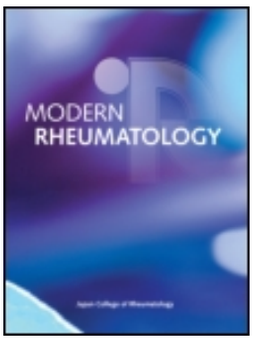

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ORIGINAL ARTICLE

Etanercept reduces the serum levels of macrophage chemotactic protein-1 in patients with rheumatoid arthritis

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Abstract This study was performed to analyze the effect of etanercept, the soluble tumor necrosis factor-α (TNF-α) receptor, on the serum levels of several chemokines including monocyte chemotactic protein-1 (MCP-1), regulated upon activation normal T expressed and presumably secreted (RANTES), and granzyme B in rheumatoid arthritis (RA) patients. Twenty-eight patients with RA were administered etanercept once or twice a week for more than 6 months. Clinical and laboratory parameters were measured and serum levels of MCP-1, RANTES, and granzyme B were determined using enzyme-linked immunosorbent assay (ELISA) kits at baseline and at 3 and 6 months after the initial treatment. In addition, the levels of MCP-1, RANTES, and granzyme B produced by cultured synovial cells stimulated with TNF-α were measured. A significant decrease in serum MCP-1 levels was observed at 3 and 6 months after initial treatment with etanercept. Serum RANTES and granzyme B levels did not show significant changes. TNF-α induced MCP-1, RANTES, and granzyme B production in cultured synovial cells from RA patients. Serum MCP-1 levels were significantly correlated with the disease activity scores of 28 joints combined with CRP (DAS28-CRP), indicating the role of MCP-1 in the pathogenesis of rheumatoid inflammation. This study demonstrated that a reduction of MCP-1 production in RA patients was a newly determined effect of etanercept. Another cascade not associated with TNF-α may induce granzyme B and RANTES production in RA patients.

Keywords Rheumatoid arthritis · Monocyte chemotactic protein-1 · Etanercept

Introduction

Tumor necrosis factor-α (TNF-α) has been reported to be associated with the pathogenesis of rheumatoid arthritis (RA) [1, 2] through inducing the production of a number of chemokines, cytokines, and reactive oxygen species [3, 4]. Previous studies indicated that an anti-TNF-α monoclonal antibody, infliximab, reduces the serum levels of interleukin (IL)-6, IL-8, monocyte chemotactic protein-1 (MCP-1), vascular endothelial growth factor (VEGF) [5, 6], IL-18 [7], matrix metalloproteinase (MMP)-1, -3, and -9 [8], IL-15, and Gro-α in RA patients [9, 10].

In addition, we previously reported that a soluble TNF-α receptor, etanercept, reduced serum IL-23 and MIP-3α (CXCL20) levels in RA patients [11]. There have been reports that TNF-α increases the gene expression of granzyme B and that TNF-α increases the production of several chemokines including RANTES [12–15]. In the current study, we investigated the changes in the serum levels of other chemokines, such as MCP-1, regulated upon activation normal T expressed and presumably secreted (RANTES), and granzyme B, which were considered to exist in TNF-α cascade according to previous reports on RA patients.

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Materials and methods

RA patients treated with etanercept

Twenty-eight patients (25 females and 3 males; mean age 61.1 ± 10.6 years; mean disease duration 12.4 ± 7.1 years; Steinbrocker's stage I: 1, II: 10, III: 15, IV: 2) who met the American College of Rheumatology diagnostic criteria for RA were included in this study between December 2005 and November 2007. This study was performed in accordance with the guidelines of the ethics committee of Heisei Memorial Hospital, and informed consent was obtained from all patients. In this study, patients who underwent treatment with etanercept for more than 6 months without side-effects were evaluated. Sixteen patients were administered prednisolone at constant mean dosage of 2.53 ± 2.02 mg/day throughout this study for more than 6 months before the etanercept administration. Etanercept was injected subcutaneously at dosage of 25 mg once or twice a week. Twenty patients received etanercept in combination with methotrexate (MTX) perorally at constant mean dosage of 4.5 ± 2.7 mg/week for more than 6 months before the etanercept administration. Eight patients were treated with etanercept alone. The patients treated with etanercept were divided into MTX-treated [MTX (+)] and MTX-nontreated [MTX (−)] groups, or prednisolone-treated [prednisolone (+)] and prednisolone-nontreated [prednisolone (−)] groups, and their data were evaluated in total and respective groups.

