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Enhanced Neutrophil Functions by Recombinant Human Granulocyte Colony-Stimulating Factor in Diabetic Patients with Foot Infections in vitro

This study was performed to evaluate the effect of granulocyte-colony stimulating factor on neutrophil functions in diabetic patients with active foot infections in vitro. Twelve diabetic patients with foot infections and 12 normal volunteers were enrolled. Neutrophils from peripheral blood were incubated with granulocyte colony-stimulating factor (G-CSF, 50 ng/mL) for 20 min. Superoxide production of neutrophils was measured by the reduction of ferricytochrome C. Neutrophil phagocytosis was assayed using *Staphylococcus aureus* and the weighted phagocytic index was calculated. Superoxide production of neutrophils in diabetic patients with foot infections was 7.7 (unit: nmol/2×10^5 cells/60 min), which was significantly lower than that in controls (12.0) (p<0.05). G-CSF increased neutrophil superoxide production to 12.1 in diabetic patients with foot infections and to 19.8 in controls (p<0.05 for each). Weighted phagocytic index in diabetic patients with foot infections was 0.77, which was not significantly different from that of the controls (0.69). Weighted phagocytic index was increased significantly by G-CSF to 0.88 in diabetic patients with foot infections and to 0.79 in controls (p<0.05 for each). In conclusion, G-CSF significantly enhanced neutrophil functions in diabetic patients with foot infections in vitro.

Key Words: *Diabetes Mellitus; Neutrophils; Granulocyte Colony-Stimulating Factor; Phagocytosis; Superoxides*

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INTRODUCTION

Foot infections occur in up to approximately 25% of diabetic patients (1). Treatment of serious foot infection remains difficult partly due to defective neutrophil function, which often requires amputation of the limbs (2, 3). Chemotaxis, phagocytic activity, bactericidal activity, respiratory burst activity, and superoxide production of neutrophils are defective in diabetic patients (4-6).

Granulocyte colony-stimulating factor (G-CSF) induces release of neutrophils from bone marrow to peripheral blood (7) and improves functions of normal and dysfunctional neutrophils (8-11). Recently, uses of recombinant human G-CSF (rhG-CSF) in non-neutropenic infection have been tried in several studies, especially in animal models, which showed that rhG-CSF increased the survival (12-20). In human studies, rhG-CSF was administered safely to neonates with infection and patients with pneumonia (21-23).

rhG-CSF has been reported to enhance myeloperoxidase activity and superoxide generation of neutrophils in diabetic patients without infection (24, 25). This study was performed to evaluate the effect of rhG-CSF on neutrophil functions in diabetic patients with active foot infections in vitro.

MATERIALS AND METHODS

Patients

Twelve diabetic patients with active foot infection were enrolled. Six were males and the median age was 62 yr (range of age: 37-76). Exclusion criteria were malignant disease, immunosuppressive therapy within 4 weeks, liver cirrhosis, pregnancy, and sepsis. The median duration of diabetes mellitus was 11.0 yr and the blood glucose was not controlled well (the median HbA_1C: 9.3%). Eight

patients had osteomyelitis and four patients had soft tissue infection only. Osteomyelitis was diagnosed according to MRI findings. Of the eight patients with osteomyelitis, five patients received amputation surgery, two received debridement surgery, and one refused surgical interventions. After the improvement of foot infection, follow-up evaluation of neutrophil function was performed in five patients out of 12. Twelve healthy volunteers (median age, 41 yr; range of age, 27-63) were enrolled as normal controls and 12 diabetic patients without infections who had more than 8 years' history of diabetes as diabetic controls (median age, 57 yr; range of age, 35-68).

Purification of neutrophils

Venous blood was collected into syringe containing heparin. Leukocyte-rich plasma was separated from erythrocytes by dextran (4.5%) sedimentation at 37℃ (26). Neutrophils were isolated from monocytic cells by Lymphoprep™ (Nycomed Pharma. Density 1.077) gradient centrifugation. Contaminating erythrocytes were lysed by hypotonic shock. Neutrophils were washed twice with phosphate-buffered saline (PBS), resuspended in PBS with 10% inactivated fetal bovine serum (FBS), and final suspension was adjusted to 2×10^6 neutrophils/mL. Cell viability was determined using trypan blue exclusion and showed more than 95% viability. Differentials were performed using Giemsa stain. Neutrophils were more than 95% of final cell suspension.

