Hematopathology / Platelet Serotonin in Children

Diagnosing Platelet δ-Storage Pool Disease in Children by Flow Cytometry

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Abstract

Bleeding problems are symptomatic of platelet δ-storage pool diseases (SPDs) such as Hermansky-Pudlak syndrome. Although at present no cure is available for δ-SPD, early diagnosis is of great importance for prophylactic and supportive treatment. This study tested the usefulness of a flow cytometric assay for platelet serotonin in children. The assay was used to diagnose δ-SPD in a 10-year-old girl. Platelet serotonin levels were significantly lower in the patient than in all healthy control subjects (10 children and 10 adults). The serotonin results were supported by traditional tests, which are transmission electron microscopy of whole mounts and adenosine triphosphate release by lumi-aggregometry. The flow cytometric serotonin assay is a major improvement to current pediatric diagnostics. The advantages of this test are small sample volume of fresh or fixed/frozen platelets, availability of objective results within 2 hours of obtaining the blood sample, and automated analysis by flow cytometry.

The δ-storage pool diseases (SPDs) are a group of disorders characterized by defective formation and/or malfunction of platelet dense granules as well as melanosomes in skin cells. Dense granules, also called δ granules, are one type of major storage pools in platelets, which contain high concentrations of adenosine diphosphate (ADP), adenosine triphosphate (ATP), calcium, phosphate, and serotonin.1 Hematologic symptoms of δ-SPDs range from mild bleeding and easy bruising to moderate bleeding complications.2 Clinical laboratories do not have an easy blood test for the diagnosis of a δ-SPD such as Hermansky-Pudlak syndrome (HPS) despite earlier publications on flow cytometric serotonin assays.1,3 The standard tests to diagnose δ-SPD are lumi-aggregometry and transmission electron microscopy (TEM).4,5 Although lumi-aggregometry is available in large clinical laboratories, confirmation with whole mount electron microscopy is usually required, a test that is not generally available.

Bleeding problems due to platelet dysfunction are usually of autosomal recessive inheritance and, therefore, rare,4 but evidence for autosomal dominant inheritance of isolated δ-SPD has also been presented.6 SPDs can have variable clinical manifestations and are likely underdiagnosed. In HPS, at least 15 genes can be involved, leading to heterogeneity in phenotypes, which is most obvious from the variability of oculocutaneous albinism. Severe cases of δ-SPD are associated with lung disease, inflammatory bowel disease, and kidney disease. The molecular mechanism of the bleeding diathesis in δ-SPD remains unknown, although in specific cases, the hematologic dysfunction has been linked to the disability of patient platelets to form dense granules7 or release the dense granule content.8,9
Pediatric patients with bleeding problems associated with albinism are often not tested for SPDs because diagnosis is still based on repeated lumi-aggregometry or electron microscopic examination of blood samples. Owing to the possible development of pulmonary fibrosis and interstitial inflammation at later stages of disease, it is highly recommended that SPDs in pediatric patients be diagnosed as early as possible.

Lumi-aggregometry uses the ability of ATP to produce bioluminescence in a sample supplemented with luciferin and luciferase. Normal platelets contain ATP in high amounts in dense granules and release it during platelet aggregation. Thrombin, the strongest biologic platelet agonist, induces complete release of 1 to 4 mmol/L of ATP from normal platelets, whereas only partial or no ATP release is seen from platelets obtained from patients with an SPD. Usually, age-matched control samples are not available for pediatric patients, making it difficult to judge whether absent lumi-aggregometry response is due to absent ATP release or a failure of the assay. Thus, at least 1 confirmatory analysis is performed 2 months after the first test. Furthermore, this test requires 1 to 2 mL of fresh platelet-rich plasma (PRP), which is not always easy to obtain from small pediatric patients with poor venous access.

Normal platelets also contain high concentrations of calcium in dense granules, which makes them opaque to the electron beam in TEM. The absence of dark spots in TEM micrographs of patient platelets is indicative of the absence of calcium storage granules and suggests δ-SPD. Although the preparation and analysis of whole mounts require only small samples that can be fixed and frozen until use, TEM is not a routine technique, and results are not easily quantifiable.