Clinical and laboratory examinations

C-reactive protein (CRP), anti-agalactosyl IgG antibody (CA-RF), and the disease activity scores of 28 joints combined with CRP (DAS28-CRP) were measured during clinical and laboratory examinations at baseline and at 3 and 6 months after the initial treatment. Serum samples and the second urinary samples from patients in the early morning were obtained just before the initial injection of etanercept at baseline and at 3 and 6 months after the initial treatment with etanercept and stored at −80°C until assayed. Serum anti-agalactosyl IgG antibody (CA-RF) was also measured with an enzyme-linked immunosorbent assay (ELISA) kit (Ei test, Eisai, Tokyo, Japan).

Measurements of the serum levels of MCP-1, RANTES, and granzyme B in RA patients treated with etanercept and control individuals

The serum levels of MCP-1, granzyme B, and RANTES in RA patients undergoing etanercept treatment and in healthy individuals were measured using an enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (MCP-1 and granzyme B ELISA kits, Bender MedSystem GmbH, Vienna, Austria; RANTES Instant ELISA kit, Bender MedSystem GmbH, Vienna, Austria). All samples were measured in duplicate.

We have previously reported that serum total, urinary total, and urinary free pentosidine decreased after etanercept treatment [16]. In the present study, the serum total and urinary total pentosidine levels were also measured for single regression analysis between these pentosidine levels and the serum levels of MCP-1, granzyme B, and RANTES.

Measurements of the levels of MCP-1, RANTES, and granzyme B produced by cultured synovial cells stimulated with TNF-α

Synovial membranes were obtained from six patients with RA at total knee arthroplasty. The synovial membranes were finely minced and then digested for 2 h at 37°C with 1 mg/ml collagenase type I (Sigma, St. Louis, MO, USA). The synovial cells were washed with phosphate-buffered saline and suspended in culture dishes with a 12-cm diameter containing Dulbecco's modified Eagle's medium (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. After 2 days, the adherent cells in the dish were incubated for 48 h after the addition of TNF-α (0, 5, or 50 ng/ml) (R&D, Minneapolis, MN, USA). The cell-free supernatants were collected and used for MCP-1, RANTES, and granzyme B assays.

Measurements of serum and urinary pentosidine levels

Takahashi et al. have previously established a method for measuring the total pentosidine levels composed of dehydrated forms and the free pentosidine of an undehydrated form, respectively [17]. The levels of serum total and urinary total pentosidine in RA patients before and after treatment with etanercept and of control healthy individuals were measured using high-performance liquid chromatography, as previously described [17].

Statistical analysis

In this study, all data are expressed as means ± standard deviation (SD). A comparison of the values obtained in the pre- and post-treatment measurements of CRP, serum CA-RF, DAS28-CRP, serum MCP-1, granzyme B, and RANTES in RA patients was performed using the Wilcoxon signed rank test. In addition, a comparison of the data at baseline in RA patients with the data in control individuals was performed using the Mann–Whitney *U* test. Single regression analysis between serum MCP-1, RANTES, and granzyme B and other parameters including CRP, ESR,

