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Effects of Granulocyte Colony-Stimulating Factor on Opsonin Receptor Expression and Neutrophil Antibacterial Activity in a Mouse Model of Severe Acute Pancreatitis

Wpływ czynnika stymulującego tworzenie kolonii granulocytów na ekspresję receptora opsoninowego i przeciwbakteryjną aktywność neutrofilów w mysim modelu ostrego zapalenia trzustki

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Summary

The antimicrobial function of neutrophils, which is dependent on opsonin receptors, deteriorates in severe acute pancreatitis (SAP). Granulocyte colony-stimulating factor (G-CSF) putatively enhanced levels of the opsonin receptors CD11b and CD32/16 in healthy human subjects, and provided protection against infection in animal models of SAP. A statistically convincing study of the effect of G-CSF on CD32/16 expression in an SAP model is lacking. We used a mouse model of SAP to investigate the association between G-CSF administration and CD32/16 levels on neutrophils and bacterial translocation. G-CSF or saline was subcutaneously injected into SAP-induced mice. The pancreases were histologically examined, and leukocytes were stained to count neutrophils. The expression of CD11b and CD32/16 on neutrophils was measured by flow cytometry, and bacterial translocation was observed by bacterial culture.

The numbers of CD11b and CD32/16-positive neutrophils were significantly elevated in the SAP mice treated with G-CSF, and the mean fluorescence intensities of these receptors on neutrophils were significantly elevated. Bacterial translocations to cavity organs were suppressed from 17% to 6% by G-CSF treatment. Our results indicated that the number of neutrophils significantly increased with increasing expression of CD11b and CD32/16 and their mean fluorescence intensities (MFIs). This inhibited bacterial translocation to other organs. These results are in accord with other studies in SAP dogs and SAP mice. Our findings suggest that G-CSF was effective in protecting against bacterial infection in SAP mice.

Keywords: **CD11b • CD32/16 • granulocyte colony-stimulating factor • pancreatitis • severe acute pancreatitis • mouse model**

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Introduction

Severe acute pancreatitis (SAP) causes the failure of local and systemic bacterial clearance, and the incidence rates of bacteremia and mortality are proportional to the severity of the disease [5]. Infected pancreatic necrosis is primarily considered a late complication in the course of acute pancreatitis, but in almost one of four patients it also occurs early [11].

Neutrophil granulocytes (neutrophils), which make up the majority of white blood cells (leukocytes), are important constituents of the innate immune system. There is evidence that the defensive functions of neutrophils deteriorate in SAP. The migration and phagocytic functions of neutrophils are strictly controlled by the opsonin receptors on their surface [1,16]. The main opsonin receptors are cluster of differentiation molecule 11B (CD11b) and CD32/16 [5,7]. CD11b and CD32/16 levels are elevated in mice with edematous pancreatitis, but attenuated in SAP [7,13,15].

Granulocyte colony-stimulating factor (G-CSF) has been shown clinically to stimulate the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils [2,3,8,14]. Other studies have endeavored to improve neutrophil function through G-CSF. G-CSF enhanced the expressions of CD11b and CD32/16 and the phagocytic functions of neutrophils in healthy volunteers [8]. The administration of G-CSF was also associated with induced CD16 expression in two patients complicated by bacterial infections, and these patients recovered from or evaded infection [10]. However, the small number of human patients and healthy volunteers in these studies limited their statistical power.

In a canine model of SAP, treatment with G-CSF was associated with a significant and sustained increase in mature granulocytes, reduced bacterial translocation, and decreased rate of distant infection [12]. In a rat SAP model, administration of G-CSF appeared to increase the number of neutrophils and enhance their antibacterial functions [4]. However, the expression of CD32/16 was not investigated in these two *in vivo* studies [4,12], and the effect of G-CSF on CD32/16 levels is unknown.

In the present study, we hypothesized that G-CSF CSF might promote CD32/16 expression on neutrophils, thus inhibiting bacterial translocation to cavity organs in SAP mice. To evaluate the effects of G-CSF on the antibacterial activity of neutrophils, we directly investigated the expression of CD32/16 on neutrophils and bacterial translocations in a mouse model of SAP.