Serotonin is another dense granule constituent and, therefore, a possible marker for δ-SPD. We tested the usefulness of an optimized, immunofluorescence-based, flow cytometric assay of serotonin for pediatric patients, and we describe the diagnosis of δ-SPD in a pediatric patient as proof of principle.

Materials and Methods

Blood Donors

The parents of a 10-year-old girl and 10 pediatric control donors, 10 to 12 years old (5 boys and 5 girls) provided written, informed consent for participation. Ten healthy adult donors (8 women and 2 men; 24–46 years old) also participated in the study after providing written, informed consent. The control donors or their parents confirmed that they had no medical condition known to affect platelets or platelet serotonin levels and had not taken any medication known to interfere with platelet function 2 weeks before donating blood. This study was approved by the institutional review committee of the University of British Columbia and Vancouver Hospitals and was conducted in accordance with the Declaration of Helsinki. All subjects were fasting at the time of phlebotomy (8:00-10:00 AM). The family of the patient with oculocutaneous albinism and mild bleeding diathesis had no history of bleeding problems. The patient did not have signs or symptoms of immunodeficiency, and growth was satisfactory.

Blood samples were drawn into Vacutainer tubes (Becton Dickinson, Oakville, Canada) with a final concentration of 3.2% sodium citrate. The whole blood samples were kept at room temperature before centrifugation at 150g for 12 minutes to obtain PRP. PRP was removed with a transfer pipette, and 1 part was kept at 37°C for aggregometry. Another part was further processed for high-pressure liquid chromatography (HPLC) with electrochemical detection (ECD) analysis or fixed in paraformaldehyde (PFA; 2% final concentration in phosphate-buffered saline [PBS; 50 mmol/L of NaH2PO4 · H2O; 5 mmol/L of potassium chloride; 1.5 mmol/L of potassium chloride; 80.1 mmol/L of sodium chloride; pH 7.4]). Fixation was carried out at room temperature for 45 minutes, and samples were subsequently frozen at –80°C until use. Figure 1 shows a detailed flow chart of the sample preparation for flow cytometric serotonin analysis.

Fluorescence Microscopy

Microscopy was performed on a Zeiss Axioplan 2 microscope (Carl Zeiss, Toronto, Canada) with phase contrast and fluorescence attachments using a 100×/1.3 NA Plan Neofluar oil immersion objective (Carl Zeiss). The microscope was equipped with a digital video camera (Digital Video Camera, Austin, TX). Fixed platelets were permeabilized with Triton X-100 (0.2% final concentration) and washed with PBS (platelet pellets have to be resuspended immediately) before addition of the monoclonal mouse antihuman serotonin antibody (DAKO Diagnostics, Mississauga, Canada). After incubation and another washing step, the secondary Alexa488-labeled goat antimouse IgG1 (Cedarlane, Mississauga, Canada) was added at a dilution of 1:20. Fluorescence and phase contrast microscopy were performed at the same time. Negative control samples for fluorescence microscopy were prepared as described above except that the incubation with the serotonin-specific antibody was omitted.

Flow Cytometry

Platelets, labeled for serotonin fluorescence microscopy as described above, were diluted with PBS buffer 1:100 and analyzed with an Epics XL flow cytometer (Beckman Coulter, Miami, FL). The fluorescence resulting from the binding of fluorescently labeled nonspecific antibody alone was set to 2% platelet-associated fluorescence (negative control).
High-Pressure Liquid Chromatography

Serotonin concentrations in platelet pellets were measured with HPLC using an XTerra reverse phase column (Waters, Franklin, MA) in conjunction with an electrochemical detector (Waters, Alliance 2690-464). For HPLC, PRP was prepared as described above. Platelets were counted on a Coulter MaxM cell counter (Beckman Coulter) to allow normalization of the results. Plasma and platelets were separated by centrifugation at 1,000g for 15 minutes at 4°C. The platelet pellet was resuspended in PBS. To protect serotonin from oxidation, 0.5 mg/mL of cysteine hydrochloride (Fisher Scientific, Whitby, Canada) was added to all samples before the addition of trichloroacetic acid (0.2N final concentration, Fisher Scientific) for the precipitation of proteins. The samples were centrifuged and the supernatants frozen. Isocratic flow at 0.25 mL/minute was used with a