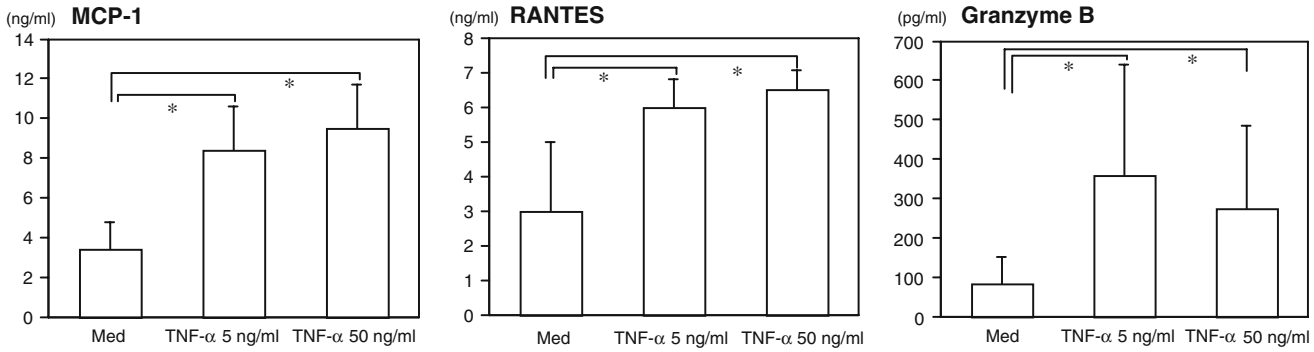

Fig. 1 RA synovial fibroblast cells (1×10^5 cells/well) obtained from knees were incubated for 48 h after the addition of TNF-α (0, 5, or 50 ng/ml). MCP-1, RANTES, and granzyme B levels of the supernatant were measured using ELISA kits. Significant differences are indicated by $^*P < 0.05$ and $^{**}P < 0.01$

DAS28-CRP, and serum total and urinary total pentosidine was also performed, The statistical significance of correlation was determined with Pearson's correlation test. *P* values less than 0.05 were considered to indicate significant difference. This calculation was performed on a Macintosh computer using the Statcel software package.

Results

Clinical and laboratory disease activity

In total RA patients, CRP levels significantly decreased from 2.81 ± 1.26 mg/dl at pretreatment to 0.86 ± 0.99 mg/dl ($P < 0.01$) and 0.89 ± 1.06 mg/dl ($P < 0.01$) at 3 and 6 months after the initial injection of etanercept, respectively (Table 1). These tendencies did not depend on MTX (+) or MTX (−), and prednisolone (+) and prednisolone (−). In total RA patients, DAS28-CRP also was significantly decreased at 3 and 6 months compared with at pretreatment, but serum CA-RF levels were not. These tendency also did not depend on MTX (+) or MTX (−), and prednisolone (+) and prednisolone (−).

Serum levels of MCP-1, RANTES, and granzyme B, and total pentosidine and urinary levels of total pentosidine

The mean serum MCP-1 levels in total RA patients receiving etanercept treatment decreased significantly from 1607 ± 1135 pg/ml at pretreatment to 848 ± 513 pg/ml ($P < 0.05$) and 913 ± 683 pg/ml ($P < 0.01$) at 3 and 6 months after the initial injection of etanercept, respectively (Table 1). The mean serum baseline levels of MCP-1, RANTES, and granzyme B in total RA patients were higher than those in control individuals. The serum levels of RANTES and granzyme B in total RA patients did not significantly change at 3 and 6 months compared with the levels measured before the etanercept treatment. The serum total and urinary total pentosidine levels in total RA patients also decreased at 6 months, as we have described previously [16]. These changes of the levels of the mean serum MCP-1, RANTES, granzyme B, and pentosidine and those of urinary total pentosidine did not depend on MTX (+) and MTX (−) groups, or prednisolone (+) and prednisolone (−).

Correlation analysis between serum levels of MCP-1, RANTES, and granzyme B and clinical and laboratory parameters

Serum MCP-1 levels showed significant correlation with DAS28-CRP, serum granzyme B, and serum total and urinary total pentosidine levels (Table 2).

The levels of MCP-1, RANTES, and granzyme B produced by cultured synovial cells stimulated with TNF-α

Cultured synovial cells from RA patients were stimulated with TNF-α. TNF-α at concentrations of 5 and 50 ng/ml significantly induced a large amount of MCP-1, RANTES, and granzyme B production in comparison with the cultured medium levels (Fig. 1).

