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)

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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 glycolysis 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, aphthous 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 NH4Cl 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-hydroxyethylpiperazine-2-ethanesulfonic acid) buffer at a concentration of 2 × 10^6/mL. Chemotactic 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 help of the Fluoroblok inserts, which are used to create a constant gradient of chemotactic stimulus across the insert in a well. The migration was recorded electronically with a computerized Fluorometer (Molzan, Zoetermeer, the Netherlands). The relative migration value obtained was calculated for each treatment as the mean of at least 3 experiments.

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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 H2O2 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 assessed18 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 medium12 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 the differential protection from functional decay by these hematopoietic factors, and—with the HTS7000+ plate reader. Maximal slope of H2O2 release was assessed over a 2-minute interval.

Table 1. Patient characteristics

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

* 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 H2O2 release in nmol H2O2/min per 10^6 cells. The mean of 2 age-matched controls measured on the same day is given in parentheses.
Neutropenia can result from diminished BM production and/or shortened half-life in the blood stream and rapid clearance from the bloodstream. Both myeloid hyper- and hypocellularity of the BM have been reported in GSD1b. Enhanced elimination of not yet differentiated neutrophils in GSD1b by macrophages via the PS receptor, in concert with CD14, deposited mannose binding lectin and/or complement fragments, 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 hepatopoietic 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 Howell-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.

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 smears, 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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References