Session Objectives

- Important tests for evaluating platelet functional disorders
- Common forms of platelet functional disorders
- Why platelet function testing is difficult to standardize and issues important to test quality
- Understand, through case-based examples, the importance of diagnostic testing for platelet functional disorders

Perspectives on Platelet Functional Disorders in 2007

- Disorders - common and important
- Uncertainties about best test practices for evaluating these conditions and about test sensitivity, specificity, positive predictive value, negative predictive value
- Also lack tools for standardizing the clinical part of the diagnostic assessment

Current Concepts on Platelet Functions Implications for Types of Potential Defects

- Congenital or acquired defects in
  - Receptors for:
    - Adhesive proteins
  - Signaling/Activation Problems
    - Receptors for important agonists
    - Signaling/secreton pathways that enhance activation (including release of dense granule contents)
    - Platelet procoagulant activity
  - Some conditions that affect platelet numbers also impair platelet function

Disorders with Impaired Platelet Function

- Tethering
- Translocation
- Stabilization
- Activation
- Collagen, von Willebrand factor, fibrin, other matrix elements
- Temporary bandage

Disclosures for Catherine P. M. Hayward

<table>
<thead>
<tr>
<th>Research Support/PI</th>
<th>N/A = No support from industry</th>
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<tr>
<td>Supported by:</td>
<td>Heart and Stroke Foundation of Ontario</td>
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N/A = Not applicable (no conflicts)
Acquired Qualitative Defects

- Drugs – antiplatelet agents are the most common
- Uremia
- Liver disease
- Cushing’s Syndrome
- Cardiopulmonary bypass
- Inhibitory antibodies
- Bone marrow disorders
  - Diverse – e.g. storage pool defects, membrane glycoprotein deficiencies

Screening Tests for Platelet Functional Disorders

- Bleeding time
  - Sensitivity limited, performance issues
  - Use – predicting response to DDAVP therapy?
- Closure Time measured by PFA-100™
  - Rapid, simple, test of shear-dependent platelet adhesion
  - Sensitivity
    - Not perfect for screening
    - 24% to >90% sensitivity for congenital platelet disorders
    - Better for studies prospectively evaluating platelet disorders
    - Better for common platelet disorders

Hayward, Harrison, Cattaneo, Ortel and Rao; the Platelet Physiology SSC of ISTH. Platelet function analyzer (PF-100) closure time in the evaluation of platelet disorders and platelet function. JTH 2006; 4: 312-9.

Platelet Disorders

Lack data from population surveys

Secretion defects are more common than dense granule deficiency

Dense granule deficiency is almost as common as von Willebrand disease

about 3-5% of referred patients at our center

CT in Diagnostic Testing for Platelet Disorders

- Potential advantages
  - Early clues about a defect if abnormal
  - Abnormal results may trigger a referral
- More evidence is needed on its most appropriate use in clinical practice related to platelet disorders
  - Sensitivity is better for VWD than for platelet disorders
  - Diagnostic Screening FURTHER TESTING NEEDED, REGARDLESS
  - Role in drug monitoring – needs further evaluation

Hayward, Harrison, Cattaneo, Ortel and Rao; the Platelet Physiology SSC of ISTH. Platelet function analyzer (PF-100) closure time in the evaluation of platelet disorders and platelet function. JTH 2006; 4: 312-9.
Antiplatelet Therapy

Dysfunction

- Platelet secretion, evaluated by release of dense granule content
- Platelet function, evaluated by aggregation tests

Include an assessment of/for:

- Drug
  - Thienopyridines: plus aspirin
  - Other NSAIDs: prasugrel, eptifibatide

Others

- Tests for procoagulant defects (appear rare, testing rarely done)
- Serum apart from tests such as the PFA-100
  - More sensitive endpoint for defective function

Complexity of Diagnostic Testing for Platelet Disorders

- Platelet number and size, platelet and leukocyte morphology
  - ~17% of referrals for testing are thrombocytopenic
- Platelet function, evaluated by aggregation tests
- Platelet dense granule deficiency:
  - Aggregation, BT, PFA-100™ CT - may be normal
- Platelet secretion, evaluated by release of dense granule contents
  - ~2 More sensitive endpoints for defective function
- Adhesion testing:
  - apart from tests such as the PFA-100™ CT, this remains in research domain
- Optional:
  - Tests for procoagulant defects (appear rare, testing rarely done - \( \beta\)-thromboglobulin, platelet consumption to screen)
  - Others:
    - Transmission electron microscopy, glycoprotein analysis, thromboelastography
  - Immunostaining for some conditions (e.g., MHY9 related disorders)