Discussion

The use of TNF-α inhibitors can affect the production levels of a variety of cytokines, chemokines, mitogens, and proteases, which may exist at a site downstream of a TNF-α cascade in RA patients [5–10]. Catrina et al. reported that the serum levels of MMP-1 and MMP-3 in RA patients decreased after etanercept treatment [18]. Previously, we reported that etanercept reduces the serum levels of IL-23 and MIP-3α and the serum and urinary levels of pentosidine in RA patients [11].

Table 1 Clinical and laboratory parameters: serum monocyte chemotactic protein-1 (MCP-1), regulated upon activation normal T expressed and presumably secreted (RANTES), and granzyme B, and serum total and urinary total pentosidine, in patients with RA treated with etanercept, performed at baseline and at 3 and 6 months after initial treatment with etanercept

	Baseline	3 months	6 months	Control
Serum RANTES (ng/ml)				
Total	14.2 ± 9.0	14.2 ± 10.5	13.9 ± 7.4	5.0 ± 2.8*
MTX (−)	12.5 ± 7.23	13.3 ± 7.87	14.1 ± 9.73	
MTX (+)	15.4 ± 10.2	14.4 ± 12.3	12.4 ± 6.58	
Prednisolone (−)	13.8 ± 6.18	25.8 ± 14.1	16.9 ± 9.54	
Prednisolone (+)	15.6 ± 10.2	12.9 ± 11.5	12.8 ± 7.52	
Serum granzyme B (pg/ml)				
Total	53.8 ± 33.8	56.7 ± 30.0	49.7 ± 19.0	6.0 ± 29.8*
MTX (−)	37.8 ± 7.6	54.4 ± 11.3	52.3 ± 13.3	
MTX (+)	64.4 ± 40.7	58.2 ± 37.3	47.8 ± 23.0	
Prednisolone (−)	62.5 ± 52.3	45.8 ± 14.8	48.8 ± 11.8	
Prednisolone (+)	50.3 ± 27.3	61.3 ± 33.9	50.1 ± 21.0	
Serum MCP-1 (pg/ml)				
Total	1607 ± 1135	848 ± 513*	913 ± 683**	162 ± 95**
MTX (−)	1091 ± 681	708 ± 385*	592 ± 358*	
MTX (+)	1950 ± 1278	941 ± 586*	1127 ± 779*	
Prednisolone (+)	1530 ± 1183	554 ± 335*	610 ± 483*	
Prednisolone (−)	1645 ± 1174	995 ± 530**	1065 ± 739*	
Serum total pentosidine (nmol/L)				
Total	223 ± 103	166 ± 98*	116 ± 28**	83 ± 10**
MTX (−)	214 ± 68.3	144 ± 46*	113 ± 13*	
MTX (+)	230 ± 131	184 ± 128	120 ± 39*	
Prednisolone (−)	266 ± 145	244 ± 164	132 ± 51*	
Prednisolone (+)	203 ± 90	138 ± 51*	111 ± 10*	
Urinary total pentosidine (nmol/mmol cre)				
Total	7.63 ± 2.51	6.31 ± 2.49*	5.74 ± 2.50*	3.2 ± 1.1**
MTX (−)	7.95 ± 3.05	6.63 ± 2.20	5.00 ± 1.06*	
MTX (+)	7.43 ± 2.32	6.09 ± 2.78*	6.20 ± 3.15*	
Prednisolone (−)	8.77 ± 3.67	7.02 ± 3.97	6.03 ± 3.14*	
Prednisolone (+)	7.11 ± 1.84	5.84 ± 1.57*	5.30 ± 1.93*	
CRP (mg/dl)				
Total	2.81 ± 1.26	0.86 ± 0.99**	0.89 ± 1.06**	ND
MTX (−)	2.62 ± 0.90	0.50 ± 0.57**	0.44 ± 0.46**	
MTX (+)	2.97 ± 1.55	1.17 ± 1.20**	1.30 ± 1.30**	
Prednisolone (−)	3.24 ± 1.71	1.11 ± 1.49**	0.80 ± 1.26**	
Prednisolone (+)	2.59 ± 1.00	0.73 ± 0.69**	0.94 ± 1.02**	
DAS28-CRP				
Total	4.53 ± 0.55	2.77 ± 0.65**	2.97 ± 0.99**	ND
MTX (−)	4.57 ± 0.41	2.71 ± 0.69**	2.81 ± 0.66**	
MTX (+)	4.51 ± 0.68	2.80 ± 0.77**	2.99 ± 1.14**	
Prednisolone (−)	4.48 ± 0.67	2.30 ± 0.75*	2.34 ± 0.53*	
Prednisolone (+)	4.56 ± 0.67	2.99 ± 0.62**	3.17 ± 0.99**	
Serum CA-RF (AU/ml)				
Total	284 ± 515	314 ± 692	277 ± 576	ND
MTX (−)	380 ± 746	500 ± 1049	400 ± 901	
MTX (+)	234 ± 229	191 ± 170	244 ± 228	
Prednisolone (−)	440 ± 906	583 ± 1255	462 ± 994	