Pretreatment of neutrophils with rhG-CSF

Isolated neutrophils (2×10^6/mL) were incubated with 50 ng/mL of rhG-CSF (Neutrogin®; Lenograstim) in PBS at 37℃ for 20 min. Neutrophils were incubated with buffer only for baseline.

Superoxide generation by neutrophils

The reaction mixture was made with ferricytochrome C (160 μM) and N-formyl-methionyl-leucyl-phenylalanine (FMLP; 1 μM) at 4℃ (27). Superoxide dismutase (300 unit/mL) was added to the mixture for control. One hundred μL of reaction mixture was added into each well of a 96-well tissue culture plate. Neutrophils (100 μL) preincubated with or without rhG-CSF were added into each well. The plates were then covered with lids and placed in a 37℃ humidified incubator for 60 min. The reduction of cytochrome C was measured by spectrophotometry (550 nm) at 3, 15, 30, and 60 min of incubation. The absorbance after 60 min was not measured because the absorbance at 90 or 120 min was similar with that at 60 min in a preliminary study. The produced superoxide was calculated from the reduced cytochrome C at each time. Each experiment was done in triplicate.

Bacterial phagocytosis

Staphylococcus aureus (ATCC 29213) was grown in Mueller-Hinton broth for 2-3 hr at 37℃ before each experiment to obtain bacteria in a log-phase growth. They were then washed with PBS (pH 7.2) three times and adjusted to 1×10^8 cfu/mL in PBS. *S. aureus* was preopsonized with normal human serum (50% vol/vol, at 37℃ for 30 min), and mixed with 10^6 neutrophils previously treated with buffer or rhG-CSF in 1 mL of PBS, in a final cell-to-bacteria ratio of 1:10 (11, 28). Preparations were rocked at 37℃ for 30 min and immediately mixed with 0.2 mL of cold *N*-ethylmaleimide (0.2 mM; Sigma, CA, U.S.A.) to inhibit additional phagocytosis. The nonphagocytosed, extracellular bacteria were lysed with lysostaphin (20 units/mL; Sigma). Preparations were cytocentrifuged and stained with modified Wright's-Giemsa (Diff-Quick; Diagnostic Systems, Gibbstown, NJ, U.S.A.). Phagocytosis was measured by direct microscopy. The weighted phagocytic index (WPI) was calculated by multiplying the number of neutrophils having 0, 1-10, 11-20, 21-30, or >30 ingested bacteria by 0, 1, 2, 3, or 4, respectively, and dividing the total score by the number of neutrophils examined (usually 100).

Expression of a surface receptor

Neutrophils (10^6/mL) were incubated with fluorescein isothiocyanate (FITC)-labeled monoclonal anti-CD64 antibody for 30 min in ice. Incubated neutrophils were washed twice with Dulbecco's modified PBS, resuspended in 1 mL of PBS, and then analyzed by FACScan flow cytometer (29).

Statistics

Data were shown as mean±standard error (SEM). Superoxide generation was compared by repeated measure ANOVA. Phagocytosis and expression of CD64 were compared by paired t test. Differences were taken to be significant at p value <0.05.

RESULTS

At baseline, superoxide production of neutrophils in diabetic patients with active foot infection was 7.7±1.06 nmol/2×10^5 cells/60 min (mean±SEM), which was sig-

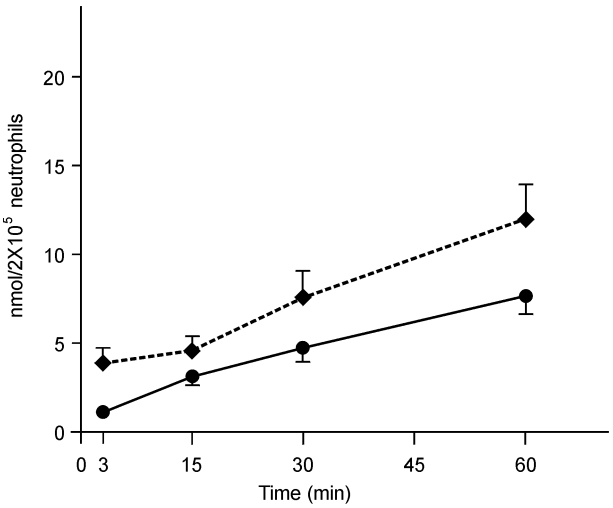

Fig. 1. Superoxide production of neutrophils in diabetic patients with foot infections and normal controls. Superoxide production is significantly lower in diabetic patients (●, solid line) than in controls (◆, dashed line) ($p<0.05$). Data are expressed as mean (nmol/2×10^5 cells) at each time. Vertical bars denote standard errors.