Material and Methods

Animals

Female Balb/c mice (8 weeks old and 22-25 g) were supplied by Cler Corporation of Japan (Tokyo, Japan). The care and handling of the animals were in accordance with the Guidelines of the National Institutes of Health of the United States. The mice were acclimated in our animal facility for one week prior to the experiments. The Surgical Research Committee and Animal Administrative Advisory Committee of Kyorin University, Japan approved this study.

Experimental Design

Thirty mice were randomly apportioned to one of the following three groups: SAP (SAP, n = 12), SAP with G-CSF treatment (SAP-G, n = 12), or a normal control group without SAP or G-CSF treatment (NC, n = 6). SAP was induced after the mice were fasted for 6 hours via 13 hourly subcutaneous injections of 0.2 mL of saline with 50 μg/kg body weight ceruletide (Sigma-Aldrich Japan, Tokyo, Japan). The G-CSF group was treated with G-CSF (120 μg/kg body weight; Nartograstim from Kyowa Hakko Kogyo, Tokyo, Japan), while the NC group received the same volume of saline, subcutaneously injected into the mice every 12 hours, beginning 2 hours after the final injection of ceruletide.

The mice were euthanized after fasting for 6 hours in the NC group, and 12 hours or 24 hours after the final injection of ceruletide in the SAP and SAP-G groups. Lavage of the peritoneal cavity was performed with phosphate buffered saline (PBS), and the peritoneal fluid was collected. Heparinized blood (100 units/mL) was obtained from the inferior vena cava. The pancreas, liver, and mesenteric tissues were harvested for pancreatic histological examination and bacterial culture at each time point.

Histological Examination

The harvested pancreases were fixed in 10% formaldehyde for 24 hours. After hematoxylin and eosin staining, the pancreases were histologically examined and pancreatitis was graded based on the degrees of acinar cell vacuolization, necrosis, and inflammatory cell infiltration according to the method of Niederau et al. [9]. The two examiners were blinded to the treatment and control groups.

Amylase Assay

Plasma amylase activity was measured 12 and 24 hours after the induction of acute pancreatitis using CNP-G7(5) as the substrate (Kanto Kagaku, Tokyo, Japan) on an automated analyzer (7070 Type, Hitachi, Tokyo, Japan).

Leukocyte Counting

Circulatory leukocytes (per mm^3) and peritoneal exudative leukocytes (total number in peritoneal lavage fluid) were stained with Turcki solution (Muto Chemical, Tokyo, Japan) and counted.

Sample Preparation and Measurement of Expression Levels of CD11b and CD32/16

The monoclonal antibodies used in this study were fluorescein isothiocyanate-conjugated rat anti-mouse CD11b, which reacts specifically with the 170-kDa α chain of Mac-1, and r-phycoerythrin-conjugated rat anti-mouse CD32/16 immunoglobulin G, which reacts specifically with a common nonpolymorphic epitope on the extracellular domains of mouse FcγRII and FcγRIII receptors immunoglobulin G (Pharmingen, San Diego, CA). Cells were considered to stain positive for CD11b or CD32/16 if they exceeded the fluorescence of 99.95% of the isotype-matched control (rat IgG2bκ) cells.

Peritoneal lavage fluids were centrifuged at 600×*g* for 5 minutes, and the peritoneal exudative cells were resuspended in 400 µL of PBS. One hundred microliters of blood or peritoneal exudate cell sample was incubated with anti-CD11b or anti-CD32/16 monoclonal antibody at 40°C in the dark for 30 minutes. Erythrocytes were then lysed with 2 mL of FACS lysis buffer (168 mM NH_4CL, 10 mM $KHCO_3$, and 10 mM EDTA-4Na). The remaining leukocytes were washed with PBS supplemented with 0.1% sodium azide and 0.1% bovine serum albumin (Gibco Laboratories, Grand Island, NY) and fixed in 0.5 mL of 1% paraformaldehyde for 5 minutes. Finally, the cells were washed with PBS and stored at 4°C in the dark until analyzed with flow cytometry.

Flow Cytometry

Flow cytometry was performed using FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). For each sample, 104 leukocytes were enumerated. Neutrophils were gated using morphologic characteristics displayed on a dot plot of forward light scatter versus side light scatter. Of these neutrophils, 95% were stained with the 7/4 antibody, which is a monoclonal antibody that reacts with a polymorphic neutrophil differentiation antigen present on neutrophils of specific mouse strains. The percentage of total neutrophils or CD marker-positive neutrophils among circulatory or peritoneal exudative leukocytes was calculated. The absolute number of CD marker-positive neutrophils was calculated as the circulatory or peritoneal exudative leukocyte count multiplied by the appropriate percentage.

