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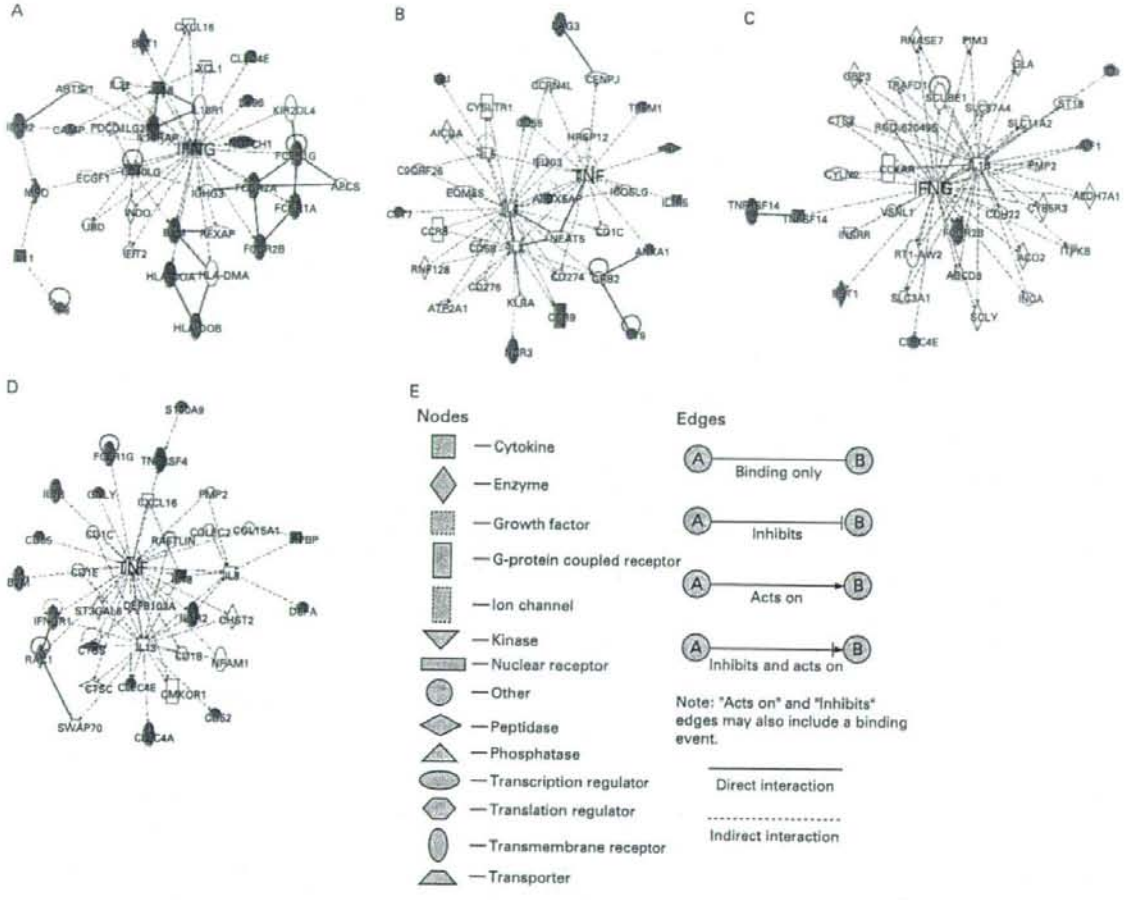

Figure 1 Network-based analysis on defence response genes. Ingenuity Pathways Network analysis, a web-delivered application (http://www.ingenuity.com), enables us to discover, visualise and explore biological interaction networks.[31] This analysis displays up to 35 genes in a single network. We chose networks containing more than 10 genes of that group as a meaningful network in systemic juvenile idiopathic arthritis (sJIA). For comparison, networks of polyarticular JIA (polyJIA) that included the same central molecule in sJIA were described. A,B. Defence response regulated by tumour necrosis factor (TNF) and interferon (IFN)γ in sJIA. C,D. Defence response regulated by TNF and IFNγ in polyJIA. E. explanation of the symbols. Genes are represented as individual nodes whose shapes represent the functional class of the gene products. Genes in coloured nodes were found in the over-represented category of defence response. Genes in uncoloured nodes were not found in the over-represented category of defence response and were depicted in the computationally generated networks on the basis of evidence stored in the Ingenuity Pathways Knowledge Base indicating a strong biological relevance to that network.

was no statistically significant difference in the methotrexate (MTX) doses between patients with sJIA and patients with polyJIA. The white blood cell counts and the proportion of neutrophils in patients with sJIA were higher than normal, but these were not significantly different between patients with sJIA and patients with polyJIA.

Gene ontology analysis on the genes differentially expressed in patients with JIA identified by DNA microarray analysis

DNA microarray analysis revealed that 3491 genes were differentially expressed in patients with sJIA compared to healthy children with statistical significance: 1273 out of 3491 genes were upregulated and the remaining 2218 genes were downregulated. Similarly, 2406 genes were differentially expressed in polyJIA: 691 genes out of 2406 genes were upregulated and the remaining 1715 genes were downregulated.

To identify the aberrant cellular functions in peripheral leukocytes from patients with sJIA and patients with pdgJIA, gene ontology analysis was performed on the genes differentially expressed in sJIA and pdgJIA using EASE.

The tables 2–5 list EASE results. EASE performs theme discovery, defined as the identification of functional categories that describes a statistically significant number of genes in a list with respect to the number of genes described by the functional categories in the population of genes from which the list is derived. We conducted EASE analysis using the Gene Ontology (GO) database (http://www.geneontology.org/GO.database.shtml) terms for the "biological process" category. An EASE score (Fisher exact test) represents the probability that an over-representation of certain functional category occurs by chance.[15] Categories with an EASE score of less than 0.05 are listed. A hierarchical relationship between biological processes was

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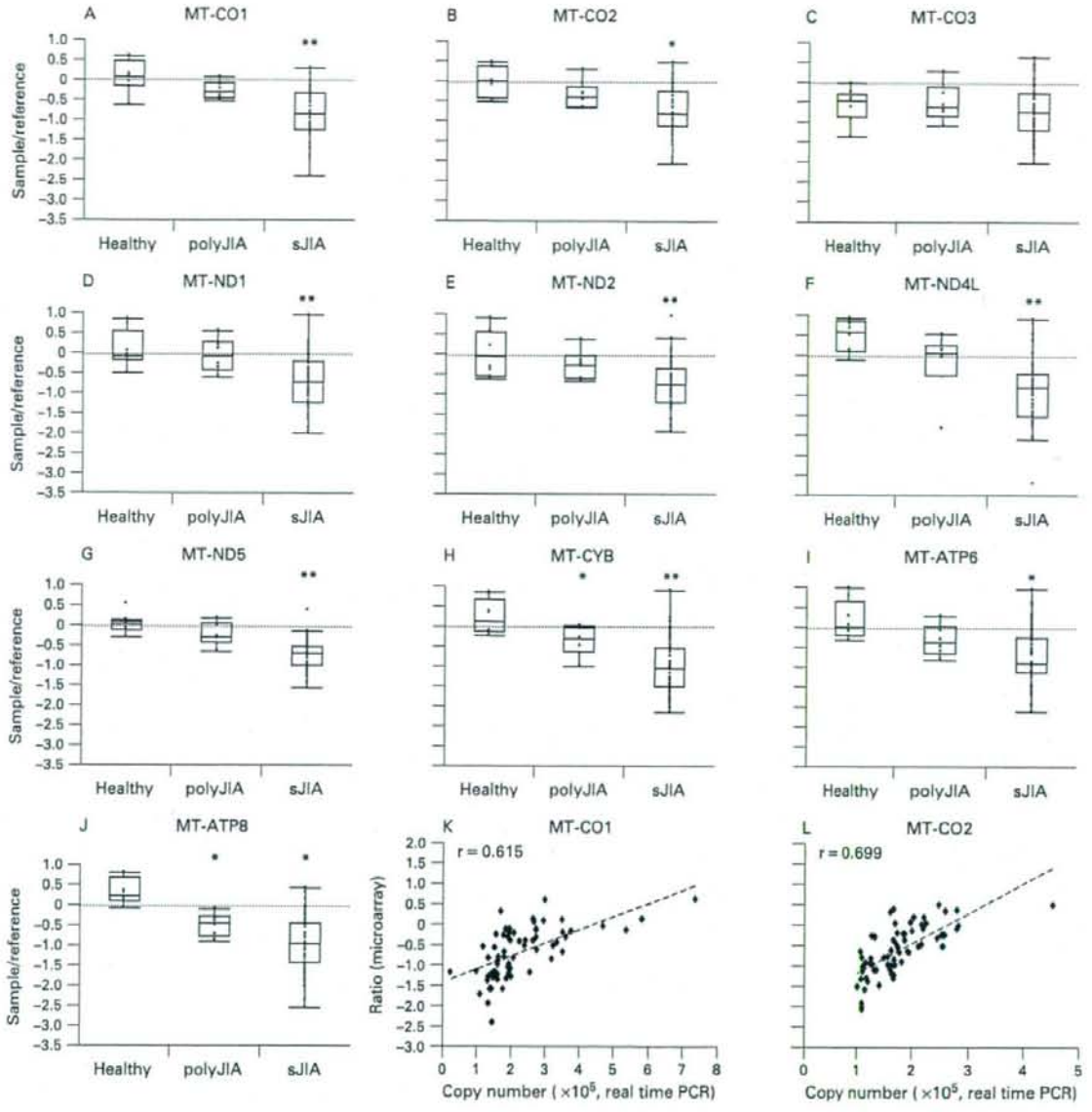

