Granulocyte-Colony Stimulating Factor Improves an Impaired Bactericidal Function in Neutrophils From STZ-Induced Diabetic Rats

NORIYUKI SATO AND HIROYUKI SHIMIZU

To evaluate whether G-CSF improves an impaired production of oxygen-derived free radicals in diabetic neutrophils, we studied the effect of G-CSF on chemiluminescence amplified by a luciferin analog (CLA-DCL) and luminol (L-DCL) in response to fMLP in neutrophils from STZ-induced diabetic rats. Both CLA-DCL and L-DCL in diabetic neutrophils were significantly reduced, and L-DCL was more sensitive to this inhibition than CLA-DCL. G-CSF did not change the basal chemiluminescence in either control or diabetic neutrophils, but it apparently primed CLA-DCL and L-DCL. Although, in diabetic neutrophils, the priming effect of G-CSF to both CLA-DCL and L-DCL was less compared with that in control neutrophils, L-DCL was more sensitive than CLA-DCL to this priming effect. Because bacterial infection is still an important cause of morbidity and mortality in diabetic patients, these data suggest that G-CSF may be useful as a drug to prevent the aggravation of bacterial infection in diabetic patients. *Diabetes* 42:470–73, 1993

Despite the great improvements brought by insulin and antimicrobial agents, bacterial infection is still an important cause of morbidity and mortality in diabetic patients (1). Neutrophils play a critical role in the host defense mechanism against various bacterial infections, and it is suggested that impaired neutrophil functions (e.g., chemotaxis, phagocytosis, and bactericidal functions) cause susceptibility to infections in diabetic patients (1–4). Recently, we (5–7) and others (8–10) have demonstrated an impaired production of oxygen-derived free radicals (e.g., O_2^- and H_2O_2-MPO-Cl^- antimicrobial system) in neutrophils from poorly controlled diabetic patients and STZ-induced diabetic rats. On the other hand, GM-CSF (11–13) and G-CSF (14) appear to stimulate the formation of granulocyte colonies from bone marrow–derived precursors and to enhance the function of mature neutrophils (e.g., O_2^- generation).

To explore the possibility that G-CSF may be useful as a drug to prevent the morbidity and mortality caused by infections in diabetic patients, we studied the effect of G-CSF on the production of oxygen-derived free radicals in neutrophils from STZ-induced diabetic rats. In this study, we used a luciferin analog (CLA; 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]-pyrazin-3-one) and luminol (L; 5-amino-2,3-dihydro-1,4-phthalazine-dione) as agents to amplify the luminescence. CLA- and L-dependent chemiluminescence appear to be highly dependent on the generation of O_2^- (15,16), which is the initial oxygen-derived free radical and a major intermediate in the formation of H_2O_2, and an H_2O_2-MPO-Cl^- system (17–20), respectively.

RESEARCH DESIGN AND METHODS

Male Wistar rats (200–250 g) were made diabetic by one intraperitoneal injection of STZ (Sigma, St. Louis, MO), 60 mg/kg body weight, dissolved in 0.1 M citric buffer (pH 4.2) as described previously (6). Animals were maintained in a temperature-controlled room (23 ± 1°C) with 14/10-h light/dark cycles. Animals had free access to the laboratory chow pellets and drinking water. Whole blood samples were collected from abdominal aorta under pentobarbital anesthesia from control (n = 6) and diabetic rats (n = 6) with heparinized syringes 3 wk after STZ injection. One milliliter of whole blood was used to

From the First Department of Internal Medicine, Gunma University School of Medicine, Maebashi, Gunma, Japan.

Address correspondence and reprint requests to Dr. Noriyuki Sato, First Department of Internal Medicine, Gunma University School of Medicine, 3-39-22, Showa, Maebashi, Gunma, 371, Japan.

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G-CSF, granulocyte-colony stimulating factor; STZ, streptozocin; CLA-DCL, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]-pyrazin-3-one dependent chemiluminescence; L-DCL, luminol dependent chemiluminescence; fMLP, formyl-methyonyl-leucyl-phenylalanine; GM-CSF, granulocyte-macrophage–colony stimulating factor; O_2^-, superoxide anion; H_2O_2-MPO-Cl^-, hydrogen peroxide-myeloperoxidase-halide; HBSS, Hanks' balanced salt solution; KC, kilocounts; MPO, myeloperoxidase.