Table 1 continued

	Baseline	3 months	6 months	Control
Prednisolone (+)	213 ± 225	190 ± 190	185 ± 221	

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, or prednisolone (+) and prednisolone (−) groups. Data of control individuals were compared with those at total baseline of RA patients using Mann–Whitney *U* test

ND not done

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

Table 2 Correlations between serum MCP-1, RANTES, and granzyme B and other clinical and laboratory parameters in RA patients who received etanercept treatment

	Serum MCP-1	Serum RANTES	Serum granzyme B
CRP	0.300	−0.0715	−0.0636
DAS28-CRP	0.363*	−0.185	0.00942
Serum total pentosidine	0.666**	−0.141	0.224
Urinary total pentosidine	0.745**	−0.164	0.0471
Serum MCP-1	–	–	–
Serum RANTES	−0.129	–	–
Serum granzyme B	0.573**	−0.0477	–

Single regression analysis between MCP-1, RANTES, and granzyme B and other clinical and laboratory parameters was performed. Sample levels and clinical parameters were evaluated at 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

* $P < 0.05$; ** $P < 0.001$

To further analyze the in vivo action of etanercept, we measured the serum levels of MCP-1, granzyme B, and RANTES, at pre and post treatment with etanercept in RA patients. These chemokines have been found to increase when stimulated with TNF-α in several kinds of cells [12–15]. Therefore there is a possibility that the production of these chemokines in vivo may be reduced by the administration of etanercept. In this study, clinical and laboratory parameters in RA patients improved significantly in response to etanercept treatment. Decreases of serum MCP-1 levels in RA patients after etanercept treatment were seen, but granzyme B and RANTES levels were not significantly changed. This is the first report describing the effect of etanercept in RA patients.

The MCP-1, known as CCL2, was designated as a monocyte chemotactic and activating factor because it stimulates chemotactic migration of human monocytes and activates them to kill tumors in vitro [19]. In addition, MCP-1 is a monocyte, B-cell, CD45RO + T-lymphocyte [20, 21], and natural killer (NK) cell [22, 23] chemoattractant belonging to the CC subfamily of chemokines, and is produced by macrophages, endothelium, synovial fibroblasts, and chondrocytes in the RA joint [14, 24, 25]. Furthermore, MCP-1 has been expressed in various types of tissues such as the thymus, spleen, kidney, liver, and lung [26]. MCP-1 has been shown to be expressed in many different inflammatory diseases, such as atherosclerosis, allergic asthma, idiopathic pulmonary fibrosis [27], and inflammatory bowel disease [28].