Figure 2 Decrease in the expression of mitochondrial DNA-encoded genes in systemic juvenile idiopathic arthritis (sJIA). A–J. Expression levels of mitochondrion DNA were represented. All data represents microarray data. All genes except for mitochondrially encoded cytochrome c oxidase subunit 3 (MT-CO3) were significantly downregulated in sJIA. Mitochondrially encoded cytochrome *b* (MT-CYB) and mitochondrially encoded ATP synthase 8 (MT-ATP8) were significantly downregulated in polyarticular JIA (polyJIA). * $p<0.05$ for sJIA vs healthy or polyJIA vs healthy (Mann–Whitney U test). ** $p<0.05$ for sJIA vs healthy and sJIA vs polyJIA. Boxes contain the 50% of values falling between the 25th and 75th percentiles, the horizontal line within the box represents the median value and the "whiskers" are the lines that extended from the box to the highest and lowest values, excluding outliers. K,L. Correlation between the mitochondrial gene expression data of microarray and quantitative PCR. For TaqMan PCR assay, cDNA synthesised from 16 ng of total RNA were used to measure the gene expression with a TaqMan Universal PCR Master Mix reagent, commercially available assays on demand probe primer sets (MT-CO1: Hs0259684_g1, MT-CO2: Hs02596865_g1) (Applied Biosystems) and 7500 Real Time PCR System (Applied Biosystems). For quantification standard of either MT-CO1 or MT-CO2, plasmid DNA including a PCR product of each gene was used. The vertical axis shows the expression levels of microarray, and the horizontal axis the copy number determined by quantitative PCR. Microarray data of MT-CO1 and MT-CO2 significantly correlated with the data of quantitative PCR. Index of correlation of MT-CO1 was 0.615, and of MT-CO2 was 0.699.

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arranged by manual operation based on term lineage of GO. A category including too many genes was omitted. Number of genes refers to genes categorised in each GO differentially expressed in JIA. "List" refers to the total number of genes either upregulated or downregulated in each type of JIA that are annotated in the EASE system. "Population" reports all genes that are annotated in the EASE system. The total number of genes in "Population" was 13 802. "Hits" shows the number of genes in the list that belong to the respective gene category. Table 2 shows GO categories significantly over-represented in upregulated genes of sJIA. Among 1273 genes upregulated in sJIA, 626 genes were annotated in the EASE system because they included genes whose functions were unknown as well as the mRNA of those expected to be expressed according to the whole genome sequence. Table 3 shows GO categories significantly over-represented in downregulated genes of sJIA. Among 2218 genes downregulated in sJIA, 1641 genes were annotated in the EASE system. Table 4 shows GO categories significantly over-represented in upregulated genes of polyJIA. Among 691 genes upregulated in polyJIA, 455 genes were annotated in the EASE system. Table 5 shows GO categories significantly over-represented in downregulated genes of polyJIA. Among 1715 genes downregulated in polyJIA, 1016 genes were annotated in the EASE system. Broad (high level) biological process included more specific (lower level) biological process.

The EASE analysis identified five major functional categories: response to stimulus, metabolism, establishment of localisation, physiological process and cell organisation and biogenesis in the upregulated genes in sJIA (table 2). In the category of response to stimulus, there were three subcategories with a statistically significant number of genes: response to external stimulus, response to biotic stimulus and defence response. These were independently categorised in gene ontology. A total of 52 genes in either the response to external stimulus or the response to biotic stimulus group also belonged to the defence response group. The remainder was categorised mainly in the response to abiotic stimulus group, which was not independently over-represented. Therefore, the functional category of defence response was predominantly over-represented in response to stimulus. Similar findings were also observed for polyJIA (Table 4).

In the category of metabolism, a subcategory of protein biosynthesis was over-represented in sJIA. Of 43 upregulated genes in the protein biosynthesis group, 20 genes were related to ribosomes (18 ribosomal proteins and 2 tRNA synthetases) and 11 genes were related to glycosylation. These genes were essential for various kinds of protein synthesis. Therefore, protein synthesis appeared to be upregulated in sJIA. The remaining genes were not classified into over-represented subcategories that might help to understand their functional abnormality. This category was also over-represented in polyJIA. Of the 42 upregulated genes in this category, 23 were related to ribosomes (21 ribosomal proteins and 2 tRNA synthetases) and 6 genes were related to glycosylation. The remaining genes in polyJIA were not classified into any over-represented subcategories, similar to sJIA.

In the category of cell organisation and biogenesis, a subcategory of mitochondrion organisation and biogenesis was over-represented. Although the population of this category of EASE consists of only seven genes, three of them were upregulated in sJIA. These three genes were SLC25A4, nuclear respiratory factor 1 (NRF1) and optic atrophy 1 (OPA1). SLC25A4 is induced at stress or damage in mitochondria. NRF1 and OPA1 are necessary for replication of the mitochondrial genome as well as synthesis of new mitochondrial components. This category was over-represented only in sJIA.

In addition, the categories of establishment of localisation and organisation-physiological process were also over-represented in sJIA, suggesting possible abnormalities in these functions.

The EASE analysis identified four major functional categories: immune system process, metabolism, cell organisation and biogenesis and cell development in the downregulated genes in sJIA (table 3). In the category of metabolism, the subcategories related to RNA metabolism, including a transcription from Pol II promoter and a RNA processing group, were over-represented, while the categories related to DNA metabolism were not over-represented (table 3). Most of the genes in the transcription from Pol II promoter group were transcription factors. Simultaneously, some genes related to transcription factors in this category were upregulated. Different expression patterns of transcription factors indicated the change of protein production, suggesting the activation of peripheral leukocytes.

The category of oxidative phosphorylation was also over-represented in sJIA. Genes categorised in oxidative phosphorylation were related to the phosphorylation of ADP to ATP that accompanies the oxidation of a metabolite through the operation of the respiratory chain, and were downregulated in sJIA (table 3). Of 13 genes downregulated in this category, 9 were encoded by mitochondrial DNA.

In polyJIA, the genes related to ATP synthesis were upregulated (table 4). These were all encoded by the nuclear genome and included NADH dehydrogenase (ubiquinone) 1β subcomplex (NDUFB) 1, NDUFB2, NDUFB3 and NDUFB6.

In addition, the categories of cell organisation and biogenesis and cell development were also identified to be over-represented in sJIA, suggesting possible abnormalities in these functions.

A network-based analysis of the genes in the defence response category

Network-based analysis was conducted of the molecules categorised in defence response to identify the relationship among these molecules and the centred molecules in the networks. Two networks were found to consist of 10 or more upregulated genes in the defence response group in sJIA. One was the network in which IFNγ was central (fig 1A). The other network was mainly attributed to TNF (fig 1B). Networks involving TNF and IFNγ were also identified in polyJIA (fig 1C,D) although the IFNγ network cascade consisted of only seven upregulated molecules in polyJIA (fig 1C). There were some differences in the molecules constituting the TNF and IFNγ networks between sJIA and polyJIA. It is noteworthy that IL18 was seen in both types of JIA (fig 1A,D). IL18 was contained in the sJIA IFNγ network and in the polyJIA TNF network.

Decrease in the expression of mitochondrial DNA-encoded genes in sJIA

In the category of oxidative phosphorylation, identified to be significantly over-represented in the downregulated genes in sJIA, most of the genes were encoded by mitochondrial DNA. Expression levels of these mitochondrial DNA-encoded genes are shown in fig 2. Except for mitochondrially encoded cytochrome *c* oxidase subunit 3 (MT-CO3), all of the genes showed a significant decrease in sJIA compared with healthy children. Furthermore, MT-CO1, mitochondrially encoded NADH dehydrogenase 1 (MT-ND1), MT-ND2, MT-ND4L,

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MT-ND5 and mitochondrially encoded cytochrome *b* (MT-CYB) were significantly downregulated in sJIA compared with those in polyJIA as well as healthy individuals. The expression levels of these genes were not influenced by the corticosteroid or MTX doses. In addition, no relation was observed between the gene expression and the proportion of either neutrophils or lymphocytes. Another mitochondrial DNA-encoded gene, MT-CO3 was downregulated but, statistically, was not significantly lower in sJIA compared to healthy children. MT-CO3 expression in healthy children was significantly lower than that of healthy adults. The expression levels of MT-CO1 and MT-CO2 determined by quantitative PCR were well correlated with those by microarray, thus verifying the data.

