Rheumatol Int (2010) 30:725–730
DOI 10.1007/s00296-009-1356-y

ORIGINAL ARTICLE

Etanercept treatment reduces the serum levels of interleukin-15 and interferon-gamma inducible protein-10 in patients with rheumatoid arthritis

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Received: 11 November 2009 / Accepted: 29 December 2009 / Published online: 10 January 2010
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Abstract Tumor necrosis factor-α (TNF-α) has an essential role in the pathogenesis of rheumatoid arthritis (RA) and has been known to induce the production of several inflammatory molecules in vivo. To analyze in vivo the active mechanism of the TNF-α blocking agent, etanercept, the serum levels of the cytokine interleukin-15 (IL-15) and the chemokines growth-regulated protein-α (Gro-α), and interferon-γ inducible protein-10 (IP-10) in RA patients were measured. Twenty-two patients with RA were administered etanercept once or twice a week for more than 6 months. The clinical and laboratory parameters were measured and serum levels of IL-15, Gro-α, and IP-10 were determined using enzyme-linked immunosorbent assay (ELISA) kits at the baseline and at 3 and 6 months after the initial treatment. Additionally, the production of IL-15 and IP-10 by cultured synovial cells stimulated with TNF-α from RA patients was determined by ELISA. A significant decrease in serum levels of IL-15 and IP-10 was observed at 3 and 6 months after initial treatment with etanercept, but not in those of Gro-α. TNF-α induced production of IP-10, but not IL-15 in cultured synovial cells from RA patients. This study demonstrated for the first time the reduction of IP-10 and IL-15 production in RA patients as active mechanisms of etanercept.

Keywords Rheumatoid arthritis · Etanercept · Interleukin-15 · Interferon-gamma inducible protein-10 · Growth-regulated protein-alpha

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Introduction

Tumor necrosis factor-α (TNF-α) has been considered to be an essential cytokine forming the pathological focus of rheumatoid arthritis (RA) [1, 2] by producing various types of chemokines, cytokines, and reactive oxygen species [3, 4]. Recently, the clinical use of a TNF-α blocking agent for RA patients has brought innovative results including regulation of joint destruction. However, the biological mechanism of TNF-α blocking agents in vivo has not been always understood clearly. As one mechanism of the action of TNF-α blocking agents, it has been hypothesized that the levels of several inflammatory chemokines and cytokines decrease. We have previously demonstrated that infliximab reduced the serum levels of interleukin-15 (IL-15) and growth-regulated protein-α (Gro-α) but not those of interferon-γ inducible protein-10 (IP-10) in RA patients [5–7].

IP-10 has various activities including stimulation of the migration of monocytes, natural killer cells and T cells [8] and has been reported to exist at higher levels in the serum and synovial fluid of RA patients [7, 9]. In addition, IP-10 is induced by a variety of inflammatory mediators including TNF-α, interleukin-1 (IL-1), and interferon-γ (IFN-γ) [10–12]. IL-15 has been reported to induce the differentiation of osteoclast progenitors into preosteoclasts [13]. Gro-α is detected in the synovial membrane in RA and is a chemokine induced by TNF-α [14, 15].

Etanercept and infliximab both block TNF-α, but do not always show the same effects in clinical use for RA patients. Here, we measured the serum levels of IL-15, Gro-α, and IP-10 before and after treatment by etanercept in RA patients and analyzed the in vivo mechanism of etanercept.

Materials and methods

RA patients receiving etanercept treatment

Informed consent was obtained from all study participants. Twenty-two patients (20 females and 2 males; mean age 61.8 ± 9.1 years; mean disease duration 15.3 ± 7.4 years; Steinblocker's stage II: 4, III: 14, IV: 4) with RA, diagnosed according to the criteria of the American Collage of Rheumatology, were evaluated in this study between October 2005 and December 2006. We evaluated the patients administered etanercept for more than 6 months. Thirteen patients received a constant dose of prednisolone (mean dose 2.4 ± 2.2 mg/day) throughout the present study. Etanercept was administered by subcutaneous injection at a dosage of 25 mg once or twice a week. Sixteen patients received etanercept in combination with methotrexate (MTX) perorally at a constant dosage of 4–10 mg/week. Six patients were treated with etanercept alone. The patients treated with etanercept were divided into MTX-treated [MTX (+)] and MTX-nontreated [MTX (−)] groups, and their data were evaluated in total and respective groups.

