Flow Cytometric Evaluation of Platelet Disorders

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DISCLOSURE

• Relevant Financial Relationship(s)
  None

• Off Label Usage
  None
Objectives

• The Concept of Flow Cytometry

• Applications of Flow Cytometry in Platelet Function Testing

• Applications of Flow Cytometry in Other Coagulation Testing
The Concept of Flow Cytometry
Platelet “Surface” Deficiencies

Glanzmann thrombasthenia: no aggregation with natural agonists

Scott syndrome: decreased prothrombin conversion

Reduced response to collagen

Altered response to stimuli: ADP (P2Y₁₂), TXA₂ (TPα), 5-HT, PAF, adrenaline...

Platelet-type VWD: spontaneous binding of VWF to GP Ibα

Bernard-Soulier syndrome: lack of adhesion to VWF and abnormal response to thrombin

History of Flow Cytometry

Lou Herzenberg - 1969 - sorter based on fluorescence (arc lamp) built after working with one of Kamentsky’s RCS systems where they built an instrument they called the Fluorescence Activated Cell Sorter (FACS)

Kamentsky - Bio/Physics Systems - 1970
commercial cytometer

1974 – present commercialization and further development
# Diagnosing Platelet Abnormalities by Flow Cytometry

<table>
<thead>
<tr>
<th>Disease</th>
<th>Deficiency</th>
<th>Testing by Flow Cytometry</th>
</tr>
</thead>
</table>
| **Glycoprotein (GP) Abnormality:**  
Glanzmann thrombasthenia  
Bernard-Soulier syndrome  
Platelet-type VWD  
Collagen receptor deficiency  
Wiskott-Aldrich syndrome | GP IIb-IIIa  
GP Ib-V-IX  
GP Ib  
GP Ia-IIa & GP IV  
Flow on lymphocytes |
| **Abnormal Granules:**  
Storage pool deficiency  
Gray platelet syndrome | Delta-granule  
| **Abnormal Signal Transduction:**  
Platelet receptor defects (congenital or acquired)  
Defects in arachidonic acid metabolism  
Defects in PLC and Calcium responsiveness | P2Y12 ADP receptor (VASP)  
COX  
PLC and Ca^{2+} | Thromb Haemost. 1999 Sep;82(3):1145-52.  
Cytometry B Clin Cytom. 2008 Mar;74(2):110-7  
| **Abnormal Procoagulant Activity**  
Scott syndrome  
Microparticle | Procoagulant  
| **Other Acquired Conditions**  
HIT  
ITP | Anti-Heparin/PF4  
Principles of Flow Cytometry
Platelet Flow Cytometry Tests

- Glycoprotein Profile
- Platelet Function Analysis
Platelet Glycoprotein Profiles
Early Examples in Literature

THE USE OF FLUORESCENCE FLOW CYTOMETRY IN THE CHARACTERIZATION OF BERNARD-SOULIER SYNDROME AND GLANZMANN'S THROMBASTHENIA

Fabrizio Fabris, Alessandra Casonato, Maria Luigia Randi, Guido Luzzatto, Giustina De Silvestro, Giuseppe Ongaro, Antonio Girolami
Platelet Function Analysis by Flow Cytometry

- PAC-1 Antibody
- Anti-P-Selectin
- Annexin V

PAC-1 Antibody

Activated GP IIb-IIIa

P-Selectin

Annexin V

Activation

P-Selectin

Anti-P-Selectin
General Procedure

Whole Blood Collection

Dilute (and Treat with Stimulus)

Stain with Fluorescent Conjugated Ab

Dilute and Fix
Applications of Flow Cytometry in Platelet Testing
Applications of Flow Cytometry to the Study of Platelets

- Specific Glycoprotein Deficiency
  - Bernard-Soulier Syndrome
  - Glanzmann Thrombasthenia
  - Collagen Receptor Deficiency

- Platelet Other Deficiency
  - Activation deficiencies to
  - Procoagulant Activity

- Monitoring of Antiplatelet Agents
  - Thienopyridine (clopidogrel)
  - GPIIb-IIIa Antagonists (Abciximab)
Case 1

• 36-year-old man for evaluation of a bleeding disorder.
• He bled for an entire month after his wisdom teeth extraction.
• At that time, his platelet count was 80,000/ul.
• **Bleeding history:**
  – No bleeding after tonsillectomy at age of 3
  – No bleeding after scalp laceration at age of 10
• **Family History:**
  – Paternal grandfather had bleeding problems following removal of skin lesions
  – Father had no significant bleeding history
  – Mother had a history of abdominal bleeding during pregnancy
  – One brother died at the age of 10 from intracerebral bleeding following a playground accident.
Coagulation Testing

