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Short communication

γ-Globulin-induced modulation with necrotic-like morphology of peripheral blood neutrophils

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Abstract

To determine the effect of intravenous immunoglobulin-administration on neutrophil function, we obtained neutrophils from patients with an acute phase of Kawasaki disease. In vitro IgG-induced modulation of neutrophils into Annexin-V-positive and propidium iodide-negative cells was observed in 20 of 28 patients in the presence of more than 300 μg/ml IgG and showed necrosis-like changes in morphologic features. However, we could not find any patients showing promotion of the sub-G1 cell fraction on DNA content analysis. The modulatory effect of in vitro IgG was not observed in neutrophils from healthy volunteers and was significantly correlated with the antifebrile effect of in vivo IgG.

Keywords: Neutrophils; Apoptosis; Necrotic morphological change; IgG; Kawasaki disease

1. Introduction

To determine the effect of intravenous immunoglobulin-administration on neutrophil function, we studied patients with Kawasaki disease. Kawasaki disease is a form of acute inflammatory systemic vasculitis occurring in infants and children under 5 years old (Kawasaki, 1974). Peripheral neutrophils increase during acute phase Kawasaki disease, and this phenomenon is thought to be associated with an abnormal immune process of hypercytokinemia as has been reported previously (Furukawa et al., 1992; Igarashi et al., 1999). Intravenous immunoglobulin-administration is now also commonly used as first line-therapy for Kawasaki disease, and is generally effective (Furusho et al., 1984). The effect of intravenous immunoglobulin-administration on neutrophils has been suspected to be one of the main factors affecting clinical improvements in Kawasaki disease (Tsujimoto et al., 2001). In fact, the apoptosis of neutrophils from Kawasaki disease was more delayed than that from volunteers as shown above. The roles of in vivo and in vitro IgG on neutrophil functions were presented in previous reports (Teeling et al., 2001, 1998).

We studied and found here the in vitro IgG induced modulation of circulating neutrophils from patients with an acute phase of Kawasaki disease and considered the modulatory effect of IgG on neutrophil function as one of the mechanisms of clinical improvement with treatment using intravenous immunoglobulin-administration.

2. Materials and methods

2.1. Neutrophil preparation and IgG

We investigated 28 patients with Kawasaki disease (14 males, 14 females ranging from 3 to 60 months old of age; mean 18 months of age), who matched the diagnostic criteria for Kawasaki disease established by the Japanese Kawasaki Disease Committee (Japanese Kawasaki Disease Research Committee). Heparinized venous blood samples were obtained from healthy volunteers and patients with Kawasaki disease with their parent's consent. Neutrophils were isolated using a two-step centrifugation technique

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through Ficoll-Hypaque (Pharmacia, Upsala, Sweden) and Mono-Poly (Flow Laboratories, Australia). Briefly, whole blood was first fractionated by density gradient centrifugation with Ficoll-Hypaque. The polymorphonuclear leukocyte-rich fraction was collected from the buffycoat, resuspended in RPMI1640 medium (SIGMA, St. Louis, MO, USA), and layered onto Mono-Poly. After centrifugation at $400 \times g$ for 30 min, polymorphonuclear leukocyte was collected from the monolayer, washed and resuspended in culture medium (RPMI1640 medium supplemented with 10% fetal calf serum (SIGMA). In all cases, the neutrophil purity including eosinophil was greater than 97% in May-Giemsa stained preparations analyzed under a microscope. Eosinophil granulocytes were easily identified as cells containing numerous small orange–red-stained intracellular granules. For this in vitro study, human IgG (Venoglobulin; Welfaid, Co. Osaka, Japan) was prepared in serum free RPMI 1640 medium.

2.2. Neutrophil culture

The neutrophils were finally adjusted to 1×10^6 cells/ml in culture medium in polystyrene tissue culture dishes (Falcon 353001, NJ, USA) and cultured in a 5% CO_2 incubator at 37 °C.

2.3. Detection of apoptotic cells

2.3.1. Morphologic analysis

Cytocentrifuged preparations of neutrophils were prepared using a Shandon cytospin centrifuge (Shandon, Runcorn, UK), stained with May-Giemsa, and assessed for morphological changes characteristic of apoptosis (typical apoptosis findings consist of nuclear condensation, vacuolation, fragmented nuclei, blebbing of plasma membranes, and decrease in cell size) by light microscopy. A minimum of 200 cells/slide was examined and graded as apoptotic/nonapoptotic as described previously (Savill et al., 1989).

2.3.2. DNA content analysis

DNA content was analyzed by flow cytometry using propidium iodide (Cycle TEST PLUS DNA Reagent Kit, Becton Dickinson). Briefly, cells were washed twice with PBS and treated with propidium iodide at 37 °C for 30 min and the fluorescence of the individual nuclei was analyzed using FACScan (Becton Dickinson, San Jose, CA, USA). The apoptotic cell nuclei of the sub-G1 fraction were countered and the data were expressed as a percentage of the sub-G1 fraction.

