Continuous subcutaneous injection reduces polymorphonuclear leukocyte activation by granulocyte colony-stimulating factor

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Sato, Yukio, Yukinobu Goto, Shoko Sato, Shunsuke Endo, and Yasunori Sohara. Continuous subcutaneous injection reduces polymorphonuclear leukocyte activation by granulocyte colony-stimulating factor. Am J Physiol Lung Cell Mol Physiol 286: L143–L148, 2004. First published October 3, 2003; 10.1152/ajplung.00248.2003.—The use of granulocyte colony stimulating factor (G-CSF) for recovery from neutropenia has been established; however, acute lung injury due to G-CSF-induced polymorphonuclear leukocyte (PMN) activation is a serious complication. This study was designed to compare the activation of PMN with single bolus administration and continuous administration of G-CSF. Healthy volunteers (age 33.8 ± 1.4 yr; n = 6) received a single bolus injection of 50 μm2/m2 of G-CSF (SI; n = 6) or continuous subcutaneous injection of 50 μm2/m2 of G-CSF for 24 h (CI; n = 6) and were followed for 48 h. Circulating leukocyte counts, markers of activation on PMN, and circulating levels of G-CSF, IL-6, and PMN elastase were measured. SI rapidly increased serum G-CSF levels, which peaked at 4 h, whereas CI gradually increased G-CSF levels, which remained at a steady level from 8 to 24 h. SI caused a rapid decrease in PMN counts at 0.5 h followed by sustained increase to peak at 12 h. CI gradually increased PMN counts, which peaked at 24 h, but the peak values were not significantly different between the groups. SI-induced activation of PMN, which was characterized by increased expression of CD11b, decreased expression of L-selectin, and increased F-actin content, led to increases in serum IL-6 and PMN elastase level. Such changes were all attenuated with CI (P < 0.05). We conclude that continuous subcutaneous injection of G-CSF resulted in a marrow response similar to that to a single injection but yielded reduced PMN activation.

Continuous subcutaneous injection reduces polymorphonuclear leukocyte activation by granulocyte colony-stimulating factor (G-CSF), a hematopoietic growth factor that enhances proliferation of polymorphonuclear leukocytes (PMN) (35), is used widely to accelerate recovery from neutropenia after cytotoxic therapy, lowers the risk of infection, and potentially enables escalation of chemotherapeutic drug dosage (9). The reported complications of G-CSF treatment include acute respiratory distress syndrome (ARDS) (5, 7), pulmonary capillary leak syndrome (19), interstitial pneumonitis (3, 11, 15, 18), and deterioration of pulmonary functions in neutropenic patients with ARDS (26). Although the incidence of these complications is not high, these complications are serious and can be fatal.

Activated PMN play an important role in initiating acute lung injury (12). They sequester in microvessels because of a decrease in their deformability (12) and an increase in their adhesive qualities (2). The decrease in deformability is mediated by rapid assembly of filamentous F-actin from soluble G-actin at the cell periphery (36), which contributes to the initial sequestration of PMN (6). The selectins slow PMN by mediating rolling, and the integrins induce firm adherence between PMN and endothelial cells (2), which prolongs sequestration of PMN (6).

G-CSF activates PMN in vivo and in vitro. Administration of G-CSF causes an immediate decrease in PMN counts (4, 17) because of PMN activation and resulting sequestration of PMN in lung microvessels (32). G-CSF induces PMN sequestration in the lung of rabbits by decreasing PMN deformability (14) and increases the expression of CD11b on PMN in vivo and in vitro (4, 20) and on monocytes in vivo (21). G-CSF pretreatment enhances endotoxin-induced increase in plasma levels of tumor necrosis factor-α induced by endotoxin in humans (23), and repeated administration of G-CSF increases plasma levels of tumor necrosis factor-α in humans (24). G-CSF also increases the plasma levels of IL-6 (8), IL-8 (34), and PMN elastase (4) in humans.