- Normal CBC except for thrombocytopenia (80,000 X10^9/L)
- Giant Platelets on peripheral blood smear
- Normal PT, APTT, TT, VWF, FVIII, FIX, and FXIII
- Bleeding Time: 9 min (1.5-8 min)
- PFA-Epi: 194s (70-165); -ADP: >300 (50-115)
- Platelet Aggregation:
  - Arachidonate  N
  - ADP  N
  - Epi  N
  - Collagen  N
  - Ristocetin (1.0 and 1.5 mg/mL) ↓
Flow Cytometry Studies

Size

GPIIa

GPIbα

GPIX

Control: Green

Patient: Red
Diagnosis: Bernard-Soulier Syndrome

- **Autosomal Recessive**
- **First described in 1948**
- **Prevalence = 1/million**
Common Clinical Features

Bleeding symptoms: usually evident shortly after birth or in early childhood.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Epistaxis</td>
<td>70%</td>
</tr>
<tr>
<td>Ecchymoses</td>
<td>58%</td>
</tr>
<tr>
<td>Menometrorrhagia</td>
<td>44%</td>
</tr>
<tr>
<td>Gingival Hemorrhage</td>
<td>42%</td>
</tr>
<tr>
<td>GI bleeding</td>
<td>22%</td>
</tr>
<tr>
<td>Posttraumatic Bleeding</td>
<td>13%</td>
</tr>
<tr>
<td>Hematuria</td>
<td>7%</td>
</tr>
<tr>
<td>Cerebral Hemorrhage</td>
<td>4%</td>
</tr>
<tr>
<td>Retinal Hemorrhage</td>
<td>2%</td>
</tr>
</tbody>
</table>

Lopez JA et al. Bernard-Soulier syndrome, Blood 91:4397
Bernard-Soulier Syndrome-GPIb-V-IX Deficiency


Pathophysiology of BSS

Wintrobe’s Clinical Hematology

14, 6 Pages: 1240-1249
Mutations of GPIb-V-IX Complex

Platelet 2007 2nd Edition-page 1034
Inherited Disorders of Platelet Function
Alan T. Nurden and Paquita Nurden
Features of BSS Hemorrhagic Diathesis

- Thrombocytopenia
- ↓ Ristocetin induced PLT Aggregation
- ↓ or absent GPIb-V-IX complex
- Classification:
  - Type 1: Quantitative/Qualitative
  - Type 2: Normal platelet count and size (Kenny D. et al. 1998 Blood 92: 175)
Treatment

- Platelet transfusion for severe bleeding
- Desmopressin
- Recombinant factor VII
- Antifibrinolytic agents
Trivia for BSS

• Rare BSS variant my have normal platelet count and size.

• Patients with DiGeorge syndrome may have missing GPIb beta.

• Degrees of GPIb and IX deficiency do not appear to correlate with bleeding severity.

• GPV is not a essential component and its abnormalities do not cause BSS.
Flow Cytometry Studies for Mailed-in Samples
Patient: 34y/o female with significant bleeding history

Mild thrombocytopenia

Normal Plasmatic Coagulation Studies

PFA 100: abnormal
Platelet Aggregation Test:
  Arachidonic Acid: Abnormal
  ADP: Abnormal
  Epi: Abnormal
  Collagen: Abnormal
  Ristocetin: Decreased
Flow Cytometry-Glycoprotein Profile

Normal control: Green
Patient: Red

GPIIbα

GPIX

GPIIb

GPIIIa

Glycoprotein Panel:
CD61-FITC/CD42b-PE
CD41-FITC/CD42b-PE
CD42a-FITC/CD61-PE
CD42a-FITC/CD42b-PE
Flow Cytometry-Activity Panel

Resting Platelets: Black
ADP (0.1mM): Green

Normal Control

Patient

P-selectin

Pac-1
Diagnosis: Glanzmann Thrombasthenia

- First described in 1918 by Glanzmann E, (J kinderkranken 1918,88:113)
- Autosomal recessive
- Clinical Description
  - Homozygous:
    - Minimal bruising to severe hemorrhages
    - Bleeding symptoms manifest rapidly after birth
    - Bleeding tendency improves with age
  - Heterozygous:
    - No bleeding
Diagnosis: Glanzmann Thrombasthenia

- GPIIb/IIIa deficiency:
  
  Normal Donor
  
  GT Patient

Diagnostic Methods for GT

- Clinical/family history
- Peripheral blood
- Routine Coagulation studies
- Platelet Function studies
  - PFA-100
  - Platelet Aggregation:
    - Arachidonic acid: ↓
    - ADP ↓
    - Epi ↓
    - Collagen ↓
    - Ristocetin NL/ ↓
- Flow cytometry: GPⅡb/Ⅲa ↓ ↓ ↓
- Mutation analysis: best for prenatal and carrier status analyses.
Pathophysiology of GT
Mutations of GPIIb and GPIIIa