Quality Assurance and Platelet Tests

- Stabile
  - Converting: Simple to operate, expertise required, rapid, inexpensive
  - Accurate & Precise: The test measures what it is supposed to measure. Reproducible, different observers agree on interpretation
- Standardized:
  - Test procedure is well-documented, standards are available, existing quality assurance program
- Normal:
  - Negative test rules out disease
  - Specific: Positive test results in disease
- Population norms to guide interpretation
- Proven utility:
  - Patients are better off as a result of undergoing the test
  - Clinically relevant:
    - Results independently correlated with clinical outcome
    - Modifiable: Altering a multifactorial treatment based on the result of the test impacts clinical outcome
    - Cost effective: Benefits of testing outweigh the direct and indirect costs of testing and follow up

Tests for Drug Resistance:

- Aspirin
  - “Unidentified”
  - Test challenging, time consuming, no standardized human-like, serum stability, choice of procedure
  - Platelet aggregation
  - Thrombin-activated platelets
  - PFA-100™, high shear aggregation with citrate
  - Platelet secretion
  - Citrome assay
  - More sensitive than BT or PFA-100™

- Eptifibatide
  - VerifyNow® Aggregation Assay
  - Possible problems re specificity

- Prasugrel
  - Platelet function testing: Quality assurance
  - JTH 2006; 4: 312-319
  - CT with anti-platelet drugs
  - March 2002

What do we find with our standardized testing?

Data for 391 Unselected Patients Prospectively Evaluated for von Willebrand Disease & Platelet Disorders

- Platelet function abnormality &/or dense granule deficiency
- von Willebrand disease
- No laboratory abnormalities found
- Abnormalities of uncertain significance

Hamilton Registry Data
March 2002

39% 17% 5%

All Labs are Not the Same……

Variability Between Clinical Laboratories in Diagnostic Testing for Disorders of Platelet Function

- Goals
  - Identify common practices and problems in the testing for disorders of platelet function
  - Enthusiastic participation!
  - 47 participating labs
Aggregation methodologies
37% of NASCOLA sites used >1 method

Final Agonist Concentration for Testing Platelet Function by Aggregation

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Survey 1</th>
<th>Survey 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>0.19 – 125 µg/mL</td>
<td>0.62 – 190 µg/mL</td>
</tr>
<tr>
<td>ADP</td>
<td>0.3 – 1000 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.1 – 1000 µM</td>
<td>0.1 – 100 µM</td>
</tr>
<tr>
<td>Arachidonic Acid</td>
<td>0.005 – 1.7 mM</td>
<td>0.0016 – 2.3 mM</td>
</tr>
<tr>
<td>TRAP</td>
<td>0.25 – 15 mg/mL</td>
<td>Low dose 0.5 mg/mL, High dose 12.5 mg/mL</td>
</tr>
</tbody>
</table>

Sources of reference intervals
26% of sites used >1 method
29% if sites had not determined their own reference range (12-250 tests/yr)
Only 1 site did qualitative, without quantitative, interpretations
Only 1 site formally evaluated data for normality in distribution
See Moffat abstract this meeting

Concerns Raised About Platelet Aggregation Testing
there were many.................................
- Labor intensive
- Lack of evidence-based guidelines
- Uncertainties – how to:
  - evaluate thrombocytopenic patients
  - interpret epinephrine aggregation
  - Challenging to obtain reliable drug histories, uncertainties about the effects of different drugs
  - Influence of pre-analytical errors
    - proper sample procurement & transport

Aggregation Testing – What is Best?
- Agonist Concentrations
  - Medians – some conformity
    - Are these appropriate concentrations?
    - Review of published literature
      - The medians are probably good concentrations for testing
- Useful strategies
  - e.g. comparing arachidonic acid/thromboxane responses
    - NASCOLA Study: 15% of labs used this comparison to sort out possible ASA/NSAID-like defects at the time of this survey
Control (red, green) vs. Patient (P) with Secretion Defect
P: also reduced aggregation with arachidonic acid and thromboxane analogue

