Elevated Serum Granulocyte Colony-Stimulating Factor Levels in Patients With Active Phase of Sweet Syndrome and Patients With Active Behçet Disease

Implication in Neutrophil Apoptosis Dysfunction

Tamihiro Kawakami, MD, PhD; Syuichiro Ohashi, MD, PhD; Yoko Kawa, PhD; Hideto Takahama, MD, PhD; Masaru Ito, MD, PhD; Yoshinao Soma, MD, PhD; Masako Mizoguchi, MD, PhD

Background: Sweet syndrome (SS), an acute inflammatory disease, has clinical and laboratory features similar to those of Behçet disease (BD). Serum levels of granulocyte colony-stimulating factor (G-CSF) are elevated in patients with SS, and exogenous administration of G-CSF has repeatedly been implicated in the causation of SS. Granulocyte colony-stimulating factor is a hematopoietic growth factor that regulates the production and differentiation of neutrophils.

Objectives: To clarify the role of elevated serum G-CSF levels in patients with active SS and active BD compared with those with inactive SS or BD and healthy controls. To then analyze neutrophil apoptosis in the active state of SS and BD; and to also investigate the influence of autologous serum on neutrophil apoptosis.

Methods: Serum G-CSF was examined in 5 patients with active SS, 7 with inactive SS, 7 with active BD, 9 with inactive BD, and 5 healthy controls by means of an enzyme immunoassay kit. We measured apoptotic cells in the neutrophil fraction of peripheral blood collections in patients with active diseases and controls by means of flow cytometry.

Results: Serum G-CSF level was significantly higher in patients with active SS than in those with inactive SS. The difference in serum G-CSF levels among patients with active and inactive BD was also significant. Serum G-CSF level was significantly higher in patients with active SS than in those with active BD. Neutrophil apoptosis was significantly higher in patients with active SS than healthy controls. This increased apoptosis rate was also seen in patients with active BD. The increased rate of neutrophil apoptosis was significantly suppressed when the neutrophils were cultured for 18 hours in the presence of autologous active SS serum. Similarly, neutrophil apoptosis was suppressed in the presence of autologous serum in patients with active BD, but not significantly so.

Conclusions: These findings indicate that increased production of G-CSF in patients with SS and BD may play an important role in the manifestation of these disorders. Given the suppression of neutrophil apoptosis in the active state in the presence of the influence of autologous serum, which includes elevated G-CSF level, we propose that serum G-CSF plays a significant role in the suppression of neutrophil apoptosis. Furthermore, G-CSF–induced suppression of neutrophil apoptosis appears to be deeply involved in the pathogenesis of SS and BD.

Arch Dermatol. 2004;140:570-574

Sweet syndrome (SS) has been described as a rare but distinct disorder, characterized by 4 clinical features: fever, neutrophilic leukocytosis, sudden onset of asymmetric erythematous, and often-painful skin lesions and dense dermal infiltrates of mature neutrophils without signs of vasculitis. Behçet disease (BD) demonstrates symptoms similar to those of SS, although the incidence and clinical courses of these 2 disorders are different. Peripheral neutrophilia, enhanced neutrophil activity, and dense dermal infiltration of cutaneous lesions have also been noted in both disorders. Neutrophils may play an important role in the pathogenesis of these 2 diseases, as neutrophils are increased in blood and skin lesions.1

Recent observations have led to the notion that particular cytokines such as granulocyte colony-stimulating factor (G-CSF) are primarily involved in the pathogenesis of SS.2 This theory has arisen from studies that have demonstrated elevation of serum G-CSF levels in comparison with other cytokines during acute exacerbation of the disease.3 The validity of this theory has been substantiated by several recently reported cases of exogenous G-CSF–induced SS.4-11 Therapy with G-CSF for granulopenia is known to induce SS.12 Further-
Granulocyte colony-stimulating factor is a hematopoietic growth factor that increases the number of peripheral neutrophils and active mature neutrophils. In steady-state hematopoiesis, G-CSF regulates the level of neutrophils in bone marrow and blood and is critical for the survival of granulocytic cells. The factor strongly influences neutrophil function by stimulating production, activation, maturation, and chemotaxis of granulocytes. Philpott et al showed that G-CSF suppressed apoptosis in vivo in a CD34+ cell population and thereby prolonged their survival.

