Brief report

Apoptotic neutrophils in the circulation of patients with glycogen storage disease type 1b (GSD1b)

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Glycogen storage disease type 1b (GSD1b) is a rare autosomal recessive disorder characterized by hypoglycemia, hepatomegaly, and growth retardation, and associated—for unknown reasons—with neutropenia and neutrophil dysfunction. In 5 GSD1b patients in whom nicotinamide adenine dinucleotide phosphate–oxidase activity and chemotaxis were defective, we found that the majority of circulating granulocytes bound Annexin-V. The neutrophils showed signs of apoptosis with increased caspase activity, condensed nuclei, and perinuclear clustering of mitochondria to which the proapoptotic Bcl-2 member Bax had translocated already. Granulocyte colony-stimulating factor (G-CSF) addition to in vitro cultures did not rescue the GSD1b neutrophils from apoptosis as occurs with G-CSF–treated control neutrophils. Moreover, the 2 GSD1b patients on G-CSF treatment did not show significantly lower levels of apoptotic neutrophils in the bloodstream. Current understanding of neutrophil apoptosis and the accompanying functional demise suggests that GSD1b granulocytes are dysfunctional because they are apoptotic. (Blood. 2003; 101:5021-5024)

Introduction

Glycogen storage disease type 1 (GSD1; OMIM 23.2200) is caused by inherited defects of the glucose-6-phosphatase (G6Pase) complex. This complex has a key role in both glycogenolysis and gluconeogenesis, converting glucose-6-phosphate (G6P) to glucose. Clinical features are hepatomegaly, growth retardation, osteopenia, and kidney enlargement with hypoglycemia, hyperlactacidemia, hyperlipidemia, and hyperuricemia. GSD1 is caused by deficiencies in the activity of the G6Pase system, which consists of at least 2 membrane proteins, glucose-6-phosphate transporter (G6PT) and G6Pase. G6PT translocates G6P from the cytoplasm to the lumen of the endoplasmic reticulum; G6Pase catalyzes the hydrolysis of G6P to produce glucose and phosphate. Therefore, G6PT and G6Pase work in concert to maintain glucose homeostasis. Deficiencies in G6Pase and G6PT cause GSD1a and GSD1b, respectively.[1-6]

Neutropenia and/or neutrophil dysfunction is a characteristic hallmark of GSD1b,[7,8] only rarely present in GSD1a types.[9] Patients with GSD1b are thus susceptible to recurrent bacterial infections, aphtous stomatitis, or inflammatory bowel disease. The mechanism of the neutropenia as well as the concomitant neutrophil dysfunction, which includes impaired chemotaxis, phagocytosis, and respiratory burst,[7,8,10-12] remain unknown, although treatment with granulocyte colony-stimulating factor (G-CSF) has considerably reduced the incidence of infections.[7,8]

Neutrophils are produced in large numbers every day in the bone marrow (BM), being predisposed to cell death by apoptosis, a process that prevents the cytotoxic contents from the neutrophil granules to be released into the surrounding tissues and facilitates the harmless elimination of cells by tissue macrophages.[13] Aging of normal neutrophils is accompanied by a progressive loss of functions, such as adherence, chemotaxis, and respiratory burst.[14,15] We investigated whether granulocyte function was impaired because of apoptosis in circulating neutrophils in GSD1b.

Study design

Neutrophil purification, functional testing, and culturing

Heparinized venous blood was collected from healthy donors and from GSD1b patients after obtaining informed consent. Granulocytes were isolated as described.[16] Purity was always more than 95%. In some experiments whole leukocyte preparations were used from which the erythrocytes were lysed by ice-cold isotonic NH_4Cl solution.[16] Culturing of neutrophils (16-18 hours) was performed exactly as described.[17]

Neutrophil migration was assessed by means of the Fluoroblok inserts (Falcon; Becton Dickinson, San Jose, CA). Cells (5×10^6/mL) were labeled with calcein-AM (1 μM final concentration; Molecular Probes, Leiden, the Netherlands) for 30 minutes at 37°C, washed twice, and resuspended in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer at a concentration of 2×10^6/mL. Chemoattractant solution (formyl-Met-Leu-Phe [fMLP], interleukin-8 [IL-8], and C5a; all at 10 nM) or medium alone (0.8 mL/well) was placed in a 24-well plate, and 0.3 mL cell suspension was delivered to the inserts (3 μm pore size) and placed in the 24-well plate. Cell migration was assessed by measuring fluorescence in the lower compartment at 2.5-minute intervals for 45 minutes with the

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HTS7000+ plate reader (Perkin Elmer, Norwalk, CT). Maximal slope of migration was estimated over a 10-minute interval.