Figure 1 Workflow diagram for the flow cytometric determination of platelet serotonin levels. Platelet-rich plasma (PRP) was fixed with paraformaldehyde (PFA) and permeabilized with Triton X-100 (TX-100). Washed platelets were fluorescently labeled for serotonin (5-HT). PBS, phosphate-buffered saline; RT, room temperature.
mobile phase consisting of 5% methanol, 3% acetonitrile, and 92% phosphate buffer (100 mmol/L of NaH$_2$PO$_4$, 0.5 mmol/L of EDTA, pH 3.8 adjusted with trifluoroacetic acid); all chemicals were purchased from Sigma. Standards for serotonin were prepared by dissolving different concentrations of serotonin hydrochloride (Sigma) in PBS. The standards were treated with cysteine and trichloroacetic acid analogous to the samples. Under these conditions, the mean ± SD retention time for serotonin was 5.2 ± 0.3 minutes at 4°C, and no interference with other substances was observed. The detector voltage was set to 650 mV, and the power range was 10 mA with the direct current setting.

**Platelet Aggregation and ATP Luminescence**

A ChronoLog dual-channel lumi-aggregometer (ChronoLog, Havertown, PA) was used to measure platelet aggregation. PRP was prepared as described above and compared with plasma. The stir speed was set at 1,000 rpm. Platelets were activated with final concentrations of 1 IU/mL of thrombin, 20 µmol/L of ADP, or 30 µmol/L of epinephrine (all reagents were purchased from ChronoLog). Luciferin-luciferase (ChronoLog) was reconstituted according to the supplier’s instructions and kept on ice. Forty microliters of luciferin-luciferase was added to 450 µL of PRP 2 minutes before stimulation with the agonist. Addition of the ATP standard (2 nmol/L) was used to calibrate the luminescence signal for semiquantitative ATP determination.

**TEM of Whole Mounts**

For whole mounts, 5 µL of PRP was spotted onto formvar-coated grids (Electron Microscopy Sciences, Fort Washington, PA) and dried at room temperature. Digital images were taken on a Hitachi H-7600 120-kV transmission electron microscope (High Technologies, Rexdale, Canada) with an AMT Advantage HR CCD camera (Advanced Microscopy Techniques, Danvers, MA).

**Results**

**Flow Cytometric Determination of Platelet Serotonin Content: Analysis of a Pediatric Patient Sample as Proof of Principle**

Figure 1 shows a diagram of all steps involved in the sample preparation to determine the platelet serotonin level. Fixed and permeabilized platelets were washed, dual-labeled with a specific mouse anti-human serotonin antibody and an Alexa488-labeled secondary goat antimouse antibody, and analyzed by flow cytometry. The platelet population was selected in the light scattering panel [Figure 2A]. The percentage of these cells that bound more than 2% Alexa488-labeled antibody appeared as a right-shifted population in the fluorescence histogram (gray-shaded area in [Figure 2B]). Only 13% of platelets from an 10-year-old pediatric patient were serotonin-positive.

**Serotonin Content in Patient and Representative Control Samples Determined by HPLC**

Quantitative confirmation of the low levels of serotonin in patient platelets was obtained by HPLC-ECD [Figure 2C] and resulted in a mean ± SD of 4.7 ± 0.2 ng serotonin per mL of PRP. The platelet count in PRP was 3.66 × 10$^{11}$/L. PRP. Normalization to 10$^9$ platelets yielded a mean ± SD of only 12.8 ± 0.5 ng of serotonin per 10$^9$ platelets in the patient sample (solid line). A representative chromatogram is shown for a pediatric control sample (dotted line) that amounted to a mean ± SD of 405.7 ± 4 ng of serotonin per 10$^9$ platelets (mean ± SD, 205.7 ± 2.2 ng/mL, with a platelet count of 5.07 × 10$^{11}$/L).
Serotonin Content in Pediatric and Adult Control Samples Compared With the Patient Sample

To demonstrate general usefulness of the flow cytometric serotonin assay for pediatric patients, we compared the results from the patient sample with the platelet serotonin levels found in 10 pediatric and 10 adult control samples. Figure 3. In addition, we measured serotonin in all samples by quantitative HPLC-ECD. Both assays correlated well and clearly indicated that the patient platelets contained some serotonin but were severely serotonin-deficient.

