

# High serum and synovial fluid granulocyte colony stimulating factor (G-CSF) concentrations in patients with rheumatoid arthritis

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## **Abstract**

### **Objective**

*To determine the relationship between serum G-CSF, RA disease activity and the levels of inflammatory cytokines.*

### **Methods**

*Sixty-one patients (5 men and 56 women; mean age;  $56.1 \pm 11.4$  [ $\pm$ SD] years, range, 22-70 years) who were selected at random and met the American College of Rheumatology criteria for RA were examined. Granulocyte-colony stimulating factor (G-CSF) levels in sera and synovial fluid were measured by solid-phase radioimmunoassay (RIA). We also measured various indices of RA disease activity and serum levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  by ELISA.*

### **Results**

*The morning stiffness, number of tender or swollen joints, ESR, Lansbury index and serum G-CSF levels in patients with active RA were significantly higher than the corresponding levels in patients with inactive RA. Serum G-CSF levels correlated significantly with morning stiffness, the number of tender or swollen joints and the Lansbury index.*

*However, there was no correlation between serum G-CSF and ESR. High levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were detected in RA patients. The number of tender or swollen joints, ESR, Lansbury index, and IL-1 $\beta$  were significantly higher in G-CSF-positive RA patients than in G-CSF-negative RA patients.*

### **Conclusion**

*Our results suggest that G-CSF produced by synovial cells stimulated by inflammatory cytokines might contribute to inflammatory arthritis in RA patients.*

### **Key words**

G-CSF, rheumatoid arthritis, cytokines.

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**Abbreviations:** CSF: colony stimulating factor; DMARDs: disease modifying anti-rheumatic drugs; ELISA: enzyme linked immunosorbent assay; ESR: erythrocyte sedimentation rate.

## Introduction

Colony-stimulating factors regulate the survival, proliferation, and differentiation of hematopoietic progenitor cells into mature cells (1). Two such CSFs are granulocyte CSF (G-CSF), which mediates the clonal proliferation and differentiation of progenitors into granulocytes, and granulocyte-macrophage-CSF (GM-CSF), which generates granulocytes and macrophages from progenitor cells by a similar process. CSFs can also act on mature hematopoietic cells, suggesting that it may play a role in immunity and inflammatory processes (1). Furthermore, G-CSF enhances the antibody-dependent cytotoxicity (2), neutrophil oxidative metabolism (3), and phagocytosis of microorganisms by neutrophils (3).

Patients with rheumatoid arthritis (RA) exhibit several abnormalities of the immunoregulatory system. For example, high concentrations of cytokines are present in the affected joints and peripheral blood, and the levels of some of these cytokines correlate with the clinical activity of RA (4). On the other hand, activated synovial fibroblast-like cells are thought to contribute to the invasive and erosive properties of the inflamed synovium of rheumatoid lesions (5). They have been shown *in vitro* to be activated by cytokines, such as interleukin-1 (IL-1), to produce a number of putative mediators of inflammation and tissue destruction, such as plasminogen activator (6), collagenase (7) IL-6 (8), and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (5, 6).

Previous studies have demonstrated that synovial fibroblast-like cells produce G-CSF in response to IL-1 and tumor necrosis factor (TNF)- with synergistic interactions being observed between cytokines (9); chondrocytes and cartilage tissue also respond to IL-1 and to a combination of IL-1 and TNF- to produce G-CSF (10). However, it has not been possible in the past to correlate G-CSF levels *in vitro* with clinical disease activity in patients with RA.

In the present study, we determined the relationship between serum levels of G-CSF and disease activity in RA, as well as the levels of various inflammatory cytokines.

## Materials and methods

### Subjects

We studied 61 patients (5 men and 56 women; mean age 56.1±11.4 (±SD) yrs., range 22-70 yrs.) who met the criteria of the American College of Rheumatology for RA (10). They were selected at random from patients attending the outpatient clinic of the Department of Internal Medicine at Sasebo Chuo Hospital, Sasebo. At the time of the study, all patients were taking non-steroidal anti-inflammatory drugs (NSAIDs) and also disease modifying anti-rheumatic drugs (DMARDs). These included D-penicillamine, bucillamine, auranofin, sulfasalazopyridine, methotrexate and tiopronin. The doses of these DMARDs were 300 mg, 200 mg, 6 mg, 1000 mg, 5 mg and 200 mg, respectively. Each patient continued to receive one of these DMARDs during the study, and their administration and dosage were held constant during the study.