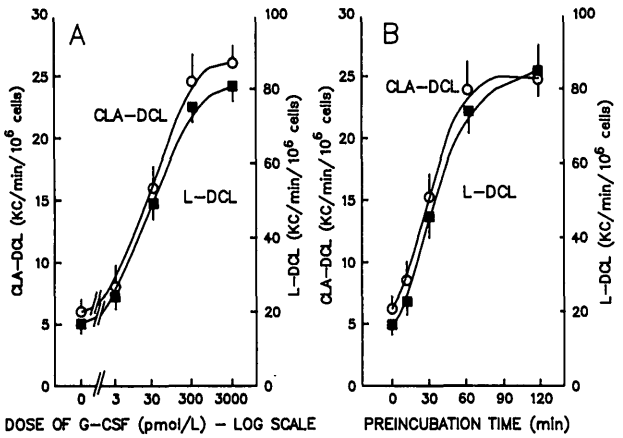

FIG. 1. Effects of dose and preincubation of G-CSF on either CLA-DCL (○) or L-DCL (■) in response to fMLP in control neutrophils. The means ± SE of 4 experiments are illustrated. *A*: Dose effect of G-CSF. Neutrophils were preincubated with the indicated concentration of G-CSF for 30 min, and CLA-DCL and L-DCL were measured as described in METHODS. *B*: Preincubation time effect of G-CSF. Neutrophils were preincubated with 30 pM G-CSF for the indicated time.

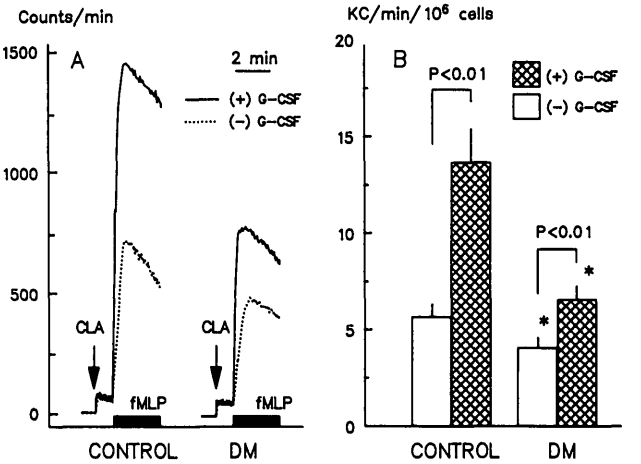

FIG. 2. Effect of G-CSF pretreatment on CLA-DCL in neutrophils from control and STZ-induced diabetic (DM) rats. *A*: Representative dynamics of chemiluminescence amplified with CLA in response to 100 nM fMLP in neutrophils pretreated with or without G-CSF. (↓), the addition of CLA; ■, the duration of fMLP stimulation (2 min). *B*: Mean and SE of replicate determination of 6 experiments. *$P < 0.05$ vs. similarly treated control rats (Student's *t* test).

measure blood glucose level, and the other blood was used for neutrophil preparation. Blood glucose level was measured by an automatic glucose analyzer with the glucose oxidase method (Model 23A, YSI, Yellow Springs, OH). Leukocytes were separated by the method described previously (21). The obtained cells consisted of 95–98% neutrophils suspended in HBSS buffer (Sigma) (1×10^6 neutrophils/ml).

After neutrophils separation, the cells were preincubated with or without 30 pM b-type recombinant human G-CSF (Chugai Pharmaceuticals, Tokyo, Japan) at 37°C for 30 min (the purity is >99%), because that dose and time were almost ED_{50}s for oxidative metabolism stimulated by fMLP (Sigma) in our experiments (Fig. 1) and in data reported previously (12,13). G-CSF was derived from Chinese hamster ovary cells (22,23), and the G-CSF solution, which was tested by the rabbit pyrogen assay, was not contaminated with lypopolysaccharides (23).

CLA-DCL and L-DCL of prepared neutrophils were measured by the method described previously (5,7). Briefly, 50 μl of CLA or L (final concentration, 50 μM) were added to the sample tube containing 100 μl of neutrophils-suspended solution and 1800 μl of HBSS buffer. After preincubation for 2 min, the neutrophils were stimulated by 50 μl of fMLP solution (final concentration, 100 nM). Chemiluminescence from neutrophils was measured by Luminescence Reader (Aloka, Model BLP 102, Tokyo, Japan). CLA-DCL and L-DCL were assessed with the peak value of chemiluminescence as $KC \cdot min^{-1} \cdot 10^6$ cells.