In the present study, we attempt to measure serum G-CSF concentrations in patients with SS and BD and to evaluate the significance of disease activity. We subsequently examine the abnormal neutrophil apoptosis mechanism attributed to neutrophils in the active state of SS or BD.

**METHODS**

**DETECTION OF G-CSF IN BLOOD SERUM**

Serum G-CSF levels were measured by enzyme immunoassay, as described by Motojima et al. Recombinant human G-CSF (rhG-CSF) was obtained (Kirin Brewery, Tokyo, Japan). Each sample and serial dilution of rhG-CSF, together with enzyme immunoassay buffer containing 2% polyethylene glycol, was added to a polyethylene tube coated with rabbit anti–rhG-CSF (IgG). These tubes were incubated for 2 hours, after which they were incubated with horseradish peroxidase–conjugated rabbit anti–rhG-GSF (Fab') solution for another 2 hours. Substrate reagent was added after washing. The reaction was stopped with 4N sulfuric acid after incubation for 1 hour in the dark, and the resulting optical density was measured at 492 nm.

**ISOLATION OF NEUTROPHILS AND APOPTOSIS ANALYSIS**

Heparinized peripheral blood from patients with active SS, patients with active BD, and healthy volunteers was individually layered on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) solution and centrifuged at 1250g for 40 minutes at 2°C. The plasma was collected and stored at −70°C. Mononuclear cell fractions were recovered from the cell band between the plasma and Ficoll-Hypaque solution. The resulting buffer coat was collected as the neutrophil-rich fraction, and neutrophils were purified by carrying out hypotonic lysis of erythrocytes. Neutrophil fractions (viability, >90%; purity, 99%) were used for the polarization assay. The neutrophil suspension was washed, filtered, and placed in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum (FCS) (GIBCO, Grand Island, NY) and 10nM HEPES buffer. Some of the culture was incubated in the presence of 20% autologous serum. All of the experiments were performed under endotoxin-free conditions.

Apopotosis was measured by flow cytometry according to a previously published procedure after 0, 3, 6, and 18 hours of incubation. After culturing, the cells were centrifuged and the pellets gently resuspended in 1.5 mL of a hypotonic solution of propidium iodide (50 µg/mL in 0.1% sodium citrate plus 0.1% Triton X-100; Sigma Chemical Co, St Louis, Mo). The tubes were placed in the dark overnight at 4°C. The propidium iodide fluorescence of individual nuclei was measured by flow cytometry by means of a standard fluorescence-activated cell sorter (FACScan; Becton-Dickinson, Mountain View, Calif).

Nuclear debris was excluded by an appropriate threshold on forward scatter in the acquisition step, and apoptotic bodies were excluded with an emission less than 10 in the red fluorescence channels (FL-3) during the analysis step. The 2 thresholds were determined to quantify the apoptotic event in the neutrophil population and to exclude material derived from its fragmentation. Ten thousand events were acquired and analyzed. All measurements were performed at the same instrument setting by the same operator.

**STATISTICAL ANALYSIS**

The 2-tailed, unpaired t test was used to compare statistical differences between 2 groups. The level of significance was set at *P*<.05 in all cases.

**RESULTS**

**MEASUREMENT OF G-CSF IN SERUM**

The mean serum G-CSF concentration was significantly higher (*P*<.01) in patients with active SS (115.4±33.2 pmol/L) vs patients with inactive SS (Figure 1). Patients with BD in the active stage had significantly (*P*=.002) higher serum G-CSF concentrations (34.1±12.8 pmol/L) than pa-
To better understand apoptosis of neutrophils in the active disease state, we incubated these cells in medium supplemented with 10% FCS and added 20% autologous serum. The solution was then analyzed by flow cytometry. Under these conditions, the rate of apoptosis of neutrophils in patients with active SS was significantly attenuated at 18 hours (P<.05; Figure 3). Autologous serum induced a marked reduction in the percentage of hypodiploid forms, which are characteristic of apoptotic nuclei, and there was a concomitant increase in the proportion of diploid nuclei. This indicates that autologous serum induces a remarkable inhibition of neutrophil apoptosis in patients with active SS.

The same methods were applied to neutrophils from patients in the active stage of BD. All assays were performed with the use of culture medium supplemented with 10% FCS and 20% autologous serum. To analyze whether proteolytic fragments generated by the action of autologous serum could be involved in the stimulation of neutrophil apoptosis, cells were incubated in 10% FCS or 10% FCS plus BD autologous serum for 0, 3, 6, and 18 hours. There was a distinct attenuation of apoptosis among nuclei treated with autologous serum for 18 hours vs those that were not (Figure 4).