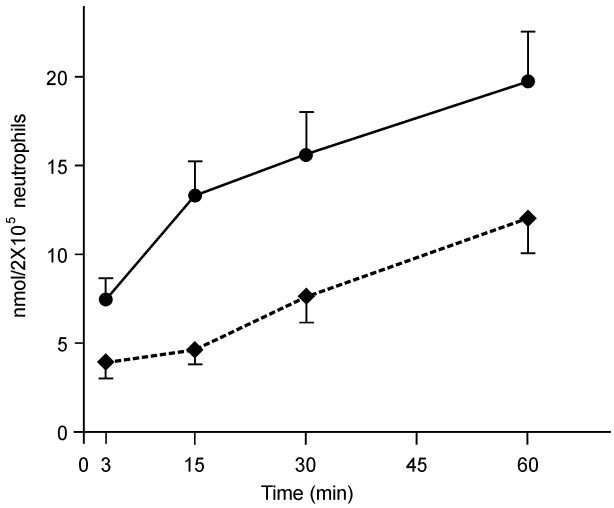

Fig. 2. The effect of rhG-CSF on the superoxide production of neutrophils in normal controls. The production of superoxide increases significantly ($p<0.05$) after the treatment of rhG-CSF (●, solid line) than before the treatment (◆, dashed line). Results are expressed as mean (nmol/2×10^5 cells) at each time. Vertical bars denote standard errors.

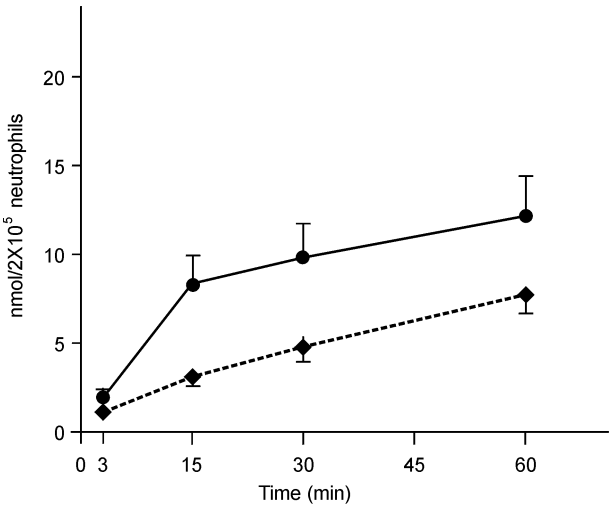

Fig. 3. The effect of rhG-CSF on the superoxide production of neutrophils in diabetic patients with foot infections. The production of superoxide increases significantly ($p<0.05$) after the treatment of rhG-CSF (●, solid line) than before the treatment (◆, dashed line). Results are expressed as mean (nmol/2×10^5 cells) at each time. Vertical bars denote standard errors.

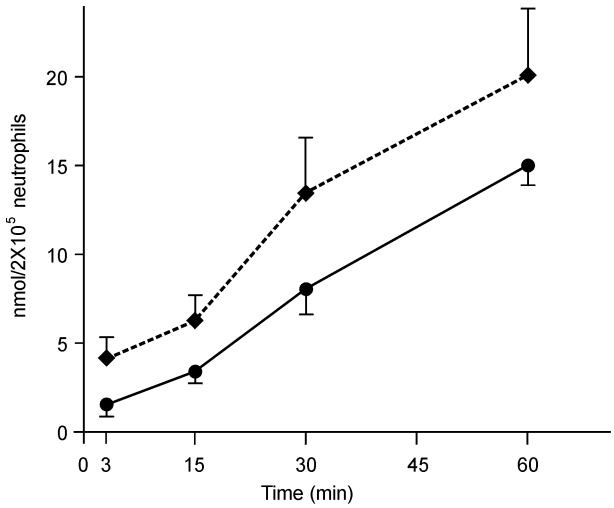

Fig. 4. Superoxide production of neutrophils in diabetic patients without infections and normal controls. Superoxide production is significantly lower in diabetic patients (●, solid line) than in controls (◆, dashed line) ($p<0.05$). Data are expressed as mean (nmol/2×10^5 cells) at each time. Vertical bars denote standard errors.