Higher concentration of agonist: shown in red (C) & black (P)

Illustration of Aggregation Findings
% aggregation with 4 μM ADP

Variability in Aggregation Tests?
data from repeat tests done on 115 patients in Hamilton

Illustration: Usefulness of Aggregation Tests

<table>
<thead>
<tr>
<th>Agent</th>
<th>Patient (P)</th>
<th>Control (red, green)</th>
<th>Reference Interval (%)</th>
<th>Aggregation %</th>
<th>Normal Results (%)</th>
<th>Deficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP 5 μM</td>
<td>5-10</td>
<td>25-60</td>
<td>36</td>
<td>71</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Collagen 5 μg/mL</td>
<td>45-101</td>
<td>35-76</td>
<td>41</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Collagen 25 μg/mL</td>
<td>68-108</td>
<td>41-12</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Epinephrine 6 μM</td>
<td>1-37</td>
<td>11-41</td>
<td>41</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Arachidonic Acid 0.6 mM</td>
<td>72-108</td>
<td>44-47</td>
<td>47</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Thromboxane analogue 1 μM</td>
<td>72-108</td>
<td>44-47</td>
<td>47</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Ristocetin 0.5 μg/mL</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Ristocetin 1.25 μg/mL</td>
<td>75-104</td>
<td>22</td>
<td>85</td>
<td>90</td>
<td>90</td>
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</tr>
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</table>

Markers above or below 0% an abnormal with thrombin

Illustration: Diagnostic Evaluation of Platelet "Luggage" Defects

Types of Luggage:
alpha (α) – protein storage container
delta (δ) – electron dense

α-granule deficiency:
Fairly common
~ 4% prevalence in our patients
Aggregation, HT, CT may be normal

δ-granule deficiency:
URAV platelets – rare
Clue – from evaluation of blood film
combined αδ-deficiency:
Rarer than α-granule deficiency

α-granules: ~ 80/platelet
δ-granules: ~ 2-5/platelet

Glanzmann Thrombasthenia
secretion absent or reduced with these agonists but normal with thrombin

Secretion Defect

Illustration: Variability in Aggregation Tests?

Figure: Usefulness of Aggregation Tests

Figure: Variability in Aggregation Tests

Figures: Diagnostic Evaluation of Platelet "Luggage" Defects

Figures: Illustration of Aggregation Findings

Figures: Control (red, green) vs. Patient (P) with Secretion Defect

Figures: Table: Illustration: Usefulness of Aggregation Tests

Figures: Illustration: Variability in Aggregation Tests?

Figures: Illustration: Diagnostic Evaluation of Platelet "Luggage" Defects
Whole Mount
Most popular method for assessing dense
granule deficiency in North America
Controls: average of 4 or more electron
dense granules per platelet

EDS
Electron Dispersion Spectral Analysis
analysis of the different dense
granule constituents

Diagnostic Evaluation of Platelet Secretion
- Secretion Defects
  - Paradox or knowledge translation gap
    - most common form of platelet disorder, yet secretion testing isn’t commonly done
  - Potential implications of NOT evaluating secretion?
    - Diagnostic label issue
    - Reduced detection of some platelet disorders?
- Methods to evaluate secretion
  - Radioactive: e.g. serotonin release
  - Nonradioactive: e.g. luminescence, other assays for nucleotides

Hamilton Platelet Secretion Testing
Second Line Investigation - Luminescence Procedure
- 36% of patients are abnormal - half of these have normal aggregation studies
- lower limit of reference range (determined using 48 controls)
- local testing done with 8 parameters, 6 agonists

Testing Platelet Function in Thrombocytopenic Patients
- 17% of patients tested in Hamilton

Data from 2002

<table>
<thead>
<tr>
<th>Reference Interval for samples with platelet counts of 250 X 10^9/L, % aggregation</th>
<th>Bernard Soulier Syndrome PRP: 29 X 10^9/L (less than 5% GP Ib/IX/V by flow)</th>
<th>Control tested at same platelet counts (PRP