There was no significant decrease in the expression of cytochrome *c* oxidase (COX) subunits encoded by the nuclear genome in sJIA such as COX subunit IV isoform 1 (COX4I1), COX5A, COX5B, COX6A1, COX6B2, COX6C, COX7A1, COX7A2, COX7B, COX7C or COX8A. Therefore, the expression of COX subunits encoded by nuclear genome was intact.

DISCUSSION

Using DNA microarray technology, we analysed the gene expression profiles of peripheral blood to identify the molecules involved in the pathogenesis of sJIA. It was found that thousands of genes were differentially expressed in patients with sJIA. Such a considerably large number of affected genes reflect that various types of cell, including lymphocytes, neutrophils and macrophages, probably contribute to the pathogenesis. Since overproduction of proinflammatory cytokines such as TNF and IL6 is reportedly involved in the disease, these soluble factors may affect the peripheral leukocytes and alter the gene expression profiles in the cells. Indeed, the genes in the defence response category constituted TNF and IFNγ network cascades. IL18 was also identified to be involved in the network cascade in sJIA. This finding confirmed previous reports that IL18 plays a pathological role in sJIA.[7] Interestingly, upregulation of some molecules in the TNF and IFNγ cascades were also observed in polyJIA, where IL18 was also involved. Although it is not clear whether or not the pathogenic antigens are common between sJIA and polyJIA, TNF, IFNγ and IL18 all seem to play common pathological roles between the two types of JIA. According to network analysis, however, the IFNγ cascade was dominant in sJIA, while TNF cascade was dominant in polyJIA. This finding might explain the fact that anti-TNF therapy was very effective for patients with polyJIA but far less so in patients with sJIA.[16] Although TNF and IFNγ network cascades were depicted in both types of JIA, the surrounding molecules in the TNF and IFNγ network cascades were not always the same. Such a difference may cause the different clinical features between the two types of JIA and be useful for the differential diagnosis.

Like defence response abnormalities, metabolic abnormalities were also evident in sJIA. In the oxidative phosphorylation group, which was over-represented only in sJIA, most of the mitochondrial molecules encoded by mitochondrial DNA were suppressed while those encoded by genomic DNA were not. Corticosteroid treatment might affect the gene expression in this category because the corticosteroid doses in sJIA were higher than those in polyJIA. However, the corticosteroid dose did not correlated with the expression of mitochondrial genes. Overall, there were no over-represented categories correlated to any other drug therapies. In addition, the increase in the proportion of neutrophils and the decrease in the proportion of lymphocytes might affect the gene expression because the mRNA from whole blood cells was analysed in this study. However, there was no correlation between the gene expression and the proportion of either neutrophils or lymphocytes. Therefore, the decrease in the expression of various genes, including key genes such as MT-CO1 and MT-CO2, should be characteristic of sJIA. MT-CO1 and MT-CO2 are subunits of COX, which is the terminal component of the mitochondrial respiratory chain and transfers electrons from reduced cytochrome *c* to molecular oxygen. The mammalian COX is an enzymatic complex of inner mitochondrial membrane composed of 13 subunits. Three of them (MT-CO1, 2 and 3) are encoded by mitochondria DNA and the remaining 10 subunits (COX4, 5A, 5B, 6A, 6B, 6C, 7A, 7B, 7C and 8) are encoded by genomic DNA. MT-CO1 and MT-CO2 form the catalytic centre of the enzyme while MT-CO3 and 10 other subunits play structural and regulatory roles.[17] Thus, MT-CO1 and MT-CO2 are key subunits of COX. Indeed, mutations in either MT-CO1 or MT-CO2 have been reported to induce severe reduction of COX activity and mitochondrial damage.[18 19] Moreover, NRF1[20] and OPA1,[21] of which the expression is induced by the synthesis of new mitochondrial components, and SLC25A4,[22] of which expression is induced by mitochondrial damage, were upregulated only in sJIA. These findings strongly support the existence of mitochondrial damage in sJIA. Since TNF induces mitochondrial damage,[23] over-production of TNF may lead to the downregulation of mitochondrial DNA-encoded genes.

MAS is a life-threatening complication observed in sJIA but not in polyJIA. While the aetiology of MAS is unknown, it is thought to be partly caused by IFNγ released from activated T cells and proinflammatory cytokines, particularly TNF, from activated macrophages.[24] Our data clearly showed that IFNγ and TNF cascades were activated. Therefore, patients with active disease are likely to develop MAS. Mitochondrial damage may also contribute to the development of MAS because MAS is often successfully treated by ciclosporin A (CsA), which suppresses cytokine release from activated T cell and stabilises the mitochondrial membrane.[25] CsA is more effective for MAS than methylprednisolone, which has no efficacy in stabilising the mitochondrial membrane.[26] Therefore, stabilisation of the mitochondrial membrane may be important to control MAS. Because TNF induces mitochondrial damage through the instability of mitochondrial membrane,[23] inhibition of TNF may protect mitochondrial damage. Success in the treatment of MAS by TNF blocker supports this idea.[27 28] However, reports that MAS has been observed in some patients with sJIA during treatment with TNF blockers suggest that factors other than TNF may contribute to mitochondrial damage and consequently to MAS.[29 30] Further study will be required to know the exact mechanisms causing mitochondrial damage and MAS.

Our data indicates that sJIA is not only an immunological disease but also a metabolic disease involving mitochondrial disorder. This study also encouraged us to use bioinformatics tools together with microarray analysis to study autoimmune diseases of which the aetiology or the pathological conditions are not clear.

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interaction and strengthens the conclusion that IL6 system has been activated to counteract SS-associated fatigue. Third, as the IL6 system leads to upregulation of DHEA, this hypothesis was further confirmed by a decrease of sIL6R during DHEA substitution treatment.

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Interleukin 11 and paired immunoglobulin-like type 2 receptor α expression correlates with the number of joints with active arthritis in systemic juvenile idiopathic arthritis

Systemic juvenile idiopathic arthritis (sJIA) is characterised by systemic inflammatory symptoms such as spiking fever, skin rash, pericarditis and hepatosplenomegaly, along with arthritis.[1] We reported the abnormal expression of genes involved in cytokine networks and mitochondrial function in patients with sJIA identified by DNA microarray.[2] The current study was performed to extend these results.

To identify genes that correlate with arthritis severity or systemic inflammation in patients with sJIA, we further analysed the 3491 genes identified by prior microarray analysis to be abnormally expressed in the peripheral blood of 51 patients with sJIA.[2] They all fulfilled International League of Associations for Rheumatology (ILAR) criteria.[3] Of these genes, 2267 were annotated in the Expression Analysis Systematic Explorer system (EASE) V. 2.0.[4] The statistical correlation between the expression level of each gene, the number of joints with active arthritis (median: 4, range: 0–39) and the serum level of C-reactive protein (CRP) (median: 4.3 mg/dl, range: 1.6–19 mg/dl), as a marker of the systemic inflammation, was analysed using the Pearson product-moment correlation coefficient.

Genes with expression levels showing a correlation index more than 0.4 or less than −0.4 with the number of joints with active arthritis and CRP levels are shown in tables 1 and 2. A total of 10 genes, including interleukin 11 (IL11) and paired immunoglobulin-like type 2 receptor α (PILRA), correlated with the number of joints possessing active arthritis. The expression levels of IL11 were upregulated in patients with sJIA compared to healthy children and positively correlated with the number of joints with active arthritis ($r = 0.48$). Because IL11 reportedly induces osteoclast formation by a receptor activator of nuclear

Table 1 Correlation of gene expression with the number of joints with active arthritis

Gene name	Index of correlation
Positive correlation:	
Interleukin 11	0.48
Plant homeodomain (PHD) finger protein 21A	0.40
Negative correlation:	
Ras-related GTP binding B	−0.51
Transient receptor potential cation channel, subfamily M, member 3	−0.45
Reticulon 3	−0.45
Paired immunoglobulin-like type 2 receptor α	−0.43
Positive cofactor 2 (PC2; multiprotein complex) glutamine/Q-rich-associated protein	−0.42
Su(var)3-9, enhancer-of-zeste, trithorax (SET) domain containing 5	−0.42
Glucosidase I	−0.41
Ubinuclein 1	−0.41

Genes identified correlated with the number of joints with active arthritis. Index of correlation was examined using the Pearson product-moment correlation coefficient test. The genes with expression levels showing a correlation index of more than 0.4 or less than −0.4 with the number of joints with active arthritis are shown.