Clinical and laboratory evaluation

Clinical and laboratory examinations including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-agalactocyl IgG antibody (CA-RF), and a Disease Activity Score of 28 joints (DAS28) were performed at the baseline and at 3 and 6 months after the initial treatment. Serum samples of RA patients were obtained just before an initial injection of etanercept at the baseline and at 3 and 6 months after the initial treatment with etanercept. The serum levels of IL-15, IP-10, and Gro-α were measured in all patients as described below.

Measurements of serum levels of IL-15, IP-10, and Gro-α

The serum levels of IL-15, IP-10, and Gro-α, and CA-RF from RA patients with etanercept treatment were measured using an enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (IL-15 and IP-10, ELISA kits, Biosource, Nivelles, Belgium; Gro-α ELISA kit, R&D, MN, USA; and CA-RF ELISA kit, Ei Test, Eisai, Tokyo, Japan). All samples were measured in duplicate. To demonstrate that this assay is not sensitive to rheumatoid factors (RF) interaction, IL-15, IP-10, and Gro-α levels of the serum from five RA patients without infliximab treatment with RF (+) were measured with or without absorption of RF by human IgG (1 mg/ml, 24 h incubation) bound protein G beads, as following our methods described previously [5]. Absorption of RF was made as follows: 100 µl of human IgG (1 mg/ml) was mixed with 100 µl of protein G beads overnight at room temperature. The beads were washed twice with PBS. The serum samples were incubated with the immunoglobulin-coupled beads (1:1 vols.) for 2 h to eliminate RF. The IL-15, IP-10, and Gro-α levels of the samples, which were incubated with or without immunoglobulin-coupled beads, were assayed. There was no significant difference in these cytokine levels between the no treated serum and the serum incubated with the immunoglobulin-coupled beads (data not shown).

Measurements of the levels of IL-15 and IP-10 produced by cultured synovial cells stimulated with TNF-α or IFN-γ

Synonival membranes were obtained from 5 patients with RA at total knee arthroplasty. The membranes were finely minced and then digested for 2 h at 37°C with 1 mg/ml of collagenase type I (Sigma, St. Louis, MO, USA). The cells were washed with phosphate-buffered saline and suspended in culture dishes with a 12-cm diameter containing DMEM with 10% fetal bovine serum for 2 weeks and then resuspended at 1×10^5 cells/well in culture dishes with a 3.5-cm diameter. The adherent cells in the dish were incubated for 48 h after the addition of IFN-γ (50 ng/ml) or TNF-α (0, 10, or 50 ng/ml) (R&D, Minneapolis, MN, USA). The cell-free supernatants were collected and used for IL-15 or IP-10 assays.

Statistical analysis

All data were shown as the mean ± SD. The values in pre- and post-treatment measurements were compared using the Wilcoxon signed rank test. Single regression analysis was performed, and the statistical significance of correlation was determined with Pearson's correlation test. P values less than 0.05 were considered to be significant in this study. All statistical analysis was performed on a Macintosh computer using the Statcel software package.

Results

Clinical and laboratory disease activity

CRP levels in total RA patients significantly decreased from 2.02 ± 0.96 mg/dl at pretreatment to 0.63 ± 0.85 mg/dl

Table 1 The serum levels of interleukin-15 (IL-15), interferon-γ inducible protein-10 (IP-10), and growth-regulated protein-α (Gro-α) in patients with RA treated with etanercept was measured at baseline, 3 months, and 6 months after initial treatment with etanercept