Confirmation of δ-SPD by Fluorescence and TEM

Discoid platelet morphologic features were seen for control Image 1A and patient Image 1F platelets. Dense granules of pediatric control platelets showed bright, specific immunofluorescence for serotonin Image 1B and Image 1C, which was comparable to the presentation in normal adult platelets. Some fluorescent staining was detectable in patient platelets Image 1G and Image 1H, but it was much less distinctive and not well defined to intracellular compartments.

Dense granules were visible as dark spots on whole mounts of the control sample Image 1D and Image 1E.
In general, the patient sample showed only a few, small spots. There were also some areas of increased electron density, such as seen in Image 1J.

**Lumi-aggregometry to Test Platelet Function in Response to Thrombin, ADP, and Epinephrine**

Patient platelets showed normal aggregation in response to 1 IU/mL of thrombin. Release of ATP from patient platelets following stimulation with thrombin was not completely absent but insufficient for reliable quantitation (Figure 4A, inset). Stimulation with 20 µmol/L of ADP resulted only in the primary wave of aggregation of patient platelets with subsequent disaggregation. Patient platelets also showed a significantly reduced response to 30 µmol/L of epinephrine. Control platelets fully aggregated in response to the high doses of agonists and showed normal ATP release. Patient platelets did not release ATP in response to ADP or epinephrine. Results from a representative control sample are shown in Figure 4 with ATP release of 3.1 nmol/L.

**Discussion**

Platelet SPDs have been associated with bleeding problems and defective granule formation or release for decades. Determination of δ-SPD has been based on the measurement of reduced adenine nucleotide release and the absence of electron dense granules by electron microscopy. Flow cytometry assays for platelets have been shown to be powerful and versatile tools. Fluorescence-based methods using acridine orange or mepacrine took advantage of the change in fluorescence of these indicators when they were loaded into dense granules because of the pH gradient. Maurer-Spurej et al developed an immunocytochemical assay for the direct detection of serotonin contained in platelet dense granules and optimized this assay before this study.

Herein, we show that this immunofluorescence assay for serotonin is particularly useful for the analysis of pediatric samples because the sample requirement is small (0.2 mL of PRP) and can be analyzed semiquantitatively by flow cytometry within 2 hours of phlebotomy. In addition, samples can be frozen and stored until analyzed or shipped on dry ice to an analytic laboratory for flow cytometric analysis. In contrast with the subjective interpretation of microscopic images, sample analysis by flow cytometry is objective and already widely used in routine clinical laboratories.

The immunofluorescence assay is a quick and reliable method for the semiquantitative determination of serotonin content. Flow cytometric analysis of fluorescently labeled normal platelets resulted in a mean ± SD of 92% ± 8% of platelets positive for serotonin in children between the ages of 10 and 12 years and of 78% ± 6% in adults. The result obtained with the patient sample of 13% positive platelets was a clear indication of serotonin deficiency.

The study is limited because only 1 sample from a pediatric patient with δ-SPD was available. However, according to the Chebyshev rules, at least 89% of normally distributed results are found within 3 SDs of the normal mean. Thus, we recommend that the lower cutoff for normal serotonin levels should be 60% serotonin positivity by flow cytometry. Umbilical cord blood from newborns is also serotonin-deficient. We
determined an average ± SD serotonin positivity of cord blood platelets by flow cytometry of 19% ± 12%, which was equivalent to 122 ± 82 ng of serotonin per 10^6 platelets (n = 3; data not shown). The results from cord blood and from the patient with δ-SPD satisfy the proposed cutoff.

Our results from control samples correlated well with previously published data. Although HPLC-ECD is a sensitive and supportive treatment, the technique is too time-consuming and sophisticated to be used for routine analyses.

We found that platelets from our patient were not completely devoid of serotonin, indicative of some capacity for serotonin storage in her platelets. This correlated with the presence of few, small, calcium-rich dots on whole mounts. Despite oculocutaneous albinism, the patient was not diagnosed with HPS because, by definition, patients with HPS completely lack platelet dense granules (W.A. Gahl, written communication, October 2005). The family of the patient decided against genetic testing.

This study illustrates that the immunofluorescence-based flow cytometric assay is a useful test for platelet serotonin in pediatric patients. The significant advantages of this test to current methods could provide early identification of many patients with δ-SPD who could then benefit from prophylactic and supportive treatment.

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