Intravascular activation of PMN by G-CSF is thought to be related to lung injuries associated with G-CSF administration. The dose and route of G-CSF administration as well as the effect of G-CSF on myeloid turnover have been studied (16). Because the effect of G-CSF on PMN activation is dose dependent (4), we hypothesized that continuous subcutaneous injection of G-CSF would cause less activation of PMN than a single subcutaneous injection. The effect of different routes of administration of G-CSF on activation of PMN has not been evaluated, and the aim of this study was to compare PMN activation and systemic inflammatory response between single bolus and continuous subcutaneous injection of G-CSF. G-CSF can be given intravenously. However, subcutaneous injection is more popular, and it is reported that intravenous injection has no advantage compared with subcutaneous injection (22). Therefore, the subcutaneous route was selected in this study. We evaluated increases of PMN count, expression of adhesion molecules on PMN and monocytes, assembly of F-actin in PMN, and serum levels of G-CSF, IL-6, and PMN elastase.

MATERIALS AND METHODS

G-CSF (Filgrastim, Kirin Brewery, Tokyo, Japan) was given to healthy volunteers (age 33.8 ± 1.4 yr). Medical history, physical examination, and routine laboratory values were normal in all subjects. None of the subjects used any medications, and none had febrile diseases in the month before the study. The subjects were under medical observation during the 2 days of the study. Written informed consent in accordance with the Declaration of Helsinki was obtained.

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from all subjects, and the protocol was approved by the Institutional Review Board of the University of Tsukuba.

The single bolus subcutaneous injection (SI) group (n = 6) received 50 μm/m² of G-CSF, the dose used for neutropenic patients after cytotoxic therapy for malignant tumor, at 9 AM (time 0). The continuous subcutaneous injection (CI) group (n = 6) received 50 μm/m² of G-CSF as a continuous subcutaneous injection for 24 h beginning at 9 AM (time 0) with a portable infusion pump (SP-3HQ, Nipro, Osaka, Japan) and special syringe (CI-3, Nipro) and tubing (SP-N, Nipro) that minimized adhesion of G-CSF. In preliminary experiments, we confirmed that G-CSF was stable in the infusion pump for at least 24 h and that the adhesion of G-CSF was negligible (data not shown). Venous blood samples were obtained immediately before the administration of G-CSF and at 0.5, 1, 2, 4, 8, 12, 24, 36, and 48 h thereafter. Blood samples for blood cell counts, differential leukocyte counts, and assessment of CD11b and L-selectin expression were collected in potassium EDTA-containing tubes. Blood samples for examination of F-actin were collected into acid-citrate-dextrose-containing tubes (9:1 by volume). Blood samples for testing of G-CSF and IL-6 levels were collected and centrifuged at 3,000 rpm for 15 min, and sera were stored at −80°C until use.

G-CSF assay. Serum levels of G-CSF were measured by Kainos Laboratories (Tokyo, Japan) with their high-sensitivity ELISA kits (Cyclysa G-CSF).

Blood cell counts. Blood cell counts were performed with a Coulter counter (T-660, Coulter Electronics, Hialeah, FL), and differential leukocyte counts were made on Wright-stained blood smears by independent, experienced observers without knowledge of the group or sampling time.

F-actin content assay. Fifty microliters of blood samples taken at 0 and 0.5 h were fixed, and the cells were permeabilized with IntraPrep permeabilization reagent (Immunotech, Coulter, Marseilles, France). PMN were stained with 1 U of BODIPY FL phallacidin (Molecular Probes), and F-actin content was measured as the mean fluorescence intensity of 5,000 cells determined with a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ) as described previously (25). PMN were identified with the typical forward- and side-scatter patterns. The change of F-actin content was expressed as percent change compared with the baseline value.

Adhesion molecule assay. The levels of L-selectin and CD11b expressed on PMN and monocytes were measured by flow cytometry as described previously (25). One hundred microliters of each sample was incubated with two micrograms of FITC-conjugated mouse monoclonal anti-human CD11b antibody (Dako Laboratories). Identical samples were incubated with FITC-conjugated mouse IgG (DAKO Laboratories) and phycoerythrin-conjugated mouse IgG2a (DAKO Laboratories) as negative controls. Erythrocytes were lysed with Immuno-lyse (Coulter), and leukocytes were fixed with Immuno-fix (Coulter). PMN and monocytes were identified with the typical forward- and side-scatter patterns, and levels of expression of L-selectin and CD11b were measured as the mean fluorescent intensity of 5,000 PMN or 1,000 monocytes. Changes in levels of L-selectin and CD11b were expressed as percent changes compared with baseline.