Table 2 Correlation of gene expression with the serum levels of C-reactive protein (CRP)

Gene name	Index of correlation
Positive correlation:	
H2A histone family, member X	0.42
Methyltransferase like 9	0.41
Complement component 1, q subcomponent binding protein	0.40
Negative correlation:	
Mucin 5B, oligomeric mucus/gel-forming	−0.47
Coiled-coil domain containing 113	−0.46
Plexin B2	−0.44
Brain-specific angiogenesis inhibitor 2	−0.43
DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57	−0.43
Cat eye syndrome chromosome region, candidate 2	−0.41
Zinc finger, DHHC-type containing 1	−0.40

Genes identified correlated with the serum levels of CRP. Index of correlation was examined using the Pearson product-moment correlation coefficient test. The genes with expression levels showing a correlation index of more than 0.4 or less than −0.4 with CRP levels are shown.

factor κB ligand (RANKL)-independent mechanism, it may be involved in joint destruction.[5] PILRA expression was down-regulated in patients with sJIA and negatively correlated with the number of joints with active arthritis ($r = -0.43$). A signal through PILRA containing immunoreceptor tyrosine-based inhibitory motifs may contribute to counterbalancing the immunoreceptor tyrosine-based activation motifs signal in osteoclast differentiation cooperated with RANKL.[6–8] Therefore, down-regulation of PILRA may also be involved in joint destruction. Additionally, eight more molecules were shown to statistically correlate with the number of joints with active arthritis, but the roles of these molecules in arthritis are still unclear.

Similarly, 10 genes correlated with CRP levels: 3 positively and 7 negatively. The former includes complement component 1q-binding protein (C1QBP), which is expressed on T cells, B cells and monocytes. A signal through C1QBP regulates the proliferation and differentiation of these cells, although the specific role of C1QBP in inflammatory responses is still to be elucidated. Since C1QBP is upregulated by inflammatory cytokines, upregulation of C1QBP may reflect the result of systemic inflammation.[9 10] Other molecules, such as mucin 5B and brain-specific angiogenesis inhibitor 2, have known functions, but others do not. Regardless, the pathological role of these molecules in systemic inflammation is not clear.

Our data indicate that IL11, PILRA and the expression of eight other molecules correlates with the number of joints with active arthritis in patients with sJIA. Further study will be required to understand their exact pathological roles in sJIA.

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Likelihood ratios as a function of antibody concentration for anti-cyclic citrullinated peptide antibodies and rheumatoid factor

Anti-cyclic citrullinated peptide (CCP) antibodies and rheumatoid factor (RF) are recommended screening tests for rheumatoid arthritis.[1] In a meta-analysis,[2] the sensitivity of anti-CCP (67%) for diagnosing rheumatoid arthritis was comparable to the sensitivity of RF (69%).[2] The specificity of anti-CCP for rheumatoid arthritis (95%) was higher than the specificity of RF (85%).[2] Consequently, the positive likelihood ratio was higher for anti-CCP (12.46) than for RF (4.86).[2]

Studies that addressed the clinical usefulness of anti-CCP used a single cut-off value and, hence, likelihood ratios were calculated based on a single cut-off. In the present letter, we illustrate how likelihood ratios for anti-CCP and RF depend on the antibody level. Our calculations were based on a clinically well defined group of patients with rheumatoid arthritis ($n = 85$), diseased controls ($n = 165$) (including psoriatic arthritis, connective tissue disease and organ specific autoimmune

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Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease

Norihiro Nishimoto, Kimio Terao, Toru Mima, Hideko Nakahara, Nobuhiro Takagi and Takahiro Kakehi

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Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti–IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease

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Interleukin-6 (IL-6) plays pathologic roles in immune-inflammatory diseases such as rheumatoid arthritis (RA) and Castleman disease. By inhibiting IL-6 receptors (IL-6Rs), tocilizumab (a humanized anti–IL-6R antibody) ameliorates the symptoms of these diseases and normalizes acute-phase proteins, including C-reactive protein (CRP). We found that tocilizumab treatment increased serum levels of IL-6 and soluble IL-6R (sIL-6R). To investigate the pathologic significance of these increases, we analyzed the kinetics of serum IL-6 and sIL-6R and the proportion of sIL-6R saturated with tocilizumab after tocilizumab administration in patients with RA and Castleman disease and then compared the results with the CRP values. Serum IL-6 and sIL-6R markedly increased after tocilizumab administration in both RA and Castleman disease. As long as free tocilizumab was detectable, sIL-6R was saturated with tocilizumab and IL-6 signaling was completely inhibited. We concluded that it is likely that sIL-6R increased because its elimination half-life was prolonged by the formation of tocilizumab/sIL-6R immune complex, and that free serum IL-6 increased because IL-6R–mediated consumption of IL-6 was inhibited by the unavailability of tocilizumab-free IL-6R. We also concluded that the increased level of free IL-6 during tocilizumab treatment closely reflects the actual endogenous IL-6 production and true disease activity. (Blood. 2008;112:3959-3964)

Introduction

Interleukin-6 (IL-6) is a multifunction cytokine that has a wide range of biological activities in various target cells and regulates immune responses, acute phase reactions, hematopoiesis, and bone metabolism.[1] IL-6 signaling is mediated by a unique IL-6 receptor system consisting of 2 functional membrane proteins: an 80-kDa ligand-binding chain (known as IL-6 receptor [IL-6R], IL-6R α-chain, or CD126)[2] and a 130-kDa non–ligand-binding signal-transducing chain (known as glycoprotein 130 [gp130], IL-6R β-chain, or CD130).[3] In cells with sufficient membrane-bound IL-6R, IL-6 binds to these receptors, the IL-6/IL-6R complex induces homodimerization of the gp130 molecule, and a high-affinity functional receptor complex of IL-6, IL-6R, and gp130 is formed.[4] In cells that do not express sufficient cell-surface IL-6R, IL-6 signal transduction starts with the binding of IL-6 to the free soluble form of IL-6R (sIL-6R), which lacks the membrane and intracytoplasmic portion of the 80-kDa membrane-bound IL-6R molecule.[3,4] Thus, either membrane-bound or soluble IL-6R can mediate IL-6 signal into cells, as long as they express gp130. Considerable amounts of sIL-6R are observed in serum and body fluids,[5,6] and sIL-6R may play physiologic roles as well as its pathologic role in immune-inflammatory and malignant diseases.[7]

Because IL-6 plays important physiologic roles, deregulated overproduction of IL-6 causes various pathologic conditions, including autoimmune, inflammatory, and lymphoproliferative disorders. It has been shown that IL-6 is involved in immune-inflammatory diseases such as rheumatoid arthritis (RA), Castleman disease, juvenile idiopathic arthritis (JIA), and Crohn disease.[8-11] Elevated serum IL-6 has been observed in patients with these diseases and the IL-6 levels correlate with disease activity,[5,10,12-14] although there are differences in IL-6 levels among the diseases.

Tocilizumab is a humanized anti-human IL-6R antibody engineered by grafting the complementarily determining regions of a mouse anti–human IL-6R antibody into human IgG1κ to create a human antibody with a human IL-6R binding site.[15] Tocilizumab binds to the IL-6 binding site of human IL-6R and competitively inhibits IL-6 signaling. A series of clinical studies have shown that inhibition of IL-6 signaling by tocilizumab is therapeutically effective in RA, JIA, Castleman disease, and Crohn disease.[16-20] In all of these diseases, tocilizumab ameliorates inflammatory manifestations and normalizes acute phase protein levels, including C-reactive protein (CRP), thus confirming the observation that IL-6 is essential for the production of CRP.

It was noticed that both serum IL-6 and serum sIL-6R increased in patients after administration of tocilizumab while the disease symptoms continued to be ameliorated, but the mechanisms of these increases and the pathologic significances of the increased levels remained obscure.

The objective of this study, therefore, was to elucidate the mechanisms and the pathologic significances of the increases in serum IL-6 and serum sIL-6R that occur when IL-6 signaling is inhibited by tocilizumab. To achieve this, we analyzed serum and blood samples collected in the previous clinical studies, and considered the results in combination with laboratory data already obtained in those studies.

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Methods

Participants and tocilizumab treatment methods

The serum and blood samples and laboratory data analyzed in this study were from the following people: (1) 20 healthy adult volunteers who received tocilizumab in a phase 1 study (N.N. and T.K., unpublished data, November 1998); (2) 15 patients with RA who were treated with tocilizumab in a phase 1/2 study in RA;[16] (3) 18 patients with RA who were treated with tocilizumab in a clinical pharmacology study in RA (K.T., T. Tsuru, M. Suzaki, T. Amamoto, H. Nakashima, S. Higuchi, T.K., and N.N., unpublished data, August 2008); and (4) 28 patients with Castleman disease who were treated with tocilizumab in a phase 2 study in Castleman disease.[19] All of these clinical studies were approved by the Japanese Ministry of Health, Labor and Welfare and also by the independent ethics committees of the respective medical institutions. Written informed consent was obtained from the patients before enrollment in accordance with the Declaration of Helsinki.