Nicotinamide adenine dinucleotide phosphate (NADPH)–oxidase activity was assessed as hydrogen peroxide production determined by an Amplex Red kit (Molecular Probes). Neutrophils (1×10^6 /mL) were stimulated with 1 μM fMLP, 1 μM platelet-activating factor (PAF), or 100 ng/mL phorbol myristate acetate (PMA), in the presence of Amplex Red (0.5 μM) and horseradish peroxidase (1 U/mL). Fluorescence was measured at 30-second intervals for 20 minutes with the HTS7000+ plate reader. Maximal slope of H_2O_2 release was assessed over a 2-minute interval.

Annexin-V, mitochondrial, and Bax staining

Annexin-V, mitochondrial, and Bax staining was performed essentially as described.[17]

Morphology

Morphology was determined after Giemsa staining of cytospin preparations. Apoptotic morphology was defined as the presence of condensed nuclei and simultaneous loss of the polysegmented nuclear appearance.

Overall caspase activity

Overall caspase activity was fluorimetrically assessed[18] in neutrophil lysates as the release of 7-amino-4-methyl-coumarin (AMC) from 50 μM acetyl-Asp-Glu-Val-Asp (DEVD) AMC (Alexis Biochemicals, San Diego, CA) over 5-minute intervals for 120 minutes by means of the HTS7000+ plate reader. Maximal slope of AMC release was estimated over a 25-minute interval.

Results and discussion

We tested neutrophil numbers and functions in 5 patients with GSD1b (Table 1).[19] In all patients, a mild-to-severe neutropenia was present. Activation of neutrophils via PMA in glucose-containing and glucose-free medium[12] confirmed the deficient respiratory burst in GSD1b upon activation of the NADPH oxidase. Directed cell motility (chemotaxis) toward neutrophil-specific stimuli (ie, C5a, IL-8, or PAF) was also diminished (Table 1). We recently studied these functional activities in healthy neutrophils during apoptosis and the protecting role of G-CSF and granulocyte-macrophage colony-stimulating factor in this process. Apart from the differential protection from functional decay by these hematopoietic factors, it became clear that the NADPH-oxidase activity is best preserved in aging neutrophils, followed by phagocytosis, and—lastly—by chemotaxis (B. Wolach et al, manuscript submitted). These findings are reminiscent of the neutrophil dysfunction in GSD1b, suggesting the possibility of a death-prone cell type in this disease.

To address this issue, we studied several apoptotic features in GSD1b neutrophils. Fresh GSD1b neutrophils displayed strong Annexin-V binding (Figure 1A); the Annexin-V^+ cells were still largely impermeable for propidium iodide (PI). In contrast, the patient's monocytes and lymphocytes in whole leukocyte preparations did not bind Annexin-V (not shown). Rescue of overnight neutrophil apoptosis by G-CSF was possible only to a limited extent (Figure 1B). Annexin-V binding indicates that GSD1b neutrophils from the circulation exposed phosphotidyl serine (PS) as an early sign of apoptosis. Specific proteolytic caspase activity inhibitable by the general caspase inhibitor zVAD-fmk was detected in the circulating GSD1b neutrophils but not in fresh control neutrophils (Figure 1C). Furthermore, in these freshly obtained cell preparations the predisposition of neutrophils to apoptosis was also demonstrated by typical apoptotic clustering of mitochondria (not shown) and redistribution of Bax protein (Figure 1D), similar to our previous findings during the process of spontaneous apoptosis in neutrophils from healthy individuals.[17]