Fig. 1. Serum granulocyte colony-stimulating factor (G-CSF) level. Single bolus subcutaneous injection (SI) immediately increased serum G-CSF levels after injection to a peak at 4 h. Continuous subcutaneous injection (CI) gradually increased serum G-CSF with a plateau from 8 h and a peak at 24 h. SI caused a higher peak compared with CI. Results are expressed as means ± SE of 6 experiments. *P < 0.05 vs. time 0.

Fig. 2. Blood cell counts. A: total leukocyte counts. SI decreased counts at 0.5 h and then increased counts to a peak at 12 h. CI increased the counts to a peak at 24 h without an initial decrease. B: polymorphonuclear leukocyte (PMN) counts. SI decreased counts at 0.5 h and then increased counts to a peak at 12 h. CI increased counts to a peak at 24 h without an initial decrease. C: monocyte counts. SI decreased counts at 0.5 h and then increased counts to a peak at 12 h. CI increased counts to a peak at 24 h without an initial decrease. The changes from 0 to 1 h are enlarged in insets. Results are expressed as means ± SE of 6 experiments. *P < 0.05 vs. time 0; **P < 0.05 vs. SI.
IL-6 assay. Serum levels of IL-6 were measured to evaluate systemic inflammatory response with a highly sensitive ELISA kit (ANALYZA IL-6 HS, Genzyme Tech, Minneapolis, MN) according to the manufacturer’s instructions.

PMN elastase assay. PMN elastase was measured with an ELISA kit (Merck Immunoassay, Darmstadt, Germany) according to the manufacturer’s instructions.

Statistics. Data were analyzed by one-way and two-way analysis of variance in a randomized block design with time as a repeating factor and donor as a grouping factor. The sequential rejective Bonferroni test was used to correct for multiple comparisons (13). A corrected $P$ value $< 0.05$ was considered significant. All values are expressed as means $\pm$ SE.

RESULTS

G-CSF level. SI increased serum G-CSF levels immediately after injection, and levels peaked at 4 h (Fig. 1; time 0: $12 \pm 5$, 4 h: $3933 \pm 526$ pg/ml; $P < 0.0001$). CI increased serum G-CSF levels gradually and remained constant from 8 h (time 0: $9 \pm 2$, 8 h: $774 \pm 90$ pg/ml; $P < 0.005$) with a peak at 24 h ($939 \pm 109$ pg/ml). SI caused a sharper and higher peak than CI ($P < 0.0005$).

Blood cell counts. SI decreased total leukocyte and PMN counts (total leukocyte: $6.5 \pm 0.4$ to $4.8 \pm 0.4 \times 10^9$/l, $P < 0.01$; PMN: $3.5 \pm 0.4$ to $2.2 \pm 0.3 \times 10^9$/l, $P < 0.05$) at 0.5 h (Fig. 2A and B) and increased both counts to peak levels at 12 h (total leukocyte: $28.0 \pm 2.1 \times 10^9$/l, $P < 0.0001$; PMN: $22.6 \pm 2.1 \times 10^9$/l, $P < 0.0001$). CI gradually increased total leukocyte and PMN counts (total leukocyte: $6.6 \pm 0.6$ to $25.0 \pm 4.0 \times 10^9$/l, $P < 0.001$; PMN: $3.3 \pm 0.4$ to $21.1 \pm 3.5 \times 10^9$/l, $P < 0.0005$) at 24 h without an initial decrease.

SI decreased monocyte counts from $0.38 \pm 0.03$ to $0.27 \pm 0.01 \times 10^9$/l at 0.5 h (Fig. 2C; $P < 0.005$) and increased counts to $0.94 \pm 0.11 \times 10^9$/l at 12 h ($P < 0.001$). CI increased monocyte counts from $0.42 \pm 0.09$ to $0.88 \pm 0.14 \times 10^9$/l at 12 h ($P < 0.05$) without an initial decrease.

