including bacteria, fungi, and *Pneumocystis jiroveci*. Etanercept may mask clinical symptoms characteristic of infections, such as fever and chills, and inhibit the production of acute inflammatory proteins, thereby causing the problem of difficulty in detecting infections at an early stage.

In addition, occurrence of tuberculosis has recently been of particular concern, although etanercept appears to be rarely associated with tuberculosis as compared with infliximab.[5,6] However, caution should be exercised when etanercept is used in Japan, where tuberculosis frequently occurs,[7] because BCG vaccinations in Japan preclude the use of the tuberculin skin test for screening at the start of drug treatment, and there are not a few patients with drug-resistant *Mycobacterium tuberculosis*.[8,9]

Although the occurrence of malignancies was the most serious among possible complications, the incidence in the more than 1100 patients was not significantly different from the expected incidence in the general population. Also, patients with active RA have been shown to have a slightly higher incidence of malignant lymphoma. In March 2003, the FDA reported that the standardized incidence ratio (SIR) for malignant lymphoma ranged from 2.3 to 3.5 in patients receiving etanercept with no statistically significant difference.[10]

Because etanercept is known to exacerbate congestive heart failure, caution should be made when etanercept is administered to patients with heart failure. Initially, clinical trials were conducted with infliximab or etanercept as a therapeutic agent for congestive heart failure because TNF-α was believed to be involved in the pathophysiology of congestive heart failure. However, individual clinical trials showed treatment failures and even worsening cases of congestive heart failure, leading to termination of these trials.[11]

In addition, rare cases of pancytopenia have been reported. Although demyelinating diseases in the central nervous system have also been reported, a causal relationship to the treatment remains uncertain. In some patients with multiple sclerosis, an increase in disease activity has been found after the treatment.[12] A recent report showed that etanercept treatment in early RA patients was well tolerated for up to 5 years.[13]

In Japan, 147 patients who were refractory to conventional DMARDs were enrolled in phase II clinical study. Patients were randomly divided into three groups, i.e., placebo group, 10 mg twice-weekly group, and 25 mg twice-weekly group, and treated for 12 weeks. Consequently, both the 10 mg group and 25 mg group yielded an almost identical ACR20 response, significantly better than the placebo group (64.0%, 65.3% vs. 6.3%, respectively). This trend was similar in both ACR50 and ACR70 response. There was no significant difference observed in severe adverse effects between the etanercept and placebo groups.

Table 1. Treatment guidelines for the use of etanercept

A. Inclusion criteria
Patients with active rheumatoid arthritis still presenting the following despite the use of one or more MHLW recommendation-level-A-DMARDs[a] (methotrexate, bucillamine, or sulfasalazine) at a normal dose for more than 3 months:
(1) Tender joints ≥6
(2) Swollen joints ≥6
(3) ESR ≥28 mm/h or CRP ≥2.0 mg/dl
Also, the patients must meet the following as having low risk for opportunistic infections:
(1) WBC ≥4000/mm^3
(2) Peripheral blood lymphocytes ≥1000/mm^3
(3) Serum β-D-glucan: negative

B. Usage
The dose of etanercept is 10–25 mg administered twice weekly as a subcutaneous injection. A patient can self-inject etanercept only after the ability to self-inject is carefully assessed and appropriate training is provided by a health professional.

C. Contraindication
1. Ongoing infection
2. Past history of serious infections in the last 6 months
3. Abnormal shadows on chest radiographs suggestive of old pulmonary tuberculosis (TB) or tuberculosis pleuritis
4. History of extra pulmonary TB or *Pneumocystis carinii* pneumonia
5. Congestive heart failure
6. Malignancy or demyelinating disease

D. Caution
1. From the point of view of screening for infection (especially TB and opportunistic infections) as well as prevention of side effects, etanercept is recommended for clinical use at medical institutes where:
(1) Chest X-rays can be obtained on the same day, and the X-ray can be interpreted by a pulmonologist, TB specialist, or radiologist
(2) Opportunistic infections can be treated
2. Comprehensive TB screening should be conducted including an in-depth patient history, chest radiographs (chest CT whenever possible) and a PPD skin test. In patients with suspected TB, based on medical history, abnormal shadows on chest radiographs suggestive of old pulmonary TB, or with a PPD skin test positive (as evidenced by redness of at least 20 mm in diameter or the presence of induration), the treatment with etanercept may be considered in addition to anti-tuberculosis drugs only if the potential benefits outweigh the potential risks.

MHLW, Ministry of Health, Labor and Welfare; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; WBC, white blood cells; TB, tuberculosis; CT, computed tomography; PPD, purified protein derivative
[a]Cited in the Diagnostic Manual and Evidence-based Treatment Guidelines

Treatment guidelines for the use of etanercept (Table 1)[14]

For the safe use of etanercept in Japan, which produces such high efficacy and potential adverse events, the internal medicine rheumatology study group of the Ministry of Health, Labor and Welfare, Japan (led by N.M., T.T., and K.E.) has developed the guidelines for treatment with etanercept, which provide indications, contraindications, and tuberculosis risk assessment, which was based on the guidelines for the use of infliximab for RA patients in Japan[9] (Fig. 1). The guidelines were approved by the board of directors of Japan College of Rheumatology.

Etanercept is indicated in patients with active RA at or above a certain level. Specifically, etanercept may be used in patients who have inadequately been controlled despite treatment of at least 3 months with the usual doses of one of the DMARDs (methotrexate, bucillamine, or sulfasalazine), which are rated as "recommendation A level" in the Diagnostic Manual and Evidence-based Treatment Guidelines[15] developed by the study group of the Ministry of Health, Labor and Welfare. Leflunomide, another DMARD rated as recommendation A, is not included in the present guidelines because of the adverse event of serious interstitial pneumonia observed in Japan.[16] Inadequate response to previous treatment is defined by the presence of at least six tender joints and swollen joints and either C-reactive protein levels of at least 2.0 mg/dl or erythrocyte sedimentation rate (ESR) of at least 28 mm/h.