nificantly lower than that in normal controls (12.0±1.96; $p<0.05$; Fig. 1). Superoxide production of rhG-CSF-treated neutrophils was significantly higher than that at baseline in normal controls (19.8±2.86; $p<0.05$; Fig. 2) and in diabetic patients with foot infection (12.1±2.23; $p<0.05$; Fig. 3). Superoxide production of neutrophils in diabetic controls (diabetic patients without active infections) was significantly lower than that of normal controls ($p<0.05$; Fig. 4).

WPI in diabetic patients with foot infections was 0.77, which was not significantly different from that in normal controls (0.69; $p=0.1$). WPI was increased significantly by rhG-CSF treatment in diabetic patients with foot infections and in normal controls (0.88 and 0.79 respectively; $p<0.05$ for each; Fig. 5). WPI in diabetic controls was 0.67, which was not very different from that in normal controls (0.69), and was also increased significantly by rhG-CSF treatment (0.81; $p<0.05$; Fig. 5). Follow-up

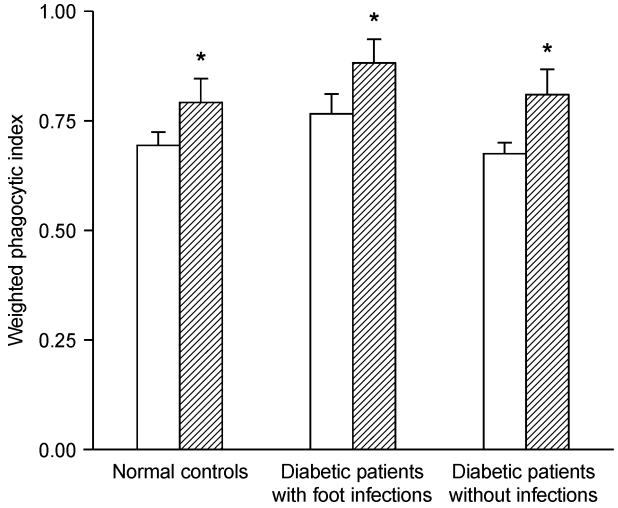

Fig. 5. The effect of rhG-CSF on the phagocytic activity of neutrophils in normal controls, diabetic patients with foot infections, and diabetic patients without infections. Phagocytic activity of untreated neutrophils (open bar) is compared with that of rhG-CSF-treated neutrophils (hatched bar). rhG-CSF enhances phagocytic activity of neutrophils significantly in each group (*$p<$ 0.05). There was no significant difference in the phagocytic activity of untreated neutrophils between the patient groups. Results are expressed as the mean weighted phagocytic index (+SEM).

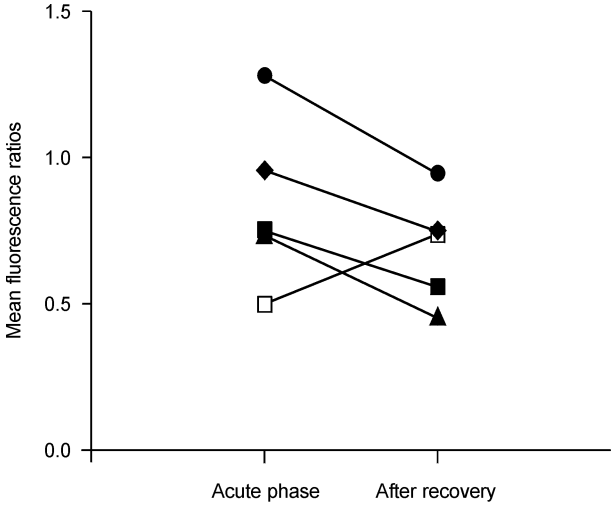

Fig. 6. Changes in phagocytic activity of neutrophils in diabetic patients with foot infections according to the treatment. Bacterial phagocytosis represented as weighted phagocytic index decreases after the resolution of the infection compared with that at the acute phase (n=5, p=0.2).