SI increased band-formed PMN counts from $0.37 \pm 0.06$ to $4.6 \pm 0.51 \times 10^9$/l at 12 h (Fig. 3A, $P < 0.0001$). CI increased band-formed PMN counts from $0.41 \pm 0.08$ to $4.1 \pm 0.59 \times 10^9$/l at 24 h ($P < 0.0005$). At the end of the study, the cumulative count of band-formed PMN, which is a surrogate

![Fig. 3. Differential counts. A: band-formed PMN counts. SI increased counts to a peak at 12 h. CI increased counts to a peak at 24 h. B: accumulative count of band-formed PMN counts. The counts at the end of the study were similar in each group. Results are expressed as means $\pm$ SE of 6 experiments. $^*P < 0.05$ vs. time 0; $^{**}P < 0.05$ vs. SI.](image)

![Fig. 4. Adhesion molecules and F-actin of PMN. A: expression of L-selectin. With SI, the expression decreased at 0.5 h, increased to a peak at 4 h, and decreased again to the lowest value at 24 h. With CI, the expression increased to a peak at 4 h and then decreased to the lowest value at 36 h. B: expression of CD11b. Both SI and CI increased the expression, which returned to baseline by 36 h. The values for the CI group from 1 to 4 h were lower than those of the SI group. C: F-actin content of PMN. SI increased the F-actin content at 0.5 h ($P < 0.05$), whereas CI did not change F-actin levels significantly. The values for the CI group were lower than those of the SI group. Results are expressed as means $\pm$ SE of 6 experiments. $^*P < 0.05$ vs. time 0; $^{**}P < 0.05$ vs. SI.](image)
marker of bone marrow output of PMN (28), was similar in each group (Fig. 3B).

**F-actin content.** SI increased the F-actin content of PMN to 155 ± 10% that of baseline at 0.5 h (P < 0.05), whereas CI did not change F-actin levels significantly (117 ± 26% of baseline at 0.5 h).

**Adhesion molecules.** SI decreased the expression of L-selectin on PMN to 87 ± 4% at 0.5 h (Fig. 4A, P < 0.01), which increased to 123 ± 7% at 4 h (P < 0.01), decreased again to 65 ± 7% at 24 h (P < 0.0005), and remained low for the rest of the study period. CI increased the expression of L-selectin on PMN to 117 ± 6% at 4 h (P < 0.05), which then decreased to 59 ± 2% at 36 h (P < 0.0005) and remained low for the rest of the study period. Both SI and CI increased expression of CD11b on PMN (Fig. 4B; SI: 189 ± 13% of baseline at 2 h, P < 0.0001; CI: 153 ± 9% of baseline at 12 h, P < 0.0005), and levels returned to baseline in both groups by 36 h. The values for the CI group from 4 to 12 h were lower than those of the SI group. Results are expressed as means ± SE of 6 experiments. *P < 0.05 vs. time 0; **P < 0.05 vs. SI.

**IL-6 level.** SI increased the serum IL-6 level from 0.84 ± 0.10 to 1.99 ± 0.35 pg/ml (Fig. 6; P < 0.05), whereas CI did not change the level significantly (time 0: 0.80 ± 0.17; 12 h: 1.24 ± 0.14 pg/ml, P = 0.08).

**PMN elastase level.** SI increased serum PMN elastase levels from 173 ± 46 to 1,058 ± 239 μg/ml at 24 h (Fig. 7; P < 0.05). CI increased these levels from 253 ± 19 to 474 ± 30 μg/ml at 36 h (P < 0.0005). The values of the CI group at 8, 24, 36, and 48 h were lower than those of the SI group (P < 0.05).
DISCUSSION

The results of this study have shown that a single bolus injection of G-CSF immediately decreases circulating PMN counts, activates circulating PMN and monocytes, and increases serum IL-6 and PMN elastase levels. This activation of circulating PMN and monocytes and the associated systemic inflammatory response have been implicated in the pathogenesis of acute lung injury. Continuous injection of G-CSF reduced these responses, whereas inducing a similar proliferative effect and bone marrow output of PMN. These findings suggest that treatment of neutropenia with continuous subcutaneous injection of G-CSF might cause fewer lung complications compared with single bolus injection.

Single bolus injection immediately increased the serum G-CSF level to a peak of ~4 ng/ml at 4 h (Fig. 1). Continuous injection of G-CSF gradually increased the serum G-CSF level, which remained at ~1 ng/ml from 8 to 24 h. The proliferative effect of G-CSF on PMN is almost saturated at a concentration of ~1 ng/ml in vitro (33); we speculate that 1 ng/ml of serum G-CSF is sufficient to increase circulating PMN counts. Excessively high concentrations of serum G-CSF may cause unnecessary and sometimes harmful activation of PMN. Therefore, it is likely that continuous injection of G-CSF maintains an effective serum concentration without a surge in blood G-CSF levels.