	Baseline	3 months	6 months
Serum IL-15 (pg/ml)			
Total	1,117 ± 784	654 ± 544**	555 ± 460***
MTX (+)	1,202 ± 899	617 ± 519**	476 ± 356**
MTX (−)	917 ± 393	740 ± 623*	683 ± 554*
Serum IP-10 (pg/ml)			
Total	395 ± 291	153 ± 213**	121 ± 163**
MTX (+)	430 ± 334	116 ± 164**	122 ± 180**
MTX (−)	300 ± 81	274 ± 271	120 ± 116*
Serum Gro-α (pg/ml)			
Total	219 ± 340	203 ± 358	247 ± 448
MTX (+)	267 ± 400	244 ± 426	298 ± 529
MTX (−)	107 ± 59	107 ± 27	120 ± 23
CRP (mg/dl)			
Total	2.02 ± 0.96	0.63 ± 0.85**	0.70 ± 1.05**
MTX (+)	1.68 ± 0.72	0.32 ± 0.22**	0.38 ± 0.41**
MTX (−)	2.82 ± 0.99	1.35 ± 1.30*	1.46 ± 1.66*
ESR (mm/h)			
Total	55 ± 17	37 ± 24**	33 ± 19**
MTX (+)	59 ± 18	36 ± 28**	36 ± 22**
MTX (−)	48 ± 11	38 ± 12*	25 ± 5.8*
Number of swollen joints			
Total	8.0 ± 4.0	3.8 ± 3.2**	3.1 ± 2.1**
MTX (+)	9.1 ± 3.7	5.1 ± 2.9**	4.0 ± 1.8**
MTX (−)	5.0 ± 3.4	0.9 ± 1.1*	1.0 ± 0.8*
Number of tender joints			
Total	2.5 ± 1.1	0.3 ± 0.7**	0.5 ± 1.2**
MTX (+)	2.6 ± 1.1	0.4 ± 0.8**	0.1 ± 0.4**
MTX (−)	2.6 ± 1.0	0.6 ± 1.0	0.3 ± 0.5*
DAS28-CRP			
Total	4.47 ± 0.50	2.70 ± 0.59***	2.20 ± 0.51***
MTX (+)	4.48 ± 0.55	2.71 ± 0.69***	2.81 ± 0.66***
MTX (−)	4.42 ± 0.38	2.60 ± 0.83*	2.94 ± 0.52*
Serum CA-RF (AU/ml)			
Total	383 ± 642	469 ± 898	476 ± 800
MTX (+)	180 ± 133	153 ± 107	217 ± 199
MTX (−)	788 ± 993	1,101 ± 1,354	904 ± 1,055

The clinical and laboratory data were also obtained at the same time

Data are shown by mean ± SD values. Data at 3 and 6 months were compared with those at baseline using Wilcoxon signed rank test. Patients treated with etanercept were divided into methotrexate (MTX) (+) and MTX (−) groups

** $P < 0.01$ versus baseline, * $P < 0.05$ versus baseline

($P < 0.01$) and 0.70 ± 1.05 mg/dl ($P < 0.01$) at 3 and 6 months after the initial injection of etanercept, respectively (Table 1). Those in MTX (+) and MTX (−) patients receiving etanercept treatment significantly decreased at 3 and 6 months after the initial injection of etanercept, respectively. ESR levels, DAS28-CRP, and number of swelling joints except for serum CA-RF levels were also significantly decreased at 3 and 6 months compared with those at pretreatment.

Measurements of serum levels of IL-15, IP-10, and Gro-α by etanercept treatment

To investigate whether these ELISA systems are sensitive to IL-15, IP-10, and Gro-α, we measured the levels of serum or SF from RA patients after the addition of human IgG for absorption of RF. The addition of human IgG (1 mg/ml) and absorption of RF did not make a statistically significant difference in the IL-15, IP-10, and Gro-α levels (data not shown).

The mean serum IL-15 levels in total RA patients receiving etanercept treatment decreased significantly from 1,117 ± 784 pg/ml at pretreatment to 654 ± 544 pg/ml ($P < 0.01$) and 555 ± 460 pg/ml ($P < 0.001$) at 3 and 6 months after the initial injection of etanercept, respectively (Table 1). Those in MTX (+) and MTX (−) patients receiving etanercept treatment significantly decreased at 3 and 6 months after the initial injection of etanercept, respectively. The mean serum level of IP-10 at pretreatment (395 ± 291 pg/ml) also decreased significantly at 3 (153 ± 213 pg/ml) ($P < 0.05$) and 6 months (121 ± 163 pg/ml) ($P < 0.01$) after the initial injection of etanercept, respectively. Those in MTX (+) patients receiving etanercept treatment significantly decreased at 3 and 6 months after the initial injection of etanercept, respectively. The mean serum IP-10 levels in MTX (−) patients significantly decreased at 6 months. The mean serum Gro-α levels in total, MTX (+), and MTX (−) patients did not show significant differences at 3 and 6 months after the initial injection of etanercept compared with the basic levels.

Correlation analysis among serum IL-15, Gro-α, and IP-10 and other clinical and laboratory parameters

Serum IL-15 levels showed significant correlation with the numbers of tender joints ($r = 0.470$, $P < 0.05$) (Table 2). Serum IP-10 levels significantly correlated with DAS28-CRP levels ($r = 0.415$, $P < 0.05$).