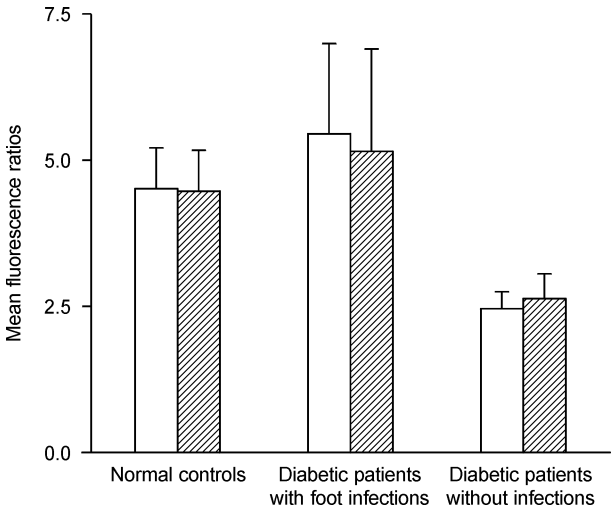

Fig. 7. The effect of rhG-CSF on CD64 expression of neutrophils (mean fluorescence ratios) in normal controls, diabetic patients with foot infections, and diabetic patients without infections. There are no significant difference in CD64 expression of neutrophils between the patient groups and before (open bar) and after (hatched bar) the treatment of rhG-CSF in each group.

WPI was measured in five patients after the recovery of foot infection, which showed decreasing tendency of WPI compared with that at acute phase of the infections although statistically not significant (p=0.2; Fig. 6).

Expression of CD64 on mature neutrophils was measured using flow cytometry. At baseline, the mean fluorescence ratio was higher in diabetic patients with foot infections (5.4) than in normal controls (4.5; p=0.1; Fig. 7). The mean fluorescence ratio of neutrophils was not sigificantly changed by rhG-CSF treatment either in diabetic patients with foot infection (5.2) or in normal controls (4.4).

DISCUSSION

Neutrophil functions are defective in diabetic patients (4-6, 24). However, most studies (4-6) have been performed in diabetic patients without infections. This study, which was done in diabetic patients with active foot infections, showed that superoxide generation of neutrophils was defective, which is consistent with previous findings.

There have been contradictory data on phagocytic activity of neutrophils in diabetic patients (4, 5, 30-32). This study showed that phagocytic activity of neutrophils was higher in diabetic patients with foot infection than that in normal controls, even though it was not statistically significant (p=0.1). It might be attributable to the presence of foot infection. Increased phagocytic activity was observed in patients with acute bacterial infection, which was normalized after the cure of infection (33). Our data also demonstrated the decreasing tendency of phagocytic activity after the recovery from foot infection in diabetic patients, although it was not statistically significant.

The high affinity receptor for immunoglobuline G

(CD64) is expressed on less than 10% of mature neutrophils, and is known to be induced in a greater degree in patients with certain infections and in patients treated with rhG-CSF (33, 34). However, there has been no data regarding the expression of CD64 on neutrophils in diabetic patients, especially with foot infections. Higher expression of CD64 in diabetic patients with foot infections than in healthy controls (p=0.1) in this study is probably caused by the presence of active infection, and may partly explain the increased phagocytic activity of neutrophils in the former group.

G-CSF has been reported to improve functions of normal and dysfunctional neutrophils (8-11). Our data showed that superoxide generation and phagocytic activity of nutrophils were both increased by rhG-CSF treatment in diabetic patients with foot infections. These seem to be the first data demonstrating the increase of phagocytic activity by rhG-CSF in diabetic patients, especially with foot infections.

In contrast to the previous findings that revealed enhanced expression of CD64 on neutrophils by rhG-CSF both in vivo and in vitro (34-38), expression of CD64 was not significantly enhanced by rhG-CSF treatment in this study. Short incubation time (20 min) in this study might have affected the degree of stimulation of the expression of CD64 on mature neutrophils, considering longer incubation times (12-72 hr) in previous studies.

In summary, rhG-CSF enhanced superoxide generation and phagocytic activity of neutrophils in diabetic patients with foot infections in vitro. These results suggest that rhG-CSF may be used to improve nonspecific immunity in diabetic patients. Further investigations are needed to evaluate in vivo effectiveness of rhG-CSF in diabetic patients as an adjunctive treatment of foot or other site infections.

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