2.3.3. Quantitative analysis by annexin-V binding

Annexin-V-binding to neutrophils was performed using an apoptosis detection kit (Annexin-V-FITC Kit, Bender Med System, Austria). Briefly, neutrophils were incubated with FITC-annexin-V and propidium iodide for 20 min at 4 °C and analyzed by two-color flow cytometry (FACScan) as previously described (Ormerod, 1998). Apoptotic cells are detected as proportions of annexin-V-positive and PI-negative cells.

The modulation effect of IgG on neutrophils was calculated as described below;

Modulation effect of IgG on neutrophils (%)=A–B;
A=%propidium iodide-negative and Annexin-V positive cell in the presence of IgG;
B=%propidium iodide-negative and Annexin-V positive cell in the absence of IgG.

The IgG-induced promotion of in vitro IgG was tentatively defined as positive when (A–B) was more than 10%.

2.4. Statistical analysis

All data are expressed as mean ± S.D. and the differences were analyzed using the Mann–Whitney test, which was used to compare non-parametric data from two sample populations. A p-value < 0.05 was considered significant.

3. Results

3.1. Spontaneous apoptosis of neutrophils

Initially, neutrophils isolated from peripheral blood were cultured without any factors for 6 h. Using DNA content analysis, the spontaneous apoptosis levels in neutrophils from patients with Kawasaki disease and healthy volunteers as a control were 25.5 ± 8.7% (n=28), and 38.2 ± 12.0% (n=6), respectively, and were significantly different (Kawasaki disease vs. control, $p=0.009<0.05$). However, we could not find any morphological differences between cultured neutrophils from controls and Kawasaki disease patients (data not shown).

3.2. IgG-induced modulation of neutrophil function

When neutrophils from Kawasaki disease patients were cultured with IgG for 6 h, in vitro modulation by IgG was clearly observed using quantitative analysis in 20 of 28 Kawasaki disease patients and a typical profile of neutrophils showing modulation into Annexin-V-positive and propidium iodide-negative cells is shown in Fig. 1A. We found that more than 300 μg/ml of an IgG induced a promotive effect based on a study in three patients showing in vitro modulation effect of IgG (Fig. 1B). In Kawasaki disease patients with IgG-induced modulation, we also found differing morphological characteristics; morphologic changes in the presence of IgG were very different from the typical findings. IgG-treated neutrophils showed not only changes in cell size but also the development of numerous