The levels of IL-15 and IP-10 produced by cultured synovial cells stimulated with TNF-α or IFN-γ

Cultured synovial cells from RA patients were stimulated with TNF-α and IFN-γ. TNF-α at the concentrations of 10 and 50 ng/ml significantly induced a large amount of IP-10 production but did not induce a significant change in the

Table 2 Analysis of correlations between serum IL-15, Gro-α, and IP-10 and other clinical and laboratory parameters in RA patients who received etanercept treatment

	IL-15		Gro-α		IP-10	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value	Correlation coefficient	*P* value
CRP	0.275	0.244	−0.199	0.379	0.465	0.244
ESR (1 h)	0.265	0.264	−0.003	0.991	0.160	0.471
CA-RF	0.358	0.122	−0.265	0.278	0.260	0.273
DAS28-CRP	0.389	0.090	−0.366	0.094	0.415*	0.048
Swollen joints	0.154	0.521	−0.348	0.135	0.210	0.340
Tender joints	0.470*	0.035	−0.220	0.331	0.213	0.334

Single regression analysis between serum IL-15, Gro-α, and IP-10 and other clinical and laboratory parameters was performed. The data of sample levels and clinical parameters used in correlation analysis were obtained at the baseline and at 3 and 6 months after initial treatment with etanercept, and data are shown by correlation coefficient. The statistical significance of correlation was determined using Pearson's correlation test

* $P < 0.05$

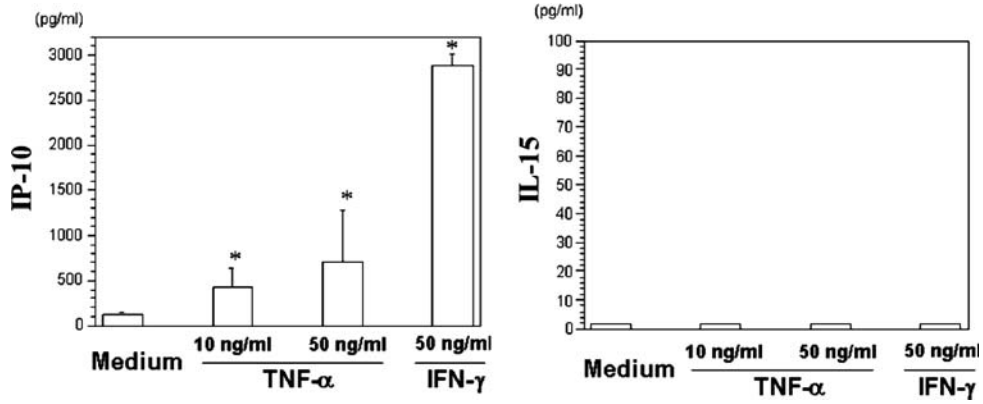

Fig. 1 The levels of IP-10 and IL-15 produced by cultured synovial cells stimulated with IFN-γ or TNF-α. Synonival membranes obtained from RA patients were finely minced and digested for 2 h at 37°C with 1 mg/ml of collagenase type I. The cells were suspended in culture dishes with a 12-cm diameter containing DMEM with 10% fetal bovine serum for 2 weeks and then resuspended at 1×10^5 cells/well in culture dishes with a 3.5-cm diameter. The adherent cells in the dish were incubated for 48 h after the addition of IFN-γ (50 ng/ml) or TNF-α (0, 10, or 50 ng/ml). The supernatants were collected and the levels of IP-10 and IL-15 were measured by ELISA kits

production of IL-15 (Fig. 1). IFN-γ at the concentrations of 50 ng/ml significantly induced a large amount of IP-10 production but did not induce a significant change in the production of IL-15.

Discussion

TNF-α blocking agents have been shown to affect the production of a variety of cytokines, chemokines, mitogens, and proteases, which may exist at sites downstream of the TNF-α cascade in RA patients [5, 6, 16]. On the other hand, it has been known that TNF-α induces the production of various cytokines and chemokines [6, 17–20]. For instance, there are some reports that IL-15, IP-10, and Gro-α are produced by TNF-α stimulation [21, 22]. Previously we demonstrated that treatment of RA patients with infliximab decreased their serum levels of IL-15 and Gro-α but did not significantly change those of IP-10 [6, 7]. In the present study, we investigated the effect of etanercept on these proteins. The serum levels of IL-15 and IP-10 in RA patients after etanercept treatment were decreased.