Microbiological Analysis

Liver, pancreas, and mesenteric tissues were homogenized by crushing and mixing them with serum. Petri dishes with blood agar medium were incubated at 37°C for 24 hours. Cells were stained with gram stain and observed under a light microscope. Gram-negative bacteria were identified using MacConkey agar supplemented with 10% lactose, and gram-positive bacteria were identified using Schaedler agar.

Statistical Analyses

Results are expressed as mean ± standard deviation (SD). Statistical analyses were performed using Student's *t*-test for variables measurement and Fisher's exact test with SPSS13 software. Statistical significance was set at $P < 0.05$.

Results

Amylase Concentrations and Pancreatic Histology

The plasma amylase concentration was higher in the SAP group than in the NC group ($P < 0.05$, Table 1). The amylase concentration did not differ between the SAP-G and the SAP groups, at each corresponding time point.

The histological examination of the NC group showed no inflammation or necrosis of the pancreatic tissues (Table 2). However, the pancreases of the SAP and SAP-G groups showed edema of the stroma, inflammatory cell invasion, and acinar cell necrosis, features that are compatible with those of SAP. Inflammation was more prominent in the SAP-G group than in the SAP group, while neither necrosis nor vacuolization was different between the two groups.

Opsonin Receptor Expression on Circulatory Neutrophils

The numbers of CD11b-and CD32/16-positive neutrophils among circulatory neutrophils were significantly higher in the SAP group than in the NC group at 12 and 24 hours after the final injection of ceruletide ($P < 0.01$; Table 3). These values in the SAP-G group were significantly higher than in the SAP group at each time point

Table 1. Plasma amylase (mean ± SD, IU/L)

	Time after final ceruletide injection		
	0 hours	12 hours	24 hours
NC	5492 ± 145	—	—
SAP	—	64383 ± 5482[a]	9745 ± 1221[a]
SAP-G	—	57116 ± 6329[a,b]	8876 ± 936[a,c]

[a]$P < 0.05$ between SAP or SAP-G and NC; [b]$P = 0.06$ between SAP and SAP-G; [c]$P = 0.19$ between SAP and SAP-G.

Table 2. Histological scores of pancreases

	Time points				
	0 h	12 h		24 h	
	NC	SAP	SAP-G	SAP	SAP-G
Necrosis	0	2	1-2	1-2	1-2
Vacuolization	0	1-2	1-2	1	1
Inflammation	±	2-3	4	2	3

The histological grading of necrosis and vacuolization refers to the approximate percentage of cells involved: 0, absent; ±, ≤ 5%; 1, 5-15%; 2, 15-35%; 3, 35-50%; and 4, ≥50%. The grade of the inflammatory alterations refers to a scale ranging from ± (the minimum) to 4 (the maximum).

Table 3. CD11b and CD32/16 expression on circulatory neutrophils (cells/mm^3)[a]

	CD11b+ cells			CD32/16+ cells		
	0 h	12 h	24 h	0 h	12 h	24 h
NC	325 ± 44	—	—	509 ± 107	—	—
SAP		1025 ± 115	683 ± 134	—	1332 ± 70	706 ± 327
SAP-G	—	4614 ± 757[b]	1682 ± 206[b]	—	4780 ± 765[b]	1748 ± 509[b]

[a]Results are shown as mean ± SD; [b]$P < 0.01$ between the SAP and SAP-G groups.

Table 4. CD11b and CD32/16 expression on peritoneal exudate neutrophilsa

	CD11b$^+$ cells			CD32/16$^+$cells		
	0 h	12 h	24 h	0 h	12 h	24 h
NC	0.6 ± 0.1	—	—	1.4 ± 0.2	—	—
SAP	—	9.0 ± 2.0 [b]	1.6 ± 0.2	—	9.0 ± 2.0 [b]	2.4 ± 0.3
SAP-G	—	30.0 ± 6.0 [c]	7.1 ± 0.6 [c]	—	40.0 ± 6.0 [c]	7.4 ± 0.6 [d]

[a]Number of cells ($\times 10^4$/total) is shown as mean ± SD; [b]$P < 0.001$, between the SAP and NC groups; [c]$P < 0.05$ and [d]$P < 0.001$, between the SAP and SAP-G groups.