Single bolus injection induced an immediate decrease in total leukocyte counts (Fig. 2). This leukopenia is caused predominantly by a decrease in PMN and a smaller decrease in monocytes. The lymphocyte counts (data not shown) did not change significantly. Single bolus injection increased the PMN counts to peak level at 12 h, whereas continuous injection gradually increased the PMN counts to a similar peak level at 24 h. Because there was no difference in the peak counts and accumulative counts of band cells between two groups (Fig. 3), which were used as surrogate markers of PMN release from the bone marrow (28), the methods of injection appeared to induce similar degrees of bone marrow output of PMN.

Single bolus injection immediately increased the F-actin content of PMN, whereas continuous subcutaneous injection did not alter F-actin content, suggesting that continuous injection did not cause the cytoskeletal and deformability changes observed with single injection. Single bolus injection immediately decreased PMN L-selectin expression. Expression then increased and then gradually decreased over time (Fig. 4B). The immediate decrease is thought to reflect L-selectin shedding due to activation of PMN (29) or selective removal of cells that express high L-selectin from the circulation by adhesion. The subsequent increase represents bone marrow release of PMN with high levels of L-selectin (30). The continuous injection gradually increased PMN L-selectin expression, suggesting that only bone marrow release of PMN occurred (30). The later decrease observed in both groups is due to shedding of L-selectin by aging of PMN in the circulation (31). Single bolus injection immediately increased CD11b expression on PMN (Fig. 3A), which is consistent with former studies (20). Continuous injection increased CD11b expression on PMN gradually; however, the peak level of expression with continuous injection was lower than that of single bolus injection, suggesting that continuous injection causes less PMN activation. These changes in the deformability and adhesive qualities of PMN caused by single bolus injection are associated with PMN sequestration in microvessels of the lung and other tissues (12). The immediate decrease in numbers of circulating PMN is the hallmark of diffuse intravascular activation of PMN (12). The fact that continuous subcutaneous injection reduces these functional changes in PMN and monocytes indicates that it reduces the intravascular PMN activation and PMN sequestration in the microvessels of the lung.

Single bolus injection increased serum IL-6 levels, which is consistent with previous reports (8). IL-6 is an important proinflammatory cytokine, and elevated levels are a marker of systemic inflammatory response (1, 27). Single bolus injection increased IL-6 levels, whereas continuous injection did not cause a significant increase in IL-6 levels, suggesting that the systemic inflammatory response is attenuated with continuous injection. Although the increase in IL-6 levels observed in this study is small and may not have physiological significance, the increase in IL-6 levels could influence the inflammatory response for patients at risk of acute lung injury. Therefore, continuous injection of G-CSF could be beneficial to attenuate inflammatory response in patients after cytotoxic therapy who are supposed to be in proinflammatory status.

Single bolus injection induced release of elastase from PMN (Fig. 7), which is consistent with previous reports (4), and continuous injection did not cause this release. Release of elastase from PMN sequestered in microvessels could damage endothelial cells and cause tissue injury (10). Our results suggest that continuous injection of G-CSF may cause less endothelial injury than single bolus injection.

In summary, our results show that single bolus subcutaneous injection and continuous subcutaneous injection of G-CSF have similar effects on myeloid proliferation and bone marrow output. However, single bolus subcutaneous injection of G-CSF activates circulating PMN and monocytes, promotes their sequestration in microvessels, and induces a systemic inflammatory response. These changes could lead to acute lung injury. Continuous subcutaneous injection of G-CSF caused lower activation of PMN and monocytes and did not generate a systemic inflammatory response. Interpretation of this study is limited because it was performed with only healthy volunteers. Therefore, the effects of continuous subcutaneous injection of G-CSF must be determined in neutropenic patients before clinical application. However, our findings suggest that a minimal essential concentration of G-CSF maintained by continuous subcutaneous injection is sufficient to treat neutropenia and will not cause unnecessary intravascular PMN activation.

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CONTINUOUS SUBCUTANEOUS INJECTION OF G-CSF