The designs of these studies were as follows: (1) phase 1 study—a randomized, single-blind, placebo-controlled, intersubject dose-escalation study in which healthy adult men received 0.15, 0.5, 1, or 2 mg/kg tocilizumab intravenously over 2 hours (n = 5/cohort), and safety, tolerability and pharmacokinetics were evaluated; (2) phase 1/2 study in RA[16]—an open-label trial in which 15 patients with active RA received 2, 4, or 8 mg/kg tocilizumab intravenously over 2 hours every 2 weeks for 6 weeks (n = 5/cohort), and safety, tolerability, pharmacokinetics, and efficacy at each dosage level were assessed on days 0, 14, 28, and 42. CRP and erythrocyte sedimentation rate (ESR) were measured as markers of IL-6R inhibition; (3) clinical pharmacology study in RA—an open-label single-dose study in which 18 patients with baseline CRP of at least 1.5 mg/dL received 8 mg/kg tocilizumab intravenously over 1 hour, and drug-disease interaction was assessed; (4) phase 2 study in Castleman disease[19]—an open-label study in which 28 patients with active Castleman disease received 8 mg/kg tocilizumab intravenously over 1 hour every 2 weeks for 16 weeks (8 times in total), and improvement in disease activity was assessed based on biochemical markers such as CRP, hemoglobin, and serum albumin, and on general fatigue (visual analog scale).

Tocilizumab is a humanized anti–human IL-6R monoclonal antibody of the IgG1κ subtype, and was supplied by Chugai Pharmaceutical (Tokyo, Japan).

Determination of free tocilizumab in serum

Serum-free tocilizumab was determined by enzyme-linked immunosorbent assay (ELISA). In brief, 100 μL human sIL-6R (SR-344, 1 μg/mL; Chugai Pharmaceutical) was added to the wells of an immunoplate precoated with mouse anti–human IL-6R antibody (MT-18; Chugai Pharmaceutical), and the plate was incubated at room temperature for 2 hours. After washing, 100 μL of 1000-fold diluted serum specimen was applied to the plate, and the plate was incubated for another 2 hours to bind the free tocilizumab to the plate. After washing, the bound tocilizumab was measured using biotin-labeled goat anti–human IgG antibody, followed by development with avidin-labeled alkaline phosphatase and p-nitrophenyl phosphate substrate. The colorimetric reaction was quantified by measuring the absorbance at 405 nm (and at 490 nm as reference) using a microplate reader. The concentration of free tocilizumab in the specimen was calculated from a calibration curve prepared from the absorbance of calibration standard solutions. The lower detection limit for free serum tocilizumab was 1 μg/mL. All serum samples were stored below −20°C, and the assay was applied within 4 weeks after drawing.

Determination of IL-6 in serum

Serum IL-6 was determined by chemiluminescent enzyme immunoassay (CLEIA)[21] using a Human IL-6 CLEIA Fujirebio (Fujirebio, Tokyo, Japan), which detects IL-6 whether it is free or bound to sIL-6R. In brief, a mixture of 20 μL of serum sample and the alkaline phosphatase–conjugated mouse anti–human IL-6 monoclonal antibody included in the kit was incubated at 37°C for 10 minutes and then added to particles covalently linked to another murine anti–human IL-6 monoclonal antibody that recognizes an epitope different from that recognized by the original antibody. After incubation for 10 minutes at 37°C, the particles were separated magnetically and washed in buffer. Subsequently, an enhanced luminol/peroxide substrate solution containing 3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy)phenyl-1,2-dioxetane disodium salt was added at 37°C, and after 5 minutes the chemiluminescence was measured using a photon counter (Lumipulse 1200′ Fujirebio). The lower detection limit for serum IL-6 was 0.1 pg/mL. All serum samples were stored below −20°C, and the assay was applied within 4 weeks after drawing.

Determination of sIL-6R in serum

Serum sIL-6R was determined by ELISA using a Quantikine Human IL-6sR kit (R&D Systems, Minneapolis, MN). We preliminarily examined and found that this assay kit could detect 3 forms of sIL-6R: sIL-6R free from IL-6 or tocilizumab, sIL-6R in a complex of IL-6/sIL-6R, and sIL-6R in an immune-complex of tocilizumab/sIL-6R (data not shown). In brief, sIL-6R in the sample was bound to a murine anti–IL-6R monoclonal antibody–coated microtiter plate and incubated with a horseradish peroxidase (HRP)–conjugated polyclonal anti–IL-6R antibody at room temperature for 2 hours. To determine the quantity of peroxidase bound, the plate was incubated with tetramethylbenzidine at room temperature for 20 minutes. The colorimetric reaction was quantified by measuring the absorbance at 450 nm (and at 540 nm as reference) using a microplate reader and the concentration of sIL-6R in the sample was calculated as described in "Determination of free tocilizumab in serum." All serum samples were stored below −20°C, and applied the assay within 4 weeks after drawing.

Determination of the proportions of tocilizumab-bound and tocilizumab-free sIL-6R in serum

Tocilizumab-bound sIL-6R and tocilizumab-unbound sIL-6R in serum samples were separated by gel filtration chromatography in a Superdex 200HR 10/30 column (300 × 10 mm internal diameter [ID]; GE Healthcare Bio-Science AB, Uppsala, Sweden) at 4°C, and the amount of sIL-6R in each fraction was determined. The eluent was phosphate-buffered saline with 0.05% Tween 20, and the fraction volume was 0.5 mL.

Determination of IL-6 mRNA expression

In the clinical pharmacology study in RA (study 3), peripheral blood for measurement of IL-6 mRNA was drawn from 18 patients at 4 time points: 7 days and immediately before, and 14 and 35 days after administration of 8 mg/kg tocilizumab.

Total RNA was extracted from the blood samples using a PAXgene Blood RNA Kit (QIAGEN, Valencia, CA) and quantified spectrophotometrically by measuring absorbance at 260 nm. The purity and integrity of the RNA extracted from each PAXgene Blood RNA tube were estimated by the ratio of 28S to 18S ribosomal RNA using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA samples that showed severe degradation were excluded from further analysis. Expression of IL-6 mRNA was measured using a DNA microarray (Human oligo chip; Hitachi Software Engineering, Tokyo, Japan). After amino allylRNA (aRNA) was synthesized from 1 μg total RNA using the Amino Allyl Message Amo aRNA kit (Ambion, Austin, TX), the Cy3 and Cy5 dyes were then used to label RNA sequences. Human peripheral blood leukocyte total RNA purchased from BD Biosciences Clontech (San Jose, CA) was used as a universal control (reference RNA) and labeled with Cy3 in all the microarray experiments so that the ratio (Cy5/Cy3) means the relative expression level to the reference in the time course experiment series. The ratio (relative expression level) was plotted against the sampling time. All RNA samples were stored below −80°C until applied the assay. The DNA microarray dataset is available at the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE12653.

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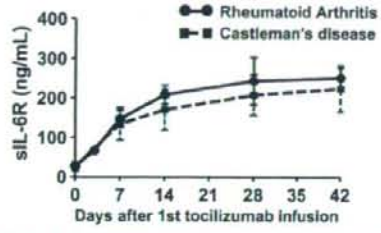

Figure 1. Serum sIL-6R after tocilizumab infusion. Tocilizumab was administered to patients with RA (●; n = 5) or Castleman disease (■; n = 28) on days 0, 14, and 28 at a dose of 8 mg/kg. Points and error bars show geometric means plus or minus SDs.

Determination of CRP

CRP was determined in all clinical studies in which the serum was collected. CRP levels were measured at each clinical study site.

Results

Elevation of serum sIL-6R after administration of tocilizumab

When patients with RA or Castleman disease were treated with 8 mg/kg tocilizumab once every 2 weeks, serum sIL-6R increased markedly in patients with a detectable serum tocilizumab concentration (1 μg/mL or more) and reached a steady state at day 42 of treatment (RA: 27.7 ± 4.4 ng/mL at baseline, 251.4 ± 24.7 ng/mL at day 42; Castleman disease: 26.4 ± 11.6 ng/mL at baseline, 224.2 ± 58.3 ng/mL at day 42; Figure 1). There was no significant difference between patients with RA and patients with Castleman disease with respect to either serum sIL-6R at baseline or serum sIL-6R at day 42. In the healthy individuals, increase in serum sIL-6R was transient because of single administration of tocilizumab (Figure 2A).