In addition, we administered NSAIDs and DMARDs; 2 RA patients had to receive prednisolone because of active disease. In addition, 2 patients were receiving prednisolone at a daily dose of 2.5 and 5 mg, respectively. Ten age- and sex-matched healthy subjects served as controls (mean age 56.5±11.6 (±SD), range 37-68 years). The study protocol was approved by the Human Ethics Review Committees of the participating institutions and signed consent was obtained from each subject.

Blood samples were obtained from all subjects and allowed to clot for 2 hours at room temperature before centrifugation. Serum samples were stored at -80°C until use. Synovial fluid samples (SF) were obtained from 10 RA patients during therapeutic joint aspiration. Paired samples of peripheral blood and SF were also simultaneously obtained during joint aspiration.

### Measurement of disease activity

RA disease activity was assessed by the duration of morning stiffness (in min), the number of tender or swollen joints, the Westergren erythrocyte sedimentation rate (ESR, in mm/hr) and a modified Lansbury index (12). Active RA was defined as the presence of at least 3 of the following 4 criteria: number of tender

joints 9, number of swollen joints 6, ESR 28 and morning stiffness 45 min. The Lansbury index was estimated based on 4 variables (ESR, morning stiffness, grip strength and joint score). Disease activity was assessed by an investigator who was blind to serum G-CSF levels.

#### G-CSF radioimmunoassay

G-CSF concentrations in synovial cell supernatants were measured by a solid-phase radioimmunoassay (RIA). This was performed in strips of flat-bottomed wells (Immulon-2, Dynatech Laboratories, Alexandria, VA), which had been coated with the IgG fraction of rabbit anti-G-CSF antibody, and blocked with BSA. Triplicate wells of samples and G-CSF standard were incubated for 5 hours before the addition of monoclonal anti-G-CSF (75A) for a further overnight incubation. Binding of antibody was detected by the addition of  $^{251}$ I-rabbit anti-mouse IgG (NEN/Dupont, Boston, MA) for 2 hours. Measurement of radioactivity was made using a gamma-counter, with a sensitivity of 0.5 ng/mL G-CSF.

#### Measurement of cytokine concentrations

Aliquots of serum and SF were frozen at  $-80^{\circ}\text{C}$  until assayed. Cytokine concentrations were determined according to the instructions provided by the manufacturers of the commercially available enzyme linked immunosorbent assay (ELISA) kits used. Ten kits were obtained from the following manufacturers: IL-1 and TNF- $\alpha$  from Otsuka Pharmaceutical Co. (Tokyo, Japan) and IL-6 from Genzyme Corporation (Boston, MA). The lower limits for detection were 20 pg/ml for IL-1, IL-6 and TNF- $\alpha$ .

#### Statistical analysis

All data were expressed as means  $\pm$  SD. Differences between groups were examined for statistical significance using the Student's t-test or paired t-test. A P value less than 0.05 denoted the presence of a statistically significant difference.

## Results

### RA disease activity

Twenty-nine patients with active RA and 32 patients with inactive RA were in-

cluded in the study. The characteristics of the two groups are summarized in Table I. There was no statistically significant difference between the two groups in age and disease duration. On the other hand, morning stiffness, the number of tender or swollen joints, the ESR and the Lansbury index in the active RA group were significantly higher than in the inactive RA group.

### Serum G-CSF and inflammatory cytokine levels

Table II shows the serum concentrations of G-CSF and inflammatory cytokines in patients with active and inactive RA and healthy subjects. We measured serum G-CSF concentrations in normal subjects with their informed consent. None of the control subjects had high levels of serum G-CSF or cytokines; all levels were at or below the sensitivity limit of each assay. There were no significant differences among the two groups of RA patients with regard to serum IL-1, IL-6 or TNF- $\alpha$ . However, serum G-CSF in the active RA group was signifi-

cantly higher than in the inactive RA group ( $p < 0.05$ ).

### Serum and synovial fluid G-CSF levels in RA

Next, we measured G-CSF concentrations in 10 paired serum and SF samples from RA patients (Fig. 1). These RA patients were regarded as having active disease according to the definition in the Materials and Methods section. G-CSF values in the SF of 3 patients were higher compared to the corresponding serum levels, but were almost similar in the remaining 7 samples. Furthermore, the mean G-CSF level in the SF for the whole group had a high tendency compared to that in the peripheral blood.