In GSD1b neutrophils, the import of G6P into the endoplasmic reticulum is decreased, thus causing a local decrease in G6P dehydrogenase activity. This enzyme, which serves to produce NADPH, determines the cellular redox status by permitting regeneration of reduced glutathione, resulting in decreased sensitivity to direct or indirect apoptosis.[1,20,21] In line herewith is the recent observation that the specific G6PT-inhibitor S3484 increases apoptosis of neutrophils, which can be rescued by preincubation of cells with the reactive oxygen species (ROS) scavenger Trolox C or with the flavoprotein inhibitor diphenyleneiodonium (DPI).[22] Whether such local changes in intracellular redox state affect the localization or activity of Bcl-2 members or caspases is as yet unclear. If so, this may explain why in GSD1a the neutrophils do not show any apoptotic feature (not shown): in GSD1a neutrophils the import of G6P into the endoplasmic reticulum is intact.

Microscopic examination of cytospins prepared from the GSD1b neutrophils revealed apoptotic changes in nuclear morphology in about 1:25 neutrophils. The early-apoptotic Annexin-V^+ neutrophils outnumbered the neutrophils with clustered mitochondria, Bax translocation, and apoptotic morphology, which were considered to be late-apoptotic. Although at low frequency, a few monocytes were found in the leukocyte preparations that had already engulfed apoptotic material (Figure 1E).

Table 1. Patient characteristics

Patient no. (sex)*	Age, y	G6PT mutation	Infection	G-CSF†	ANC, ‡ per uL	Chemotaxis§ C5a	Chemotaxis§ IL-8	Chemotaxis§ PAF	NADPH-oxidase activity‖ PMA − glucose	NADPH-oxidase activity‖ PMA + glucose
1(F)	17	228G > A 1211-1212 del CT	Stomatitis; IBD	Yes	540	60	36	49	1.4 (1.23)	1.1 (2.01)
2(F)	9	Homozygous 1211-1212 del CT	Stomatitis	Yes	240	47	NT	21	1.6 (1.11)	1.5 (2.24)
3(M)	8	627C > T 1211-1212 del CT	None	No	190	41	NT	68	1.2 (1.08)	1.2 (2.12)
4(F)	2.5	624G > A 1184G > T	Stomatitis; ENT infections	No	750	28	NT	45	1.4 (1.38)	1.5 (1.98)
5(M)	1.2	550T > G 1212T > C	Stomatitis; ENT infections; Skin abscesses	No	760	30	49	44	0.9 (1.17)	1.1 (2.01)

*M indicates male; F, female.

†G-CSF 3 μg/kg every other day.

‡ANC indicates absolute neutrophil count (normal, > 1500 per μL).

§Chemotaxis is expressed as percentage of mean maximal slope of 2 age-matched controls measured on the same day; NT indicates not tested.

‖NADPH-oxidase activity is expressed as maximal slope of H_2O_2 release in nmol H_2O_2/min per 10^6 cells. The mean of 2 age-matched controls measured on the same day is given in parentheses.