Statistical analysis was made with Student's *t* test. Viability of cells measured by Trypan blue staining was >97% at both the beginning and end of the experiments. All values are expressed as means ± SE. Experiments were repeated three times with essentially identical results each time.

RESULTS

The mean blood glucose level from diabetic rats was 31.5 ± 1.1 mM and was significantly higher than that from control rats (4.9 ± 1.0 mM, $P < 0.001$). From control neutrophils preincubated without G-CSF, fMLP caused a prompt rise in chemiluminescence (Figs. 2*A* and 3*A*). CLA-DCL and L-DCL were 5.7 ± 0.7 and 21.5 ± 1.6, respectively (Figs. 2*B* and 3*B*). In the neutrophils from diabetic rats, CLA-DCL and L-DCL were 4.1 ± 0.6 and 11.8 ± 0.9, respectively. Both CLA-DCL and L-DCL in neutrophils from diabetic rats were significantly lower than those from control rats, and L-DCL was clearly more inhibited than CLA-DCL (Fig. 4*A*) (CLA-DCL = 70.9%

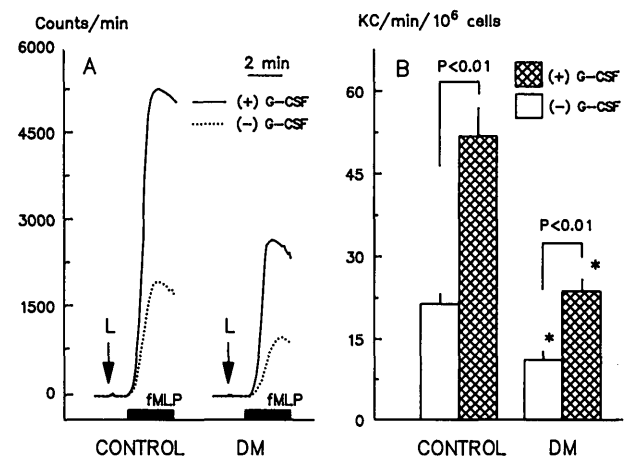

FIG. 3. Effect of G-CSF pretreatment on L-DCL in neutrophils from control and STZ-induced diabetic (DM) rats. *A*: Representative dynamics of chemiluminescence amplified with luminol in response to 100 nM fMLP in neutrophils pretreated with or without G-CSF. (↓), the addition of luminol (L); ■, the duration of fMLP stimulation (2 min). *B*: Mean and SE of replicate determination of 6 experiments. *$P < 0.05$ vs. similarly treated control rats (Student's *t* test).

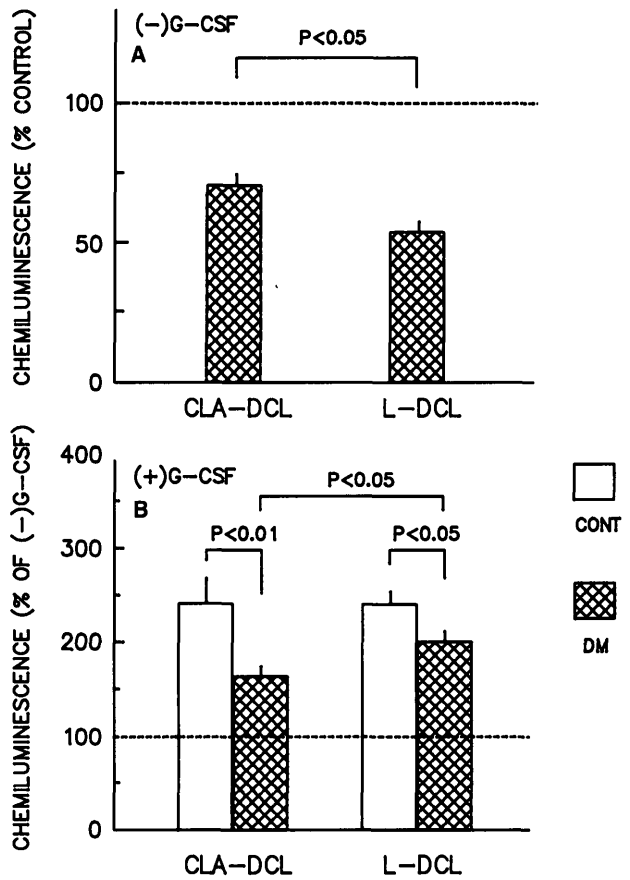

FIG. 4. Effect of G-CSF pretreatment on either CLA-DCL or L-DCL in *response to fMLP in neutrophils from control and STZ-induced* diabetic (DM) rats. Data are based on that illustrated in Figs. 2 and 3. *A*: Effects of diabetic condition on CLA-DCL and L-DCL. Data are expressed as a percentage of the responses from normal control neutrophils. Neither diabetic nor control neutrophils were pretreated with G-CSF. *B*: Comparing effects of G-CSF on DCL (CLA-DCL and L-DCL) between control and diabetic neutrophils. Data are expressed as a percentage of the responses achieved without G-CSF pretreatment (100% = responses achieved without G-CSF pretreatment).