### Relationship between disease activity and serum G-CSF levels

RA disease activity was assessed by the duration of morning stiffness, the number of tender or swollen joints, the ESR and the Lansbury index. As shown in Figure 2, serum G-CSF levels correlated significantly with the index of morning

**Table I.** Characteristics of the study populations (values are expressed as means  $\pm$  SD).

RA patients	Active	Inactive
Number	29	32
Age (years)	56.8 $\pm$ 14.6	57.5 $\pm$ 15.7
Sex (M/ F)	2/27	3/29
Disease duration (months)	96.5 $\pm$ 72.8	120.3 $\pm$ 111.3
Morning stiffness (min.)	84.8 $\pm$ 45.4*	11.9 $\pm$ 13.8
No. of tender or swollen joints	19.7 $\pm$ 12.0*	4.4 $\pm$ 4.1
ESR (mm/h)	65.0 $\pm$ 40.0**	33.1 $\pm$ 20.0
Lansbury Index (%)	62.8 $\pm$ 20.0*	26.4 $\pm$ 10.0

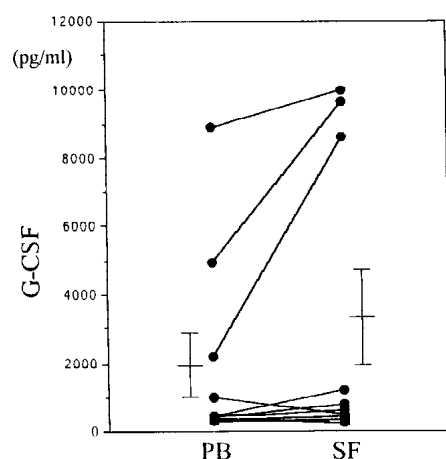
The Lansbury Index was calculated based on 4 variables (ESR, morning stiffness, grip strength and joint score). \* $p < 0.0001$ , compared to patients with inactive RA; \*\* $p < 0.0002$ , compared to patients with inactive RA.

**Table II.** Serum G-CSF and inflammatory cytokines from patients with active or inactive RA and healthy subjects (data are expressed as means  $\pm$  SD pg/ml).

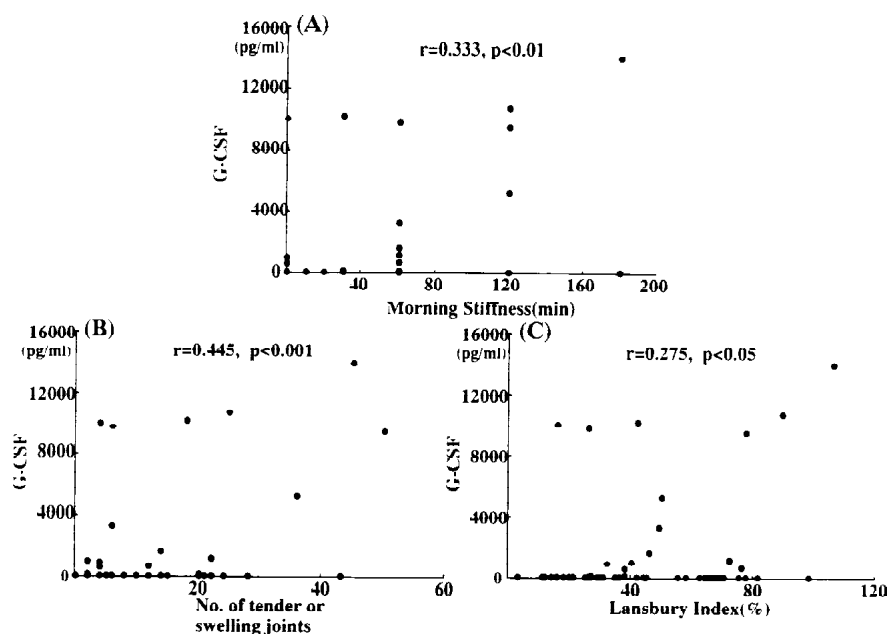
	Active RA	Inactive RA	Healthy subjects
n	29	32	20
G-CSF	2,365.7 $\pm$ 772.5*	484.3 $\pm$ 310.8	103.5 $\pm$ 61.2**
IL-1	123.6 $\pm$ 41.4	70.2 $\pm$ 30.1	21.3 $\pm$ 8.9**
IL-6	156.4 $\pm$ 29.6	110.7 $\pm$ 5.6	110.4 $\pm$ 36.5**
TNF- $\alpha$	663.4 $\pm$ 285	378.9 $\pm$ 165	23.5 $\pm$ 4.6**

\* $p < 0.05$  compared to patients with inactive RA; \*\* $p < 0.01$  compared to active or inactive RA.