**COMMENT**

The findings from this study demonstrated that G-CSF levels in peripheral blood are significantly higher in active SS and active BD vs the inactive stage. The G-CSF is a growth factor that promotes the production and maturation of myeloid cells and, in particular, the proliferation and differentiation of neutrophil progenitors both in vitro and in vivo. Treatment with G-CSF can exacerbate preexisting inflammatory conditions, presumably by stimulating neutrophil proliferation and activity at sites of chronic inflammation. High levels of G-CSF in active SS or BD seem to be a useful indicator of the
activity level in these diseases. Consequently, we propose that G-CSF may induce the active phase of SS via stimulatory effects on the production and function of neutrophils. The molecular basis for enhanced neutrophil function in the active state is unknown. Elevated serum G-CSF levels suggest that the cytokine physiologic activity of this compound may be involved. This hypothesis is supported in part by the present results concerning G-CSF. We further speculated that elevated G-CSF concentrations in inflammatory states may produce the associated neutrophilia. The maintenance of neutrophil numbers seen in active SS and BD is an important feature in response to inflammatory stimuli. Neutrophil chemotaxis has been found to be enhanced, and this has been attributed to the increased level of a chemotactic attractant in the serum.

Both BD and SS yield similar laboratory findings, but the clinical course of SS is different from that of BD. In SS, most symptoms appear at the same time, while 1 or more symptoms of BD appear in the acute phase. After the initial symptoms appear, it takes several years for other symptoms to appear in BD, which is a chronic inflammatory disease with remissions and relapses. The patients with active SS had significantly higher serum G-CSF levels than the patients with active BD. This finding suggests that increased G-CSF serum levels play a prominent role in the worsening of SS, and at least some part in the worsening of BD. The elevated G-CSF levels in active BD may play a direct role in the disease process. Although we have documented quantitative G-CSF abnormalities in patients with active SS or BD, the difference in cause between SS and BD, including the role of G-CSF, remains unclear.

An inverse relationship between serum G-CSF level and active phase in our patients suggests a physiologic role of G-CSF in neutrophil homeostasis. The G-CSF prolongs survival of neutrophils while enhancing chemotaxis, phagocytosis, superoxide generation, and other metabolic functions. We evaluated the correlation between serum G-CSF level and neutrophil apoptosis in patients with active SS, those with active BD, and healthy controls. This indicates that neutrophil apoptosis is accelerated in the active phase of disease. Furthermore, these data, based on incubation in FCS medium alone, suggest that apoptosis plays a large role in dramatically reducing neutrophilia in the active phase.

The inclusion of autologous serum led to a marked reduction in the rate of neutrophil apoptosis, and this was most prominent at 18 hours. Adding autologous serum to active SS or BD neutrophils was a more potent inhibitor of neutrophil apoptosis than FCS alone, suggesting a specific role for G-CSF in the regulation of neutrophil homeostasis. Apoptosis has been explained by a variety of neutrophil functional defects. The neutrophil apoptosis system seems to become less effective with progression of disease activity. This suggests that dysfunction associated with neutrophil apoptosis is an early, biologically relevant risk factor for active progression of the disease. The possible suppression of apoptosis in abnormal neutrophils by G-CSF requires further investigation. Furthermore, by better understanding the pathogenesis of this disorder, it may be possible to develop more effective treatment strategies for SS and BD through the induction of neutrophil apoptosis.

Accepted for publication May 28, 2003.

This study was supported by grants from the Scientific Research Fund of the Ministry of Education, Science, Sports and Culture, Tokyo, Japan (Grant-in-Aid for Scientific Research No. 14570828) and the Basic Dermatological Research from Shiseido Co Ltd, Tokyo.

Corresponding author and reprints: Tamihiro Kawakami, MD, Department of Dermatology, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa, 216-8511, Japan (e-mail: tami@marianu-u.ac.jp).

REFERENCES


17. Philpott NJ, Prue RL, Marsh JW, Gordon-Smith EC, Gibson FM. G-CSF-mobilized CD34+ peripheral blood stem cells are significantly less apoptotic than unstimulated peripheral blood CD34+ cells: role of G-CSF as survival factor. Br J Haematol. 1997;97:146-152.