($P < 0.01$). The numbers of CD11b-and CD32/16-positive neutrophils were about 4-fold higher in the SAP-G group than in the SAP group ($P < 0.01$); the mean fluorescence intensity (MFI) of CD11b at 12 and 24 hours ($P < 0.01$) and the MFI of CD32/16 only at 24 hours were significantly greater in the SAP-G group compared with the SAP group ($P < 0.05$, Fig. 1).

Opsonin Receptor Expression on Peritoneal Exudative Neutrophils

The numbers of CD11b-positive peritoneal exudative neutrophils increased more than 15-fold ($P < 0.001$), and CD32/16-positive peritoneal exudative neutrophils more than 6-fold ($P < 0.001$), at 12 hours in the SAP group compared with the NC group, and these increased 3- to 4-fold ($P < 0.05$) in the SAP-G group compared with the SAP group (Table 4). The MFIs of CD11b and CD32/16 were higher in the SAP-G group than in the SAP group ($P < 0.05$) 24 hours after the final injection of ceruletide (Fig. 2).

Microbiological Findings

The various tissue cultures showed three main bacterial species: *Enterococcus faecalis*, *Proteus mirabilis*, and *Escherichia coli*. For each sampled organ, there were no significant differences in the number that tested positive for bacterial translocation between the NC and SAP groups, or between the SAP and SAP-G groups (Table 5).We com-

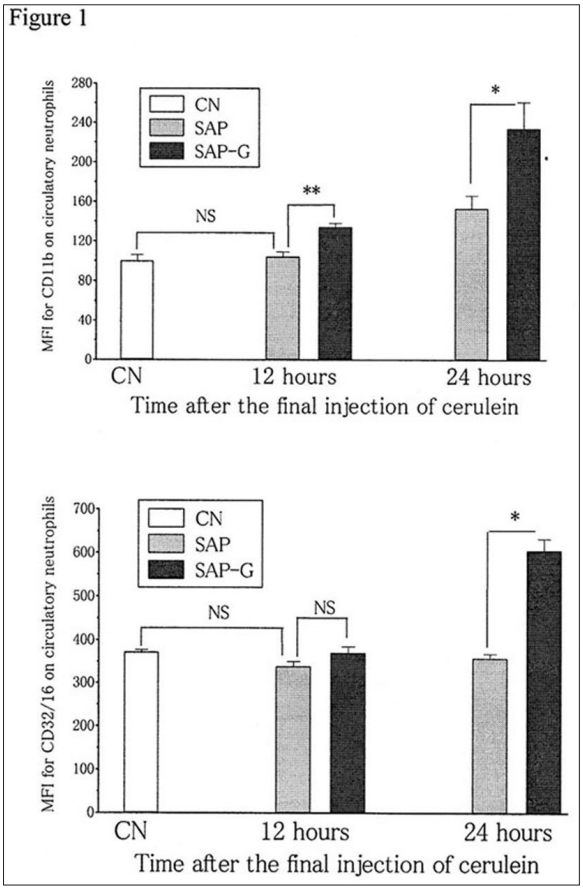

Fig. 1. MFIs of opsonin receptor expression on circulatory neutrophils. MFI is shown as mean ± SD. *$P < 0.05$; **$P < 0.01$. Above, MFI for CD11b; Below, MFI for CD32/16. NS denotes no statistically significant difference