## Amounts of G-CSF in Sera and Synovial Fluid from Patients with RA



**Fig. 1.** Concentrations of G-CSF in 10 paired sera and synovial fluid (SF) samples from patients with RA. Note the high concentrations of G-CSF in peripheral blood (PB) and SF samples. The errors bars show the means  $\pm$  standard error (SE) for each group. Concentrations in the PB and SF were  $1,931 \pm 901$  and  $3,234 \pm 1358$ , respectively. There was no significant difference between the PB and SF ( $p = 0.11$  calculated by Student's *t*-test).



**Fig. 2.** Relationship between indices of disease activity and serum G-CSF levels in patients with RA. (A) Correlation between serum G-CSF levels and morning stiffness; (B) correlation between serum G-CSF levels and the number of tender or swollen joints; (C) correlation between serum G-CSF levels and the Lansbury index.

**Table III.** Comparison of RA disease activity in the sera of G-CSF-positive and G-CSF-negative groups (values are expressed as means  $\pm$  SD).

Indices	Positive (n = 18)	Negative (n = 42)
Morning stiffness (min.)	61.1 $\pm$ 12.3	41.2 $\pm$ 7.3
No. of tender/swollen joints	16.7 $\pm$ 3.6*	9.7 $\pm$ 1.4
ESR (mm/h)	63.8 $\pm$ 10.7*	42.0 $\pm$ 4.2
Lansbury Index (%)	53.7 $\pm$ 6.2*	40.0 $\pm$ 3.4
IL-1 (pg/ml)	214.2 $\pm$ 72.3**	46.5 $\pm$ 14.8
IL-6 (pg/ml)	158 $\pm$ 44.1	122.3 $\pm$ 9.7
TNF- (pg/ml)	934.1 $\pm$ 423	345.9 $\pm$ 142.3

\* $p < 0.05$  compared to patients with G-CSF-negative RA; \*\* $p < 0.005$  compared to patients with G-CSF-negative RA.

stiffness, and the number of tender or swollen joints. However, there was no correlation between serum G-CSF and ESR (data not shown). Furthermore, serum G-CSF levels correlated significantly with RA disease activity as expressed by the Lansbury index (Fig. 2).

#### Serum G-CSF and inflammatory cytokines in RA patients and controls

High G-CSF concentrations were detected in 18 of the 61 RA patients. The mean serum G-CSF level in 61 patients with RA ( $1,379 \pm 416$  pg/ml) was significantly higher than in 20 healthy subjects. There was no relationship between serum G-CSF levels and therapy with any particular agent, but the use of various anti-rheumatic drugs by these patients precluded confident analysis (data not shown). On the other hand, IL-1 was detected in 22, IL-6 in 11 and TNF- in 37 of the 61 serum samples from RA patients ( $95.6 \pm 25.3$  pg/ml,  $132 \pm 14.6$  pg/ml and  $514 \pm 161$  pg/ml, respectively).

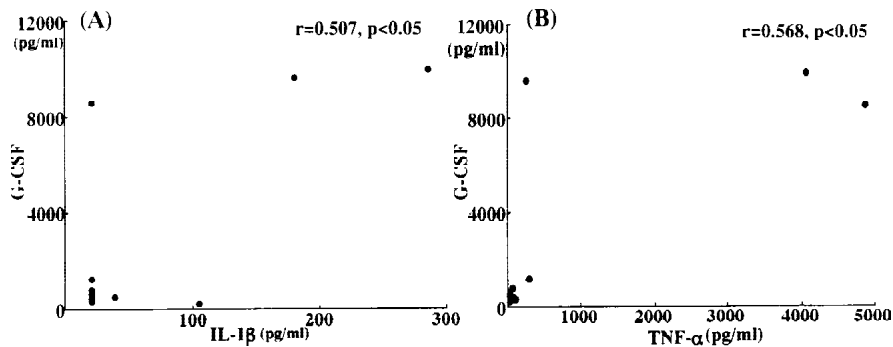
We also analyzed the data based on the presence of high versus normal levels of serum G-CSF levels in RA patients. Table III shows differences in various clinical indices and serum cytokine levels in G-CSF-positive and -negative patients with RA. The number of tender or swollen joints, ESR, Lansbury index, and IL-1 in G-CSF-positive group were significantly higher than the corresponding values in G-CSF-negative group (Table III). However, there was no significant difference in the other clinical indices between the 2 groups.