Relationship between free tocilizumab and the proportions of tocilizumab-free and tocilizumab-bound sIL-6R in serum

Total serum sIL-6R peaked 14 days after a single administration of 2 mg/kg tocilizumab to healthy individuals in the phase 1 study. Serum sIL-6R was tocilizumab-free before administration of tocilizumab, but after administration of tocilizumab, more than 95% of the sIL-6R molecules were bound in a sIL-6R/tocilizumab immune complex as long as the free tocilizumab concentration remained detectable in serum (at least 1 μg/mL). Representative data are shown in the Figure 2A. Similar data were obtained in the sIL-6R, which increased after repetitive administration of tocilizumab to patients with RA in the phase 1/2 study (Figure 2B). When free tocilizumab concentration fell below the detection limit in serum, the level of sIL-6R/tocilizumab complex decreased, and the proportion of free sIL-6R increased (Figure 2A,B).

Relationship between free tocilizumab and CRP level in serum

CRP is a representative acute-phase reactant, and change in CRP correlates with severity of inflammation. It has been shown that inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor induce the production of CRP by hepatocytes, and that IL-6 is essential for the production of CRP.[22]

Tocilizumab normalized the CRP level in patients with RA in the phase 1/2 study[16] as long as the free tocilizumab, which is capable of binding IL-6R and of inhibiting IL-6 actions, remained

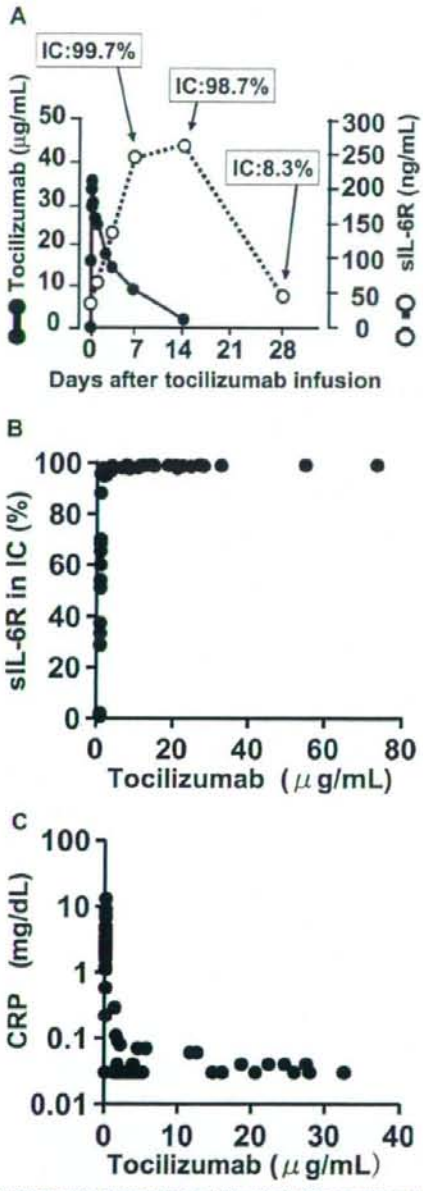

Figure 2. Relationships between free tocilizumab, sIL-6R, percentage of sIL-6R bound to tocilizumab, and CRP in serum. (A) Relationship between serum free tocilizumab (●), serum sIL-6R (○), and the percentage of sIL-6R bound to tocilizumab in an immune complex (IC). Serum sIL-6R (○) includes all sIL-6R: free, bound to tocilizumab, and bound to IL-6. Increased sIL-6R after tocilizumab infusion formed an immune complex with tocilizumab. Almost all sIL-6R was bound to tocilizumab while serum-free tocilizumab was detectable (1 μg/mL or more). This figure shows a representative data from the phase 1 study in healthy individuals. (B) Relationship between serum tocilizumab and percentage of sIL-6R bound to tocilizumab (phase 1/2 study in RA). More than 95% of sIL-6R was bound to tocilizumab while serum-free tocilizumab remained 1 μg/mL or more. (C) Relationship between serum tocilizumab and CRP. Serum CRP was normalized as long as the free tocilizumab concentration remained 1 μg/mL or more (phase 1/2 study in RA). The sensitivity of CRP assay in the present study was 0.03 mg/dL, and the normal range was less than 0.2 mg/dL.

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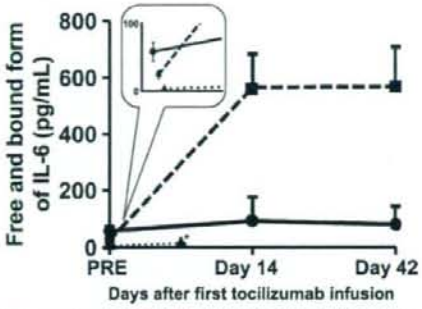

Figure 3. Change in serum IL-6 after administration of tocilizumab. Serum IL-6 increased after tocilizumab infusion and reached steady state at different levels in patients with RA (●; n = 5), patients with Castleman disease (■; n = 28), and healthy volunteers (△; n = 4). Points and error bars show geometric means plus or minus SEs. *Samples from healthy volunteers were drawn on day 7.

above 1 μg/mL in serum (Figure 2C). This shows that tocilizumab effectively inhibits IL-6 signaling when it is detectable in serum.

Serum IL-6 after administration of tocilizumab

Serum IL-6 concentrations after a single dose of tocilizumab in healthy adult volunteers after the start of dosing (once every 2 weeks) in patients with RA, and in patients with Castleman disease were compared. At baseline, serum IL-6 was significantly higher in RA and Castleman disease than the normal range (< 4 pg/mL). Note that serum IL-6 was significantly higher in RA than in Castleman disease (58.4 ± 13.8 pg/mL vs 24.5 ± 6.5 pg/mL [geometric mean ± SE]; $P < .05$), yet serum CRP was significantly higher in Castleman disease than in RA (8.7 ± 5.0 mg/dL vs 5.4 ± 1.7 mg/dL; $P < .05$) and serum IgG was also higher in Castleman disease than in RA (5220 ± 1957 mg/dL vs 1516 ± 409 mg/dL; $P < .05$).

In healthy volunteers, serum IL-6 showed a significant increase at day 7 after tocilizumab administration (3.0 ± 0.6 pg/mL at baseline, 9.3 ± 1.0 pg/mL at day 7). In patients with RA, serum IL-6 showed a greater increase at day 14 and had not changed at day 42 (58.4 ± 13.8 pg/mL at baseline, 92.8 ± 82.4 pg/mL at day 14, 89.7 ± 63.7 pg/mL at day 42; n = 5). In patients with Castleman disease, serum IL-6 showed an even more significant increase at day 14, even though it was significantly lower in Castleman disease than in RA at baseline, and had not changed at day 42 (24.5 ± 6.5 pg/mL at baseline, 564.8 ± 127.6 pg/mL at day 14, 567.0 ± 141.0 pg/mL at day 42; n = 28; RA vs Castleman disease: $P < .001$ at day 14 and at day 42; Figure 3). These data clearly indicate that the degree of increase in serum IL-6 after IL-6R inhibition is not the same in RA and Castleman disease.

Furthermore, there observed much stronger correlation between the CRP at baseline and the serum IL-6 increasing after IL-6R blockade than that between the CRP at baseline and the serum IL-6 before IL-6R blockade in patients with RA and in Castleman disease (Figure 4).

IL-6 mRNA expression in peripheral blood cells before and after administration of tocilizumab

In order to know whether the IL-6R inhibition augmented the IL-6 production through the elimination of possible negative feedback by IL-6 on IL-6 production, changes in IL-6 mRNA expression after tocilizumab administration in peripheral blood cells were examined. However, there was no significant difference in IL-6 mRNA

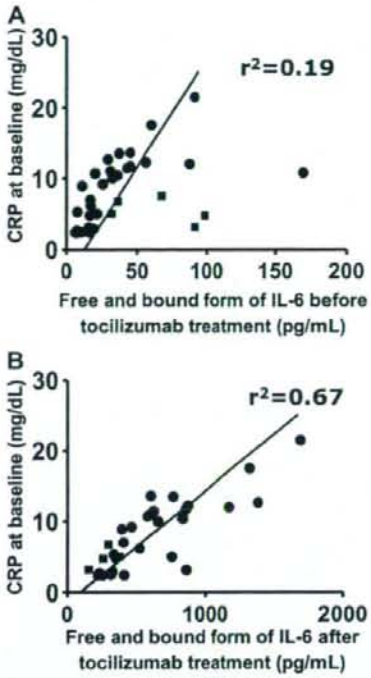

Figure 4. Correlation between serum CRP at baseline and serum IL-6 at baseline or after tocilizumab administration. ■ and ● show patients with RA and patients with Castleman disease, respectively. The *x*-axis shows (A) serum IL-6 at baseline (r^2 = .19) and (B) serum IL-6 after tocilizumab infusion (r^2 = .67).

expression at these 4 sampling time points, so administration of tocilizumab did not increase the production of IL-6 (Figure 5).