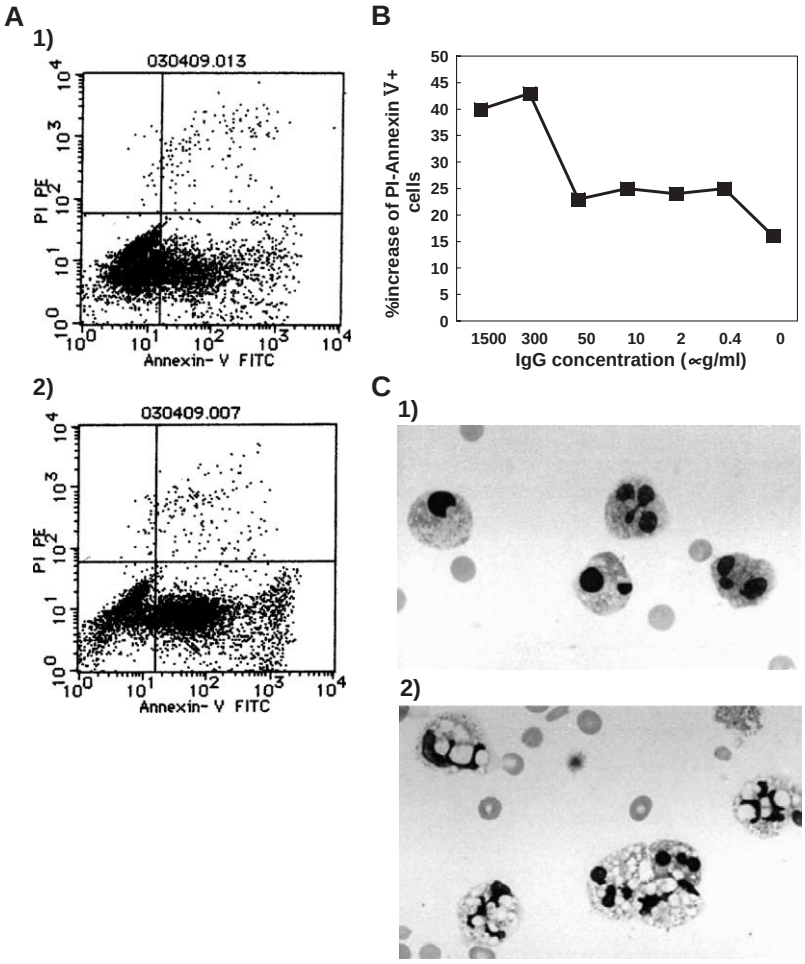

Fig. 1. Effect of in vitro IgG on neutrophils from patient with Kawasaki disease. (A) Quantitative analysis by annexin-V binding. The percentage of cells in the left lower (live), right lower (early apoptosis), and the right upper (primary necrotic and late apoptotic/secondary necrotic) quadrants is indicated. IgG-treated neutrophils were modulated to show early apoptosis (propidium iodide-negative and Annexin-V positive neutrophils; (1) with medium alone 28%, (2) with IgG 49%). (B) Effect of in vitro IgG-concentration on neutrophil modulation. Neutrophils were cultured with various concentration of IgG for 6 h, and then analyzed using flow cytometry. The values are the mean value of triplicate determinations in one experiment on three patients showing the promotive effect of in vitro IgG. More than 300 μg/ml IgG clearly modulated neutrophil function. (C) Morphological changes in neutrophils treated by in vitro IgG. Neutrophils were treated with IgG (2 mg/ml) for 6 h. (1) Neutrophils from a healthy volunteer. Apoptotic neutrophils showed typical changes. (2) Neutrophils from patient with Kawasaki disease. IgG-treated neutrophils showed changes in cell size and the development of numerous vacuoles throughout the neutrophil cytoplasm. (Original magnification, ×1000).

vacuoles throughout the neutrophil cytoplasm (Fig. 1C). However, we could not find any patients showing promotion of the sub-G1 cell fraction on DNA content analysis (data not shown). These modulation effects of in vitro IgG as shown above were not observed on neutrophils from healthy volunteers.

3.3. Modulation effect of in vitro IgG on neutrophils and the antifebrile effect of intravenous immunoglobulin-administration

We studied correlation between the antifebrile effect of intravenous immunoglobulin-administration and the modulation effect of in vitro IgG on neutrophil using quantitative analysis by annexin-V binding. Antifebrile effect was tentatively defined as disappearance of fever within 48 h after intravenous immunoglobulin-administration. We confirmed that antifebrile effect positively correlates with the promotive effect of in vitro IgG on neutrophil modulation ($p = 0.028 < 0.05$) (Table 1).

Table 1
In vitro IgG-induced modulation of neutrophil function and intravenous immunoglobulin-induced antifebrile effect

	Antifebrile effect of intravenous immunoglobulin-administration	
	Response	No response
In vitro IgG-induced modulation		
Response	14	2
No response	6	5

The antifebrile effect of intravenous immunoglobulin-administration and the promotion of a modulatory effect of in vitro IgG converting neutrophils into propidium iodide-negative and Annexin-V-positive cell were analyzed in 27 patients treated with immunoglobulin (1 g/kg for one day). Responsiveness was defined in Materials and methods and Results.

4. Discussion

We examined the effect of in vitro IgG on peripheral blood neutrophils. We chose Kawasaki disease as a model case and obtained the following findings. In vitro IgG-induced modulation of neutrophils from Kawasaki disease patients into propidium iodide-negative and Annexin-V-positive cells, but modulation into the sub-G1 cell fraction was not clear. The patients' netrophils were suspected of possessing responsiveness to IgG due to being sensitized by certain cytokines. In fact, hypercytokinemia in Kawasaki disease was reported previously. Various cytokines and chemical agents are generally known to influence neutrophil; TNF-α induces functional modulation, and GM-CSF delays apoptosis (Brach et al., 1992).

Apoptosis is usually characterized by specific phenomena such as chromatin condensation and membrane blebbing. However, in IgG-treated Kawasaki disease patient neutrophils, we found morphologic changes consisting of large cell size and the development of numerous vacuoles throughout the neutrophil cytoplasm in the presence of IgG, which differed from the typical findings of apoptosis. Although those cells were positive for Annexin-V and negative for propidium iodide, these cells were not contained in the sub-G1 fraction. Recently, Takei et al. (1996) reported that phorbol myristate acetate (PMA) rapidly induced neutrophil death showing features distinct from those typical of either apoptosis or necrosis. Furthermore, Kitanaka and Kuchino (1999) also described new models of programmed cell death showing necrotic-like morphology. We considered that IgG-induced morphologic changes with vacuoles and other findings were very similar to those of program cell death showing necrotic-like morphology although further examination on the mechanisms was necessary.

We now speculate that intravenous immunoglobulin-administration, which is currently the best protocol for Kawasaki disease therapy, induces neutrophil modulation and relieves hyperactivated conditions in leukocytosis by neutrophil modulation including specific morphological changes. The in vitro IgG-modulatory effect correlated with the concentration of IgG. Supporting this result, intravenous immunoglobulin-administration using higher levels of IgG shows a better effect on treatment of Kawasaki disease. Administration of 2 g/kg IgG/day is currently used, although 1 g/kg for 1 day or 400 mg/kg for 5 days were previously common. The in vitro IgG-modulatory effect also correlated with the antifebrile effect. In conclusion, we strongly suspected that intravenous immunoglobulin-administration directly induced the modulation of pathological neutrophils based on the above results. Furthermore, we suspect that these findings could be also observed in pathological neutrophils from disorders other than Kawasaki disease.

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