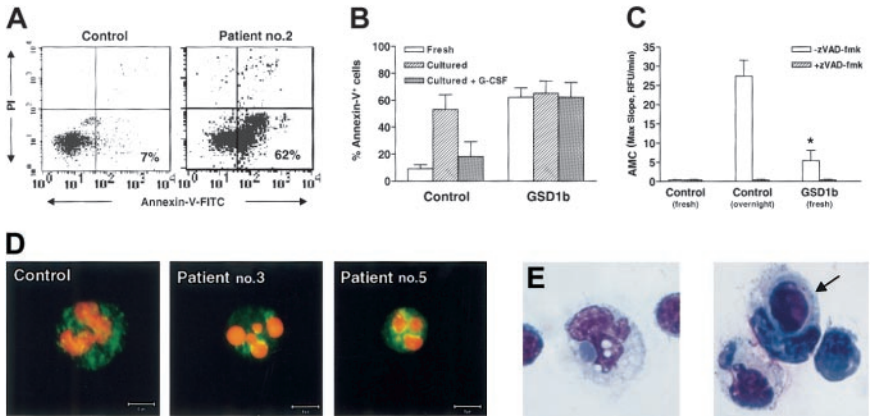

Figure 1. Granulocytes from GSD1b patients display apoptotic features. (A) Freshly purified neutrophils from a patient with GSD1b (n = 5) and an age-matched control (n = 10) were stained by Annexin-V–fluorescein isothiocyanate (FITC) and PI, and analyzed by FACScan. Representative dot plots are shown. Values indicate the proportion of Annexin-V$^+$ cells. (B) The summarized data of all measurements performed in fresh cells and cells cultured overnight in the absence or presence of exogenous G-CSF (100 ng/mL; n = 5). Bars represent mean ± SD of Annexin-V$^+$ cells in percentage of total number of neutrophils. (C) Overall caspase proteolytic activity in cell lysates prepared from 0.5 × 10^6 fresh healthy control neutrophils and neutrophils cultured overnight (~ 70% apoptotic cells as measured by Annexin-V–FITC staining and morphologic examination; n = 5) as well as from freshly isolated neutrophils of 3 GSD1b patients was measured by cleavage of the fluorogenic caspase substrate DEVD-AMC. To check the specificity of the caspase proteolytic activity, the general caspase inhibitor zVAD-fmk (10 μM final concentration; Alexis Biochemicals) was added to the assay mixture. The maximum speed (maximal slope) of AMC release was used as a measure of caspase activity. Results are presented as mean ± SD maximal slope. *$P < .01$ (by Student *t* test) versus control fresh neutrophils. RFU indicates relative fluorescence units. (D) Fresh neutrophils from a healthy control and from patients with GSD1b were stained with antibody specific for Bax (green), counterstained with PI (1 μg/mL; red) to visualize nuclear morphology, and analyzed by confocal microscopy. Note that in the control image a punctate-dispersed distribution of Bax and polysegmented nucleus with normal chromatin are present. In contrast, both patients' images (nos. 3 and 5) reveal Bax clustering and typical apoptotic nuclei with condensed chromatin (for further comparison with normal neutrophils, see also Maianski et al[17]). Representative images from 5 controls and 5 GSD1b patients are shown. Bar is 5 μm. (E) Whole leukocyte preparation from patients with GSD1b (1.5-2 × 10^5 cells) was obtained after erythrocyte lysis. Cytospins stained with Giemsa solution were analyzed by light microscopy. Engulfment of apoptotic material (left panel) and intact cellular remnant (arrow, right panel) in monocytes are shown. Original magnification, × 400.

Neutropenia can result from diminished BM production and/or shortened half-life in the blood stream and rapid clearance from the circulation. Both myeloid hyper- and hypocellularity of the BM have been reported in GSD1b.[8,23] Enhanced elimination of not yet fully differentiated neutrophils in GSD1b by macrophages via the PS receptor, in concert with CD14, deposited mannose binding lectin and/or complement fragments,[13,24] probably occurs in the BM prior to neutrophil egress. Rapid elimination of neutrophils in the BM could be an explanation for the discrepancy between BM cellularity and the neutropenia, irrespective of G-CSF administration. Noneliminated senescent neutrophils could subsequently appear in the circulation (Figure 1). Whether clearance by the hepatosplenic macrophage system contributes to GSD1b-associated neutropenia is unknown. We have not observed abnormal red cells in the blood smears (such as target cells or Howel-Jolly bodies) indicative of a dysfunctional macrophage function. Moreover, splenomegaly may become apparent only when the spleen is suddenly overloaded by increased clearance of apoptotic cells during infections and/or exaggerated BM production. G-CSF administration can also affect splenic size by extramedullary hematopoiesis, sometimes complicated by hypersplenism requiring dose reduction or splenectomy.[25]

In sum, neutrophils in GSD1b show a striking tendency of cell death in the circulation (with PS exposure), detectable caspase activity, perinuclear clustering of mitochondria, and translocation of Bax. Moreover, we observed phagocytic removal by monocytes at low frequency in the blood samples, which indicates that the apoptotic bodies in GSD1b can be recognized and actively engulfed. G-CSF treatment in GSD1b does not prevent the induction of apoptosis in circulating neutrophils. We have studied neutrophils from children with infections (active pneumonia or septicemia), or with other neutropenic syndromes (autoimmune neutropenia, cyclic neutropenia, and Shwachman-Diamond syndrome), but to date never observed circulating apoptotic neutrophils in these patients (not shown).

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