Previous studies demonstrated that MCP-1 levels were elevated in the plasma, synovial fluid, and synovial tissue of patients with RA [29, 30], and also that the levels of MCP-1 in the synovial fluid of RA patients were higher than in serum, reflecting local production [31, 32]. Recent studies have confirmed that MCP-1 acts as a marker for RA joint inflammation due to its significant correlation with the number of swollen joints and the Ritchie Articular Index [29, 30]. Plasma MCP-1 has been reported to be a candidate marker for monitoring the clinical efficacy of drug treatment in RA patients [29]. Our study also indicated that serum MCP-1 levels were significantly correlated with DAS28-CRP. Furthermore, serum MCP-1 levels were correlated with serum granzyme B. Thus MCP-1 may be a key chemokine in inflammatory status in RA and may have a relationship with a cytokine and chemokine network in vivo. The injection of MCP-1 antagonist was shown to markedly reduce the severity of arthritis and the infiltration of monocytes, and pretreatment with this antagonist prevented the development of experimental arthritis [33, 34]. In a randomized controlled trial with an anti-CCL2 (MCP-1) monoclonal antibody in patients with RA [35], there was no detectable clinical benefit of an anti-CCL2 monoclonal antibody compared with placebo. Therefore, it is unclear whether MCP-1 becomes a target for RA treatment.

Several cytokines have been demonstrated to increase the production of MCP-1 [12–14, 36]. IL-15 stimulates monocytes to produce MCP-1 and IL-8 production [37]. IL-1β and TNF-α-stimulated mesangial cells express MCP-1 messenger RNA (mRNA) and MCP-1 activity [12]. TNF-α, IFN-γ, and IL-1β stimulate the production of MCP-1 by mesothelial cells [13]. In RA, IL-1β and TNF-α stimulated the expression of MCP-1 mRNA and de novo MCP-1 synthesis by cultured synovial cells [14]. In our study,

TNF-α at concentration of 5 and 50 ng/ml induced the production of MCP-1, RANTES, and granzyme B by cultured rheumatoid synovial cells. Although the concentration of 50 ng/ml of TNF-α is high, this concentration may be able to exist locally in the synovium in vivo. Therefore the cascade by which TNF-α induces MCP-1 exists in RA patients, and etanercept blocks the production of this cascade.

There is a report that the administration of infliximab caused a reduction in serum RANTES and MCP-1 in RA patients [38]. In addition, monoclonal anti-TNF-α antibody has been shown to reduce the expression levels of MCP-1 in synovial cells in RA patients [39]. The CC chemokines, including RANTES and MCP-1, attract and activate a variety of cells, including monocytes, macrophages, lymphocytes, eosinophils, and basophils, and have been implicated in chronic inflammatory disease [40–43]. Recent data from animal models suggest that both RANTES and MCP-1 play important roles in the pathogenesis of arthritis [43–45]. In adjuvant-induced arthritis in the rat, increased levels of RANTES have been found in both the blood and the joints, and synovial levels of RANTES have been found to correlate with clinical symptoms of joint inflammation [43, 45]. Boiardi et al. [46] observed high levels of serum RANTES in a series of adult RA patients during the active stage of the disease, and MTX treatment significantly lowered the serum RANTES levels. High serum levels of RANTES after 6 months of MTX treatment seem to be predictive of radiologic erosion after 1 year in the same patient cohort [46]. Furthermore, the administration of anti-RANTES antibody has been shown to prevent the onset of arthritis and to greatly ameliorate arthritis symptoms once the disease develops [45]. In our study, etanercept treatment did not reduce the serum RANTES levels. RANTES production may depend on molecules other than TNF-α in vivo.

Granzyme B is expressed in NK cells and T cells [47]. Granzyme B is produced by rheumatoid synovial cells, degrades proteoglycans of cartilage, and plays an essential role in synovial inflammation and joint destruction [48]. The high levels of granzyme B in RA have already been reported [49], as described in our study. In our study, although TNF-α was able to induce the production of granzyme B in RA-cultured synovial cells, serum granzyme B levels were not significantly affected by etanercept treatment. This may suggest that another cascade not associated with TNF-α induces granzyme B production in RA.

Our study demonstrated that etanercept reduced the production of MCP-1 in RA patients. In this study, chemokines such as RANTES or granzyme B did not show reduced levels by etanercept treatment. Therefore, blocking therapy for these chemokines, when combined with etanercept administration, may be effective for RA treatment.

Conflict of interest statement None.

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