Orphanet Journal of Rare Diseases 2006, 1:10
Trivia about GT

• Treatment:
  – Platelet transfusion for severe bleeding

• Acquired Conditions:
  – ITP or other autoimmune conditions (Tholouli E, Br J Haematol 2004, 127:209)

• Disease Severity: The Unresolved issue.
  – Broad spectrum
  – Bleeding Severity does not correlate with degrees of GPIIb/IIIa deficiency
Our Experience of Platelet Function Analysis by Flow Cytometry
2006-present

<table>
<thead>
<tr>
<th>Disorders</th>
<th>Cases Number</th>
</tr>
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<tbody>
<tr>
<td>BSS:</td>
<td>4</td>
</tr>
<tr>
<td>GT:</td>
<td>5</td>
</tr>
<tr>
<td>GP Ia-IIa:</td>
<td>2</td>
</tr>
<tr>
<td>Gray Platelet Syndrome</td>
<td>5</td>
</tr>
<tr>
<td>Macrothrombocytopenia NOS</td>
<td>7</td>
</tr>
<tr>
<td>Signal pathway deficiency</td>
<td>3</td>
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<tr>
<td>Other Disorders</td>
<td>8</td>
</tr>
<tr>
<td>Normal</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
</tr>
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</table>
Problems of Current Platelet Flow Cytometry Practice

• Lack of Standard Operation
  – Pre-analytical: Sample tubes (Citrate and ACD)
  – Reagents: antibodies and fluorochromes
  – Lack of standarized fluorescent quantification
  – SOP

• Lack of Normal Range
  – Due to lack of standarized quantification guidelines
  – Age, gender, concomitant conditions

• Esoteric Practice
  – High Complexity: Mailed in specimen
Standardization of Platelet Flow Cytometry Studies

- Sample collection: Citrate or ACD
- Panels:
  - GP screen
  - GP quantification
System Description

**Glycoprotein screening assays:**
- by mean fluorescent intensity
- Whole blood sample in citrate (3.2%) or ACD tubes (A or B)
- Conjugated antibodies to
  - CD41 (GPIIb)
  - CD61 (GPIIIa)
  - CD42a (GPIX)
  - CD 42b (GPIba)
  - CD49a (IaIIa)
- Gated with Forward and Side light scatter histogram.
- The mean fluorescent equivalences (MFE) are calculated from a standard curve of calibrator beads.
### Histogram Statistics

File: 80827.001  
Log Data Units: Linear Values  
Sample ID: RCP calib  
Patient ID:  
Tube: Untitled  
Panel: Untitled Acquisition Tube List  
Acquisition Date: 27-Aug-08  
Gate: G1  
Gated Events: 29984  
Total Events: 42438  
X Parameter: FL1-H (Log)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Left, Right</th>
<th>Events</th>
<th>% Gated</th>
<th>% Total</th>
<th>Mean</th>
<th>Geo Mean</th>
<th>CV</th>
<th>Median</th>
<th>Peak Ch</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>1, 991</td>
<td>29984</td>
<td>100.00</td>
<td>70.65</td>
<td>1202.12</td>
<td>254.14</td>
<td>129.20</td>
<td>4410</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>1, 1</td>
<td>4985</td>
<td>16.63</td>
<td>11.75</td>
<td>3.84</td>
<td>3.46</td>
<td>45.38</td>
<td>3.55</td>
<td>4</td>
</tr>
<tr>
<td>M2</td>
<td>52, 13</td>
<td>4846</td>
<td>16.16</td>
<td>11.42</td>
<td>81.07</td>
<td>80.59</td>
<td>10.92</td>
<td>80.58</td>
<td>82</td>
</tr>
<tr>
<td>M3</td>
<td>136, 400</td>
<td>5104</td>
<td>17.02</td>
<td>12.03</td>
<td>231.17</td>
<td>230.55</td>
<td>7.27</td>
<td>230.82</td>
<td>237</td>
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<tr>
<td>M4</td>
<td>400, 1165</td>
<td>4979</td>
<td>16.61</td>
<td>11.73</td>
<td>606.19</td>
<td>604.88</td>
<td>6.67</td>
<td>609.76</td>
<td>615</td>
</tr>
<tr>
<td>M6</td>
<td>3051, 6264</td>
<td>5029</td>
<td>16.77</td>
<td>11.85</td>
<td>4294.34</td>
<td>4290.19</td>
<td>4.34</td>
<td>4332.30</td>
<td>4410</td>
</tr>
</tbody>
</table>

#### Example of MFE calculation

**SPHERO CALIBRATION GRAPH**

(PerCP Channel)

![Graph](image)