#### Correlation between serum G-CSF and inflammatory cytokines in RA

We also examined the correlation between serum G-CSF and IL-1, IL-6 and TNF- levels in patients with RA. As shown in Figure 3, serum G-CSF levels correlated significantly with IL-1 and TNF-. However, there was no correlation between serum G-CSF and serum IL-6 levels ( $r = 0.505$ , NS).

#### Discussion

Previous studies have suggested that G-CSF in synovial tissues may play a role in RA disease activity by activating neutrophils present in the synovial fluid of



**Fig. 3.** Relationship between serum G-CSF and inflammatory cytokine levels in RA patients. (A) Correlation between serum G-CSF levels and IL-1 $\beta$ ; (B) correlation between serum G-CSF levels and TNF- $\alpha$ .

RA patients (3). CSF production by various cells, such as fibroblasts, endothelial cells and monocytes (13-15), must be tightly regulated *in vivo* to induce rapid changes in leukocyte numbers, but also possibly to limit the magnitude of the inflammatory response and to prevent tissue damage (16).

Most studies investigating the actions of cytokines on synovial cells have analyzed those of individual cytokines. However, given that a number of cytokines have been detected in the rheumatoid synovium (17, 18), it is likely that these cytokines would act in concert on joint cells, such as synovial cells. There is a general agreement that cytokines play an important role in the pathogenesis of RA, especially with regard to IL-1, which exerts a wide variety of biological activities, such as its stimulating effects on fibroblast proliferation, bone resorption, acute phase reactants, PGE<sub>2</sub> production and collagenase production, and its destructive effect on chondrocytes (19). A correlation between plasma IL-1 levels and RA activity has also been reported (20). TNF- $\alpha$  has a similar biological activity to IL-1 and has been implicated in RA pathogenesis, as well (21-23).

In the present study, we examined the effects of several cytokines present in rheumatoid lesions on CSF production. Our results showed that only IL-1 and TNF- $\alpha$  were active; in other words, there seems to be some specificity in the control of synoviocyte CSF synthesis by cytokines. PDGF, for example, which is mitogenic for synoviocytes (24), cannot activate these cells to produce CSF. Synovial cells therefore can be included in the list of cell types (which includes

other cells of mesenchymal origin) that can respond to IL-1 and TNF- $\alpha$  by increasing G-CSF production.

When our RA patients were divided into two groups based on the presence or absence of G-CSF, significant differences between the RA patients became apparent. The number of tender or swollen joints, the ESR, the value of Lansbury index, and serum IL-1 concentrations in G-CSF-positive RA patients were significantly higher than in G-CSF-negative RA patients. These results strongly suggest that high G-CSF activity might contribute to the active inflammatory disease process in RA patients.

Previous studies have indicated that in juvenile rheumatoid arthritis there is a poor relationship between ESR levels and clinical activity (25). In contrast, serial analysis of serum concentrations of G-CSF during the clinical course of RA indicated that serum levels of this factor might be a useful marker of abnormal immune activation in RA (26). Thus our results, together with those of previous studies, suggest that the determination of G-CSF may help to identify those patients in whom active therapy should be considered, and could be useful for monitoring the response to treatment. Furthermore, serial studies of individual RA patients might be helpful to determine whether serum levels of G-CSF could be used as a marker of the response to therapy or to detect periods of heightened inflammatory activity during the course of this chronic illness. We have shown that in patients with RA, serum G-CSF levels are higher than in healthy subjects. More importantly, the concentrations correlated with clinical

and laboratory evidence of disease activity in a subgroup of RA patients. In individuals tested at different times, there were significant correlations between the G-CSF and ESR and platelets.

Our results suggest that cytokine-stimulated synovial fibroblasts may be a source of G-CSF production in the joints of patients with inflammatory arthritis. Consequently, granulocytes may be activated, leading to perpetuation of the inflammatory process and pathologic destruction in these lesions. Our results imply that G-CSF might be a clinically useful indicator of RA activity

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