Neutrophil cell death: mechanisms and regulation
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Chapter VII

Apoptotic neutrophils in the circulation of patients with glycogen storage disease type 1b (GSD1b)
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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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 polynucleated 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 medium2 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.1,2,3,21 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 Trollox 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).

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**Table 1. Patient characteristics**

| Patient no. (sex)* | Age, y | G6PT mutation | Infection | G-CSF† | ANC, × 10^9 per μL | Chemotaxis§ | NADPH-oxidase activity†† | PMA | PMA
|-------------------|--------|----------------|----------|--------|-------------------|------------|-------------------------|------|------|
| 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 | 627G > 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 | 760 | 28 | NT | 45 | 1.4 (1.08) | 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 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.
Neutrophenia 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.\(^8\) Enhanced elimination of not yet apoptotic cells during infections and/or exaggerated BM production and neutrophilia, irrespective of G-CSF administration. Moreover, splenomegaly may become apparent only after sepsis, 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

11. McCawley LJ, Korchak HM, Douglas SD, et al. In vitro and in vivo effects of granulocyte colony-stimulating factor on neutrophils in glycogen storage disease type 1b: granulocyte colony-stimulating factor therapy corrects the neutropenia and the defects...