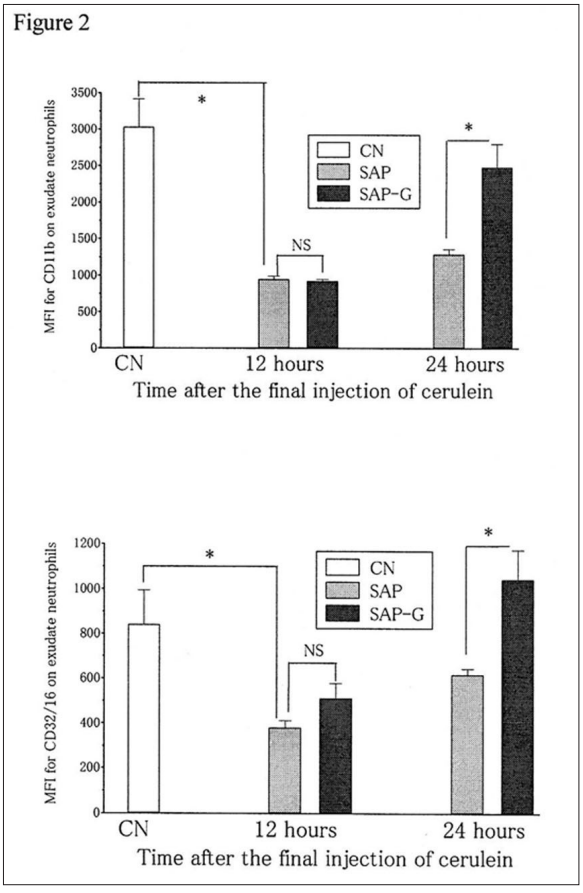

Fig. 2. MFIs of opsonin receptor expression on peritoneal exudative neutrophils. MFI is shown as mean ± SD. *$P < 0.05$ and **$P < 0.01$. Above, MFI for CD11b; Below, MFI for CD32/16

Table 5. Bacterial translocation into various tissues[a]

		Time points		
		0 h	12 h	24 h
Pancreas	NC	1/6	—	—
	SAP	—	2/6	1/6
	SAP-G	—	3/6	0/6
Mesenteric tissue	NC	0/6	—	—
	SAP	—	2/6	1/6
	SAP-G	—	1/6	0/6
Liver	NC	0/6	—	—
	SAP	—	1/6	1/6
	SAP-G	—	1/6	1/6
Total	NC	1/18 (6%)	—	—
	SAP	—	6/18 (33%)[b]	3/18 (17%)
	SAP-G	—	5/18 (28%)	1/18 (6%)

[a]Number of positive cases/number of examined cases. [b] $P < 0.05$ between NC and SAP group.

pared the rates of bacterial translocation in three types of organs. The positive rates were 6% in the NC group; 33% at 12 hours and 13% at 24 hours in the SAP group; and 28% at 12 hours and 6% at 24 hours in the SAP-G group. The positive rate of bacterial translocation at 12 hours in the SAP group was significantly higher than in the NC group ($P < 0.05$). The positive rates of bacterial translocation were lower in the SAP-G group than in the SAP group at 12 and 24 hours, although the differences were not statistically significant.

Discussion

To evaluate the effect of G-CSF on neutrophil function, we administered G-CSF to SAP mice, and investigated the expression levels of CD11b and CD16/32. Our results indicated that the number of neutrophils significantly increased, and these increases were accompanied by increased expression of CD11b and CD32/16 in neutrophils and their respective MFIs, after the administration of G-CSF. These results are in accord with the observation that G-CSF treatment increased the number of neutrophils and CD11b and CD32 expression on neutrophils in healthy volunteers and SAP dogs and SAP mice [4,10,12].

Our results also showed that G-CSF reduced bacterial translocation, although the reduction was not statistically significant compared to the control group. In fact, it was found previously that G-CSF significantly increased and maintained granulocytes, and decreased the rate of distant organ infection in SAP dogs [12]. Further studies are necessary with larger populations of experimental animals, a longer observation time, and multiple administrations of G-CSF, because the effects of G-CSF on leukocytes and neutrophils were more effective at two days post-injection, when administered to a swine model of intra-abdominal sepsis twice a day [4].

Interestingly, G-CSF increased the numbers of neutrophils and macrophages infiltrating areas of acute myocardial infarction and promoted the absorption of necrotic tissues [9]. Therefore, G-CSF treatment accelerates the healing of myocardial infarction wounds and the regeneration of myocardial tissues. Accordingly, our finding that G-CSF treatment increased the number of neutrophils infiltrating pancreatic tissues supports the need for further studies regarding whether these infiltrating neutrophils will promote the absorption of necrotic pancreatic tissues and induce the regeneration of pancreatic acinar cells.

In conclusion, G-CSF treatment in SAP mice increased the number of neutrophils, enhanced the expression of opsonin receptors of circulatory and peritoneal exudative neutrophils, and inhibited bacterial translocation to cavity organs. Our findings suggest that G-CSF may prevent bacterial infection in SAP through the increase of the number of neutrophils and expression of opsonin receptors. This study should promote the development of a treatment, or even a cure, for SAP using G-CSF.

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