Discussion

This study demonstrates that IL-6R inhibition with tocilizumab results in an increase in the levels of serum IL-6 and serum sIL-6R. The normalization of serum CRP, however, shows that IL-6 signaling was inhibited as long as free tocilizumab was detectable in serum. This matches the finding that a very high percentage of

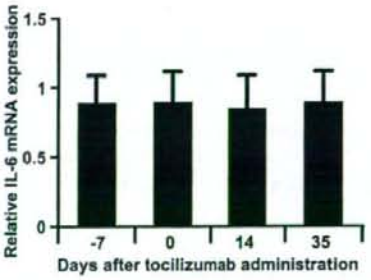

Figure 5. Relative expression of IL-6 mRNA. The relative expression levels of IL-6 mRNA compared with the reference calculated by the logarithm base 2 of ratio (Cy5/Cy3) at each time point were plotted. Expression of IL-6 mRNA in peripheral blood cells from 18 patients with RA was measured using a DNA microarray before and after administration of tocilizumab. Bars and error bars show means plus SEs.

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serum sIL-6R (> 95%) was bound to tocilizumab while free tocilizumab was detectable in serum. Since CRP is mainly produced by hepatocytes, which express cell-surface IL-6R, membrane-bound IL-6R would be also fully occupied by tocilizumab. CRP is therefore a useful surrogate marker for tocilizumab levels that are high enough to inhibit the effects of IL-6 in patients.

Since immune complex formation of antigen and antibody prolongs the elimination half-life of antigen in serum,[23] the increase in serum sIL-6R seen after administration of tocilizumab is probably due to the formation of a sIL-6R/tocilizumab immune complex. This hypothesis is supported by the decrease in the C3, C4, and CH50 levels after tocilizumab administration that we observed in another study (data not shown) because complement factors are consumed during the elimination process of immune complexes.[23]

It is noteworthy that there was no difference between RA and Castleman disease with respect to serum sIL-6R either at baseline or after tocilizumab administration. This suggests that there is no difference in sIL-6R production between these 2 diseases.

For serum IL-6, on the other hand, the level after tocilizumab administration differed greatly between the diseases as well as between individual patients (Figures 3,4). This suggested to us that the degree of increase in serum IL-6 after inhibition of IL-6R by tocilizumab may reflect different levels of endogenous IL-6 production in these diseases and in individual patients.

The serum IL-6 level depends on the balance between IL-6 production and elimination, so tocilizumab could potentially have caused serum IL-6 to increase either by stimulating production or by inhibiting elimination. One possibility is that tocilizumab might stimulate the production of IL-6 if its blockade of IL-6 signaling inhibits a negative feedback effect of IL-6 on IL-6 production. This seems unlikely, however, because serum IL-6 did not continue to increase but remained steady between day 14 and day 42, and because there was no significant increase in IL-6 mRNA expression in peripheral blood cells after administration of tocilizumab. The relevance of the latter observation may be limited, however, by the fact that we did not examine IL-6 mRNA expression in cells of the affected joints of patients with RA or the affected lymph nodes of patients with Castleman disease, which are important sources of IL-6 in these diseases.

Another possible explanation for the increase in serum IL-6 after tocilizumab administration is that tocilizumab may inhibit the clearance of IL-6 from serum. There are 2 possible elimination pathways of IL-6 from serum: one is receptor-mediated clearance via the binding of IL-6 to IL-6R; the other is direct degradation of IL-6 protein. The main elimination pathway may be receptor-mediated clearance. If so, this would explain why free IL-6 accumulates in serum when IL-6R is occupied by tocilizumab. IL-6 levels reach a steady state when the IL-6 production rate matches the IL-6 degradation rate.

We would like to explain this mechanism of action with the help of the schematic bathtub model illustrated in Figure 6. In this model, endogenous IL-6 production is represented by the stream of water flowing from the faucet into the tub at a constant rate that depends on the level of true disease activity. Receptor-mediated IL-6 clearance is represented by water flowing out of the bathtub drain. Direct degradation of IL-6 is represented by minor water flowing out from the side small drain in this figure. When the bathtub drain is plugged, the water level will depend on the flow rate from the faucet. Likewise, when IL-6R is inhibited by tocilizumab, the serum IL-6 level will reflect the actual level of endogenous IL-6 production that correlates with the level of true

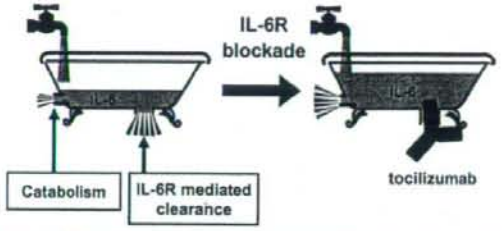

Figure 6. Schematic model of the mechanism by which serum IL-6 is increased when IL-6 receptor is blocked by tocilizumab. The bathtub model explains the elimination of IL-6 from serum before and after administration of tocilizumab. The rate of water flowing from the faucet into the tub (the IL-6 production rate) remains constant. Before tocilizumab administration, the rate of water flowing out of the bathtub (the elimination of receptor-bound IL-6 from serum and IL-6 catabolism) is also constant, so the water level (serum IL-6 level) remains constant. In the second diagram, the flow of IL-6 from the bathtub is greatly restricted by a "plug" (IL-6R–mediated elimination is inhibited by tocilizumab). The water level increases and then remains constant at a higher level (serum IL-6 increases to a new steady-state level when the IL-6 production rate matches the IL-6 degradation rate).

disease activity while inflammatory symptoms are ameliorated by the inhibition of IL-6 signaling through IL-6R.

In practice, the correlation between CRP (an indicator of resultant inflammation) at baseline and serum IL-6 level after administration of tocilizumab was much closer than that between CRP at baseline and serum IL-6 level before tocilizumab administration. Furthermore, serum IL-6 was much higher in patients with Castleman disease than in patients with RA after tocilizumab administration, even though serum IL-6 in patients with Castleman disease was lower than that in patients with RA at baseline. The difference in increased IL-6 level between RA and Castleman disease after tocilizumab treatment closely reflected the difference between RA and Castleman disease in baseline inflammatory activity and in laboratory abnormalities such as increased CRP and IgG values, whereas the IL-6 levels before tocilizumab treatment did not. The fact that IL-6 was lower in Castleman disease than in RA at baseline indicates that the elimination of serum IL-6 is much faster in Castleman disease than in RA without tocilizumab treatment, and the fact that this faster elimination in Castleman disease was greatly slowed by tocilizumab suggests that it is receptor-mediated elimination.

We conclude that the serum IL-6 level during inhibition of IL-6R by tocilizumab represents the actual endogenous production of IL-6 and the true disease activity of patients with different diseases much better than the serum IL-6 level before tocilizumab treatment. If the causal factors of IL-6 overproduction are neutralized by adequate therapy (the faucet of the bathtub is closed), the serum IL-6 level will decrease by natural protein degradation. Decrease in serum IL-6 during tocilizumab treatment may therefore indicate disease remission and may allow us to safely discontinue tocilizumab treatment without the risk of an acute flare. This idea remains to be confirmed in future studies.

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Authorship

Contribution: N.N. wrote the manuscript; N.N. and K.T. planned the study and wrote the study protocol; N.N., T.M., and H.N. treated and documented the patients; K.T., N.T., and T.K. organized and monitored the study; and K.T. performed the statistical evaluation.

Conflict-of-interest disclosure: K.T., N.T., and T.K. are employees of Chugai Pharmaceutical, whose product (tocilizumab) was studied in the present work. N.N. and T.M. received a consulting fee as medical advisers from Chugai Pharmaceutical. N.N. is on the scientific advisory board of Hoffmann-La Roche. H.N. declares no competing financial interests.

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Long-term safety and efficacy of tocilizumab, an anti-interleukin-6 receptor monoclonal antibody, in monotherapy, in patients with rheumatoid arthritis (the STREAM study): evidence of safety and efficacy in a 5-year extension study

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Long-term safety and efficacy of tocilizumab, an anti-interleukin-6 receptor monoclonal antibody, in monotherapy, in patients with rheumatoid arthritis (the STREAM study): evidence of safety and efficacy in a 5-year extension study

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Running head: Anti-IL-6R antibody therapy in RA

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ABSTRACT

Objectives. To evaluate the safety and efficacy of 5-year, long-term tocilizumab monotherapy for patients with rheumatoid arthritis.

Methods. In an open-label, long-term extension trial following an initial 3-month randomised phase II trial, 143 of the 163 patients who participated in the initial blinded study received tocilizumab monotherapy (8 mg/kg) every 4 weeks. Concomitant therapy with NSAIDs and/or oral prednisolone (10 mg daily maximum) was permitted. All patients were evaluated with American College of Rheumatology (ACR) improvement criteria, disease activity score (DAS)28, and EULAR response, as well as for safety issues.