To avoid potential opportunistic infections, patients should have a peripheral leukocyte count of 4000/mm^3 or more, peripheral lymphocyte count of 1000/mm^3 or more, and a negative test for blood β-D-glucan. These criteria are based on the findings that cellular immunity plays an important role in opportunistic infections caused by *Mycobacterium tuberculosis* or fungi such as *Pneumocystis jiroveci*, and that these infections are likely to occur in patients with decreased peripheral lymphocyte counts.[17] A test for blood β-D-glucan has been included because β-D-glucan, a component of fungi, may be diagnostic of fungal infections, especially infections with *Pneumocystis jiroveci*.

The recommended dosage and administration of etanercept in Japan is 10–25 mg given once daily and twice weekly as a subcutaneous injection. In this aspect, once-weekly administration of 50 mg etanercept in patients with active RA patients has been approved in the United States, and this dosing regimen was shown to be equivalent to 25 mg etanercept twice weekly in terms of safety, efficacy, and pharmacokinetics.[18] Patients will switch to self-injection

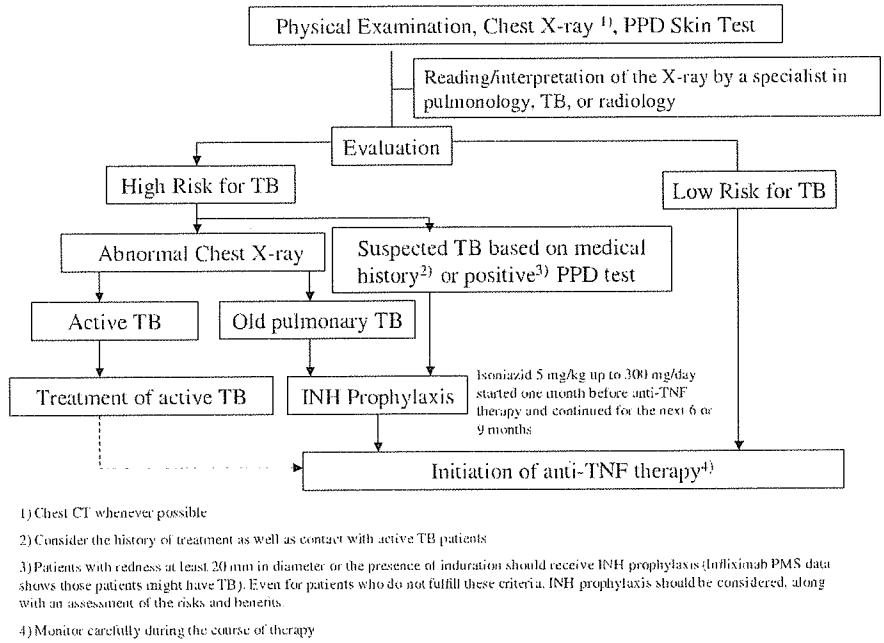

Fig. 1. Tuberculosis risk evaluation chart of the use of anti-TNF therapy. *TNF*, tumor necrosis factor; *INH*, isoniazid; *PMS*, postmarketing survery

after they are assessed as capable of conducting self-injections and receive adequate instructions. Etanercept may be used as monotherapy as the drug was administered so in clinical trials in Japan. In Europe and the United States, however, etanercept in combination with MTX has been demonstrated to provide greater efficacy in TEMPO.[1] Thus, the combination of etanercept with MTX should be considered in patients with highly active disease in Japan.

Etanercept is contraindicated in patients with active infections or a history of serious infections within the previous 6 months. In addition, careful assessment of the risk of tuberculosis should be made. Specifically, the following three examinations should be performed before treatment initiation: interview with respect to family and past history of tuberculosis, chest radiography, and purified protein derivative (PPD) skin test (Fig. 1). Chest radiographs should preferably be interpreted by a specialist in pulmonology, tuberculosis, or radiology. When abnormalities are suspected on chest radiography, computed tomography of the chest should be performed. Etanercept is contraindicated in patients with abnormalities in chest radiographs such as linear opacities, calcification ≥5 mm, and pleural thickening suggesting old pulmonary tuberculosis, and individuals infected with pulmonary or extrapulmonary tuberculosis. However, treatment with etanercept may be considered with antituberculous agents only if the potential benefits outweigh the potential risks. In patients with a positive PPD skin test (as evidenced by erythema of at least 20 mm in diameter or the presence of induration) or opacities suggesting old pulmonary tuberculosis on chest radiographs, treatment with isoniazid 0.3 g/day should be initiated at least 1 month prior to administration of etanercept and continued for the subsequent 9 months. However, no definite guidelines are available that address how to deal with isoniazid-induced hepatic impairment and isoniazid-resistant *Mycobacterium tuberculosis*, as well as how long isoniazid treatment should be given. Etanercept is also contraindicated in patients with previous *Pneumocystis jiroveci* pneumonia, congestive heart failure, malignancies, or demyelinating disease.

To sum up, in this article we focused on the guidelines for the use of etanercept that have been introduced in Japan since spring 2005. In Japan, because only about 100 patients received etanercept in clinical trials, it remains uncertain whether etanercept yields clinical benefit and adverse events with a similar frequency as observed in Europe and the United States. Nonetheless, we will conduct an all-cases postmarketing surveillance using the above-mentioned treatment guidelines, review the results, and revise the guidelines as needed.

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