and L-DCL = 54.9% of control rats). However, the dynamics of chemiluminescence in neutrophils from diabetic rats were similar to those from control rats (Figs. 2*A* and 3*A*).

In neutrophils of either the control or diabetic group, the 30-min pretreatment of 30 pM G-CSF apparently enhanced the fMLP-induced rise in both CLA-DCL and L-DCL without changing the dynamics (Figs. 2*A* and 3*A*). In the control group, G-CSF enhanced CLA-DCL and L-DCL by the same level (Fig. 4*B*). In the diabetic group, both CLA-DCL and L-DCL clearly were less enhanced by G-CSF than those from control neutrophils. Furthermore, the enhancement capacity of G-CSF in the diabetic group was clearly higher in L-DCL (202 ± 9%) than in CLA-DCL (165 ± 10%) (Fig. 4*B*).

DISCUSSION

The importance of several oxygen-derived free radicals in the antimicrobial activity of neutrophils has been recognized (24). Most of the increased oxygen uptake is used to form O_2^-, which is a major intermediate in the formation of H_2O_2. This generated H_2O_2 participates in the well-established H_2O_2-MPO-Cl^- system (19,24). In this study, both CLA-DCL and L-DCL in response to fMLP were decreased in STZ-induced diabetic rats. CLA-DCL appears to be highly dependent on the generation of O_2^-, because it is reported that azide did not affect CLA-DCL (15,16) and L-DCL dependents on an H_2O_2-MPO-Cl^- system (17–20). Thus, these results were consistent with our previous observation that the generation of O_2^- and an H_2O_2-MPO-Cl^- system was reduced in poorly controlled diabetic patients (5) and STZ-induced diabetic rats (6). Furthermore, the fact that L-DCL was more suppressed than CLA-DCL in this study suggests that the H_2O_2-MPO-Cl^- system is more impaired than O_2^- generation. This impaired H_2O_2-MPO-Cl^- system may contribute to greater susceptibility to infection in the diabetic state because O_2^- by itself is known to be weakly antimicrobial (8); the H_2O_2-MPO-Cl^- system is one of the most powerful antimicrobial systems (19); MPO activity was decreased in poorly controlled diabetic patients; and a significant negative correlation was found between HbA_1 and MPO activity (5).

G-CSF apparently enhanced fMLP-induced CLA-DCL and L-DCL in neutrophils we examined in this study. Extensive evidence from a number of laboratories indicates that GM-CSF (11–13) and G-CSF (14) act as priming agents on neutrophils, enhancing O_2^- generation in response to fMLP. However, no studies report about the effect of G-CSF on the generation of oxygen-derived free radicals subsequent to O_2^-. Our data suggest that G-CSF primes neutrophils for not only O_2^- production but the subsequent H_2O_2-MPO-Cl^- system as well.

Despite the great improvement brought by insulin and antimicrobial agents, bacterial infection is still an important cause of morbidity and mortality in diabetic patients (1), and no clinical and experimental drug trials are being conducted to find agents to improve the impaired neutrophil functions. This is the first study demonstrating that G-CSF primes the diabetic neutrophils that impair the generation of oxygen-derived free radicals. Since its discovery as a potent activator of priming the matured neutrophils for oxygen-derived free radicals, G-CSF has been used in clinical trials to improve resistance in patients with acquired immunodeficiency syndrome (25), myelodysplastic syndrome (26), and agranulocytosis (27). Although our data indicated that G-CSF could not enhance CLA-DCL and L-DCL in response to fMLP in diabetic neutrophils at the same level of those in control neutrophils, the priming ability of G-CSF was more effective in L-DCL than in CLA-DCL in diabetic neutrophils. This study shows that G-CSF is useful as a drug to improve the impaired diabetic neutrophil functions, especially an impaired H_2O_2-MPO-Cl^- system, and suggests that G-CSF may help to prevent morbidity and mortality from bacterial infections in diabetic patients.

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