Results. One hundred and forty-three patients were enrolled in the open-label, long-term extension trial and 94 (66%) patients had completed 5 years as of March 2007. Thirty-two patients (22%) withdrew from the study due to adverse events and one patient (0.7%) due to unsatisfactory response. Fourteen patients withdrew because of the patient's request or other reasons. The SAE rate was 27.5 events per 100 patient-years, with 5.7 serious infections per 100 patient-years, based on a total tocilizumab exposure of 612 patient-years. Of the 88 patients receiving corticosteroids at baseline, 78 (88.6%) were able to decrease their corticosteroid dose, and 28 (31.8%) discontinued corticosteroids. At 5 years, 79/94 (84.0%), 65/94 (69.1%) and 41/94 (43.6%) of the patients achieved ACR20, ACR50, and ACR70 improvement criteria, respectively. Remission defined as DAS28 less than 2.6 was achieved in 52/94 (55.3%) of the patients.

Conclusion. In this 5-year extension study, tocilizumab demonstrated sustained long-term efficacy and a generally good safety profile.

Key words: rheumatoid arthritis, clinical trial, interleukin-6, tocilizumab, long-term treatment

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Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by persistent synovitis and progressive joint damage [1]. Although the causes of RA are not fully understood, constitutive overproduction of interleukin-6 (IL-6), a multifunctional cytokine that regulates the immune response, inflammatory reaction, and bone metabolism, is thought to play a major pathological role in RA [2].

Tocilizumab is a humanised anti-human IL-6 receptor (IL-6R) monoclonal antibody [2], which has been demonstrated to improve the signs and symptoms of RA [3-9] and prevent radiographic progression [10] in previous clinical trials. Those controlled trials provided evidence for a rapid reduction in disease activity in response to tocilizumab in patients with active RA as measured by American College of Rheumatology (ACR) responses, disease activity scores (DAS) and a modified health assessment questionnaire (MHAQ) [5-9]. The efficacy was dose-related and 8 mg of tocilizumab per kilogram provided a marked clinical benefit. The success in the treatment of patients with RA using tocilizumab confirmed that IL-6 plays an important pathological role in RA and, further studies were therefore required to determine the long-term safety and efficacy of tocilizumab treatment. We report here the safety and efficacy of tocilizumab in a 5-year long-term extension study.

METHODS

Patients. This study was registered with http://www.clinicaltrials.gov (NCT00144651). The study protocol was approved by the Ministry of Health, Labor and Welfare of Japan, and by the ethical committee of each institute, and patients gave their written informed consent.

The eligibility criteria and the study design of the initial 12-week, randomised, double-blind, placebo-controlled study have been reported previously [5]. Briefly, eligible patients were 20 years of age or older and fulfilled the 1987 criteria for RA of the American Rheumatism Association [11] with a disease history of longer than 6 months. All subjects had been insufficient responders to treatment with at least one disease-modifying antirheumatic drug (DMARD) or immunosuppressant. Patients had active disease at the time of enrollment into the initial controlled trial as defined by the presence of six or more swollen joints, six or more tender joints, and one of the following two criteria: a Westergren erythrocyte sedimentation rate (ESR) of at least 30 mm/hour or a C-reactive protein (CRP) level of more than 1.0 mg/dL. Patients receiving prednisolone (10 mg daily maximum) and/or nonsteroidal anti-inflammatory drugs (NSAIDs) were eligible if the dose had not increased during the washout period of 1 month. Doses of both medications remained stable during the blinded study period of 12 weeks. Patients who had received tocilizumab or placebo twice or more were given the opportunity to receive tocilizumab in this open-label extension trial.

In the extension study, the use of prednisolone (10 mg daily maximum) and one NSAID was permitted. Sexually active premenopausal women were required to have a negative urine pregnancy test at entry and to use effective contraception during the study period.

Treatment. Patients were randomised to receive either placebo, or 4 or 8 mg per

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kilogram body weight (mg/kg) of tocilizumab every 4 weeks in the initial blinded 12-week trial. In the first 12 weeks of the open-label extension study, patients received 8 mg/kg of tocilizumab every 4 weeks and thereafter dose reduction and treatment interval changes (minimum 2 weeks) were allowed.

Efficacy assessments. Disease-activity was assessed at baseline and every visit during the initial blinded trial and the first 12 weeks of the extension study, and thereafter every 3 months. All patients were evaluated with ACR improvement criteria, DAS28 and EULAR response. The DAS28 was calculated using the ESR. Clinical assessments included the following: complete counts of swollen and tender joints (49 joints evaluated; cervical spine and hips evaluated only for tenderness); physician's and patient's global assessment of disease status, on a visual-analog scale from 0 (asymptomatic) to 100 (severe symptoms); patient's assessment of pain on a scale from 0 (no pain) to 100 (severe pain); functional disability measured with a modified health assessment questionnaire (MHAQ); ESR; and CRP levels [12]. Treatment time was calculated beginning with the first infusion of tocilizumab, excluding the time receiving placebo.

Safety assessments. Safety was assessed for all patients who received at least one dose of tocilizumab in the extension study. Serious adverse events (SAE) were defined as events that were fatal or life-threatening, leading to permanent or significant disability or incapacity, a congenital anomaly or birth defect, or requiring prolonged an inpatient hospitalization. Adverse events were classified using Medical Dictionary for Drug Regulatory Affairs (MedRA version 8.0).

Statistical Analysis. Patients who had remained in the study and had completed visit reports were analysed. No imputation was used for missing data. A paired t-test was employed to detect statistically significant differences in disease activity and functional outcomes from baseline. Statistical analyses were performed with SAS version 8.2 TS2M0 (SAS Institute, Cary, NC). Continuation rate, defined as the cumulative percentage of patients still receiving medication, was analysed by the Kaplan-Meier method. Analysis of adverse event was performed with the person-year method.

RESULTS

Characteristics of the Patients. One hundred and forty-three patients were enrolled in the open-label, long-term extension trial. One hundred eight patients (76%) had completed 3 years and 94 patients (66%) had completed 5 years, as of March 2007 (Figure 1). The median duration of treatment with tocilizumab was 66.7 months (range 0.95 - 73.2).

Thirty-two patients (22%) withdrew due to adverse events. Only one patient (0.7%) withdrew due to unsatisfactory response. Other reasons for withdrawals were as follows; 8 for patient's personal requests, 1 for emergence of anti-tocilizumab antibodies, and 5 for other reasons.

The baseline demographic and clinical data are summarized in Table 1. Their mean age

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was 54 years and the mean disease duration was 9.9 years. Patients had very active disease at baseline, in terms of increased number of tender and swollen joint counts and elevated ESR of 68.7 mm/h and CRP levels of 4.7 mg/dL. Furthermore, the baseline DAS28 was 6.7.

Table 1. Demographics and baseline clinical characteristics of patients with RA who received tocilizumab at any time during the blinded period or open-label extension of the tocilizumab study

	Tocilizumab (n = 143)
Demographics	
Age, years	54.3 ± 11.1
No. of men / No. of women	34/109
Clinical characteristics	
RA duration, years	9.9 ± 8.4
No. of failed DMARDs, mean (range)	4.5 (1-11)
Functional Class†, I/II/III/IV	10/93/40/0
RA Stage†, I/II/III/IV	3/34/56/50
Tender joint count, 0-49 scale	20.3 ± 10.3
Swollen joint count, 0-46 scale	14.5 ± 8.7
ESR, mm/hour	68.7 ± 29.9
CRP, mg/dL	4.7 ± 3.3
DAS28	6.7 ± 1.0

Values are mean ± SD unless stated otherwise.

The data were calculated from the baseline of the double-blind trial (4mg/kg group, 8mg/kg group) and from the extension trial (placebo group).

DMARDs = disease-modifying antirheumatic drugs; Tocilizumab = humanised anti-interleukin-6 receptor antibody; RA = rheumatoid arthritis; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints

† RA functional status determined by American College of Rheumatology criteria. RA stage determined by Steinbrocker's criteria.

Safety. One hundred forty-eight SAEs were reported in 77 patients (53.8%) for an overall rate of 27.5 events per 100 patient-years. Table 2 shows SAEs (occurring in at least 1% of patients). Joint surgery related to RA was the most common SAE, and occurred in 20 patients (14.0%). In addition, a variety of musculoskeletal disorders were reported as SAEs which were classified as non-related to tocilizumab.

Serious infections were reported in 25 patients (17.5%) at a rate of 5.7 events per 100 patient-years. The most frequently reported infections were as follows: pneumonia (9 patients, 1.5 events per 100 patient-years), herpes zoster (7 patients, 1.1 events per 100 patient-years),