Example
Quantitative glycoprotein screen test by Biocytex:
- Whole blood in citrate or ACD tube.
- Anti-CD61 (IIIa), -CD42a (GPIX) and -CD49a (GPIa/IIa) antibodies, and then stained with conjugated secondary antibody.
*The MFI will be compared with a standard curve derived from a standard primary antibody coated beads.
*Copy numbers of glycoproteins will be extrapolated from the standard curve.
Biocytex GP Screen

\[ \text{Platelet} = \text{GP} = \text{Mouse Anti-GP ab} = \text{FITC-anti-mouse ab} \]

Add anti-mouse IgG-Fluorescein conjugate

The beads give 4 peaks of fluorescence. One creates a standard curve of MFI vs. molecules/platelet(bead). GP/platelet is read off of the curve.
Standard curve

<table>
<thead>
<tr>
<th>bead peak</th>
<th>MCF</th>
<th>Mur IgG/bead</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18.7</td>
<td>490</td>
</tr>
<tr>
<td>B</td>
<td>380.71</td>
<td>13000</td>
</tr>
<tr>
<td>C</td>
<td>1243.53</td>
<td>38000</td>
</tr>
<tr>
<td>D</td>
<td>2907.62</td>
<td>96000</td>
</tr>
</tbody>
</table>

GP screen standard curve

\[ y = 32.915x - 572.58 \]

\[ R^2 = 0.9986 \]

5/3/2008

<table>
<thead>
<tr>
<th>sample</th>
<th>marker</th>
<th>MCF</th>
<th>GP sites/platelet</th>
<th>normal range (X10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>GPIIIa</td>
<td>1413.25</td>
<td>45,944.1</td>
<td>53±12</td>
</tr>
<tr>
<td>T3</td>
<td>GPIb α</td>
<td>972.46</td>
<td>31,435.6</td>
<td>38±11</td>
</tr>
<tr>
<td>T4</td>
<td>GPIa</td>
<td>126.9</td>
<td>3,604.3</td>
<td>5±2.8</td>
</tr>
</tbody>
</table>
Applications of Flow Cytometry in Other Coagulation Testing

VWF Ristocetin (VWF:RCo) Activity by Flow Cytometry
Problems of Current VWF:RCo Assays by Platelet Aggregation /Aggregometry Method

- Insensitive to Type 2 VWD
  - VWF:RCo/Ag ratio <0.5-0.7* (NL= 1.0±0.26)

- High Coefficient of Variation (15-30%)**
  - No calibrated reagent platelets
  - Complicated procedure
  - Large reaction volume (1mL)
  - Unreliable when VWF:Ag <15-20 IU/dL
  - Labor intensive

** Steve Kitchen et al. Semin Throm Hemost 2006
VWF: RCo Activity by Flow Cytometry

Chen et al. JTH Volume 6, Number 2, February 2008, pp. 323-330
VWF:Ristocetin Testing Procedure

- 0.5 μl plasma
- RT incubation for 45 minutes
- 1:20 dilute with IBS+1.0 mg/mL Ristocetin
- Flow cytometry
Validation of VWF:RCo by Flow Cytometry Method

![Graph showing VWF:RCo:AG ratios for normal donors and VWD patients, with aggregation and flow cut-off levels indicated.](image-url)
## Advantages of VWF:RCo by Flow Cytometry Method

<table>
<thead>
<tr>
<th></th>
<th>Flow Cytometry</th>
<th>Aggregation/Aggregometry</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VWF:RCo/Ag ratio for Type 2 VWD</strong></td>
<td>0.5 (more sensitive)</td>
<td>0.5-0.7 (less sensitive)</td>
</tr>
<tr>
<td></td>
<td>More specific</td>
<td>Less specific</td>
</tr>
<tr>
<td><strong>Patient Sample Volume</strong></td>
<td>1-2 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td><strong>Reagent Platelets 1 unit Platelet</strong></td>
<td>Calibrated (fixed)</td>
<td>Donor variation (fresh)</td>
</tr>
<tr>
<td></td>
<td>2500 tests (12 months)</td>
<td>80 tests (Daily)</td>
</tr>
<tr>
<td><strong>Linear Range Lowest VWF level</strong></td>
<td>3~150 IU/dL</td>
<td>12.5-100 IU/dL</td>
</tr>
<tr>
<td><strong>CV%</strong></td>
<td>5-10%</td>
<td>15-30%</td>
</tr>
<tr>
<td><strong>Complexity</strong></td>
<td>More complexed</td>
<td>Complex</td>
</tr>
<tr>
<td><strong>Automation</strong></td>
<td>Yes (with autoloader)</td>
<td>No</td>
</tr>
</tbody>
</table>
Summary

• The Concept of Flow Cytometry

• Applications of Flow Cytometry in Platelet Function Testing

• Applications of Flow Cytometry in Other Coagulation Testing
Acknowledgments

- Dr. Whyte G. Owen
- Dr. William L. Nichols
- Dr. Rajiv K. Pruthi
- Dr. John A. Heit

- Randall S. Miller
  - Flow cytometry specialist