IL-15 stimulates the differentiation of osteoclast progenitors into preosteoclasts [13] and acts at the early stage of osteoclastogenesis. IL-15 has been shown to be responsible for local T cell activation and to stimulate the proliferation of T cells prepared from peripheral blood and synovial fluid samples from RA patients [23]. The synovial fluid of patients with RA was shown to have elevated levels of IL-15 [24], and IL-15 was detected on synovial fibroblasts of RA [23]. In a collagen-induced (CIA) arthritis model known to be an animal model for RA, the soluble IL-15 receptor alpha-chain suppressed the development of CIA [25]. Antagonistic IL-15 mutant/Fcgamma2a fusion protein,

which targets the IL-15 receptors, also blocked disease progression in a CIA model [26]. Thus, IL-15 may play a crucial role in the pathogenesis of RA including osteoclastogenesis, which is a cause behind the joint destruction of RA [13]. The decrease of IL-15 production in RA patients after etanercept treatment may elucidate its ability to moderate joint destruction and the existence of the cytokine cascade by which IL-15 is induced by TNF-α in RA patients in vivo. But in our present study, TNF-α stimulation for cultured synovial cells from RA patients did not induce IL-15 production. Ernestam et al. previously reported that the levels of IL-15 in synovial membranes were not significantly unchanged after treatment with infliximab [27]. Taken together, these findings suggest that the production of IL-15 may be indirectly induced by the stimulation of another molecule induced by TNF-α.

IP-10 has been known to be induced by various inflammatory mediators including IL-1, TNF-α, IFN-α, and IFN-γ, in monocytes, neutrophils, keratinocytes, fibroblasts, endothelial cells and synovial cells [28–34]. TNF-α enhances phenotypic and functional maturation of human epidermal Langerhans cells and induces IP-10 production [10]. IP-10 has various biological activities, including stimulation of monocyte, natural killer cell and T cell migration, bone marrow progenitor maturation, modulation of adhesion molecule expression, and inhibition of angiogenesis [35]. Furthermore, it was recently reported that IP-10 stimulated the expression of RANKL and TNF-α in CD4+ T cells and showed osteoclastogenic potential in co-cultures of CD4+ T cells and osteoclast precursors [36]. In a CIA model, the levels of RANKL and TNF-α were decreased by an antibody to IP-10, and IP-10 has been shown to be involved in bone erosion in inflamed joints [36]. Indeed, RA synovial fluid (SF) contained greater amounts of IP-10 compared with osteoarthritis SF [37]. IP-10 has been detected on infiltrating macrophage-like cells, and fibroblast-like cells in the RA synovium [38]. Thus, IP-10 is considered to have a relationship with bone destruction that is essential for the pathogenesis of RA. Our data indicated that TNF-α induced the production of IP-10 in the RA synovial membrane in vivo and that etanercept reduced the serum levels of IP-10 in RA patients; therefore, the cascade by which IP-10 is induced by TNF-α stimulation may exist in vivo. The discovery of the reduction of serum levels of IP-10 in RA patients by etanercept treatment is new to this report.

Gro-α levels were not significantly changed after etanercept treatment. However, in our previous study, serum Gro-α levels were significantly decreased after infliximab treatment. Although there is a report that the synovial fluid levels of Gro-α are high in RA patients, the function of Gro-α in the pathogenesis of RA is not yet understood.

Previously, treatment of RA patients with infliximab did not result in a significant change of serum IP-10 levels but decreased the serum Gro-α levels [6, 7]. Although both of etanercept and infliximab are TNF-α blocking agents, they do not always show identical effects in clinical use, even on the same RA patient. This may result from a different mechanism in TNF-α blocking. For example, infliximab has a reverse signal through transmembrane TNF-α on the cell surface [39, 40]. CA-RF levels in RA patients were decreased significantly by infliximab in our previous study, but not by etanercept in the present study [7]. Although the in vivo mechanism of the TNF-α blocker etanercept is not completely understood, the present study provides an important clue. In the present study, we investigated the change of serum levels of IL-15, IP-10, and Gro-α using etanercept; as an additional study, we need to measure the levels of these proteins in the synovial membrane. This may elucidate further the function of the respective TNF-α blocking agents.

Acknowledgments In this study, I appreciate the cooperation of the division of host defense at the department of infectious disease in Hamamatsu University School of Medicine for the measurement of the levels of a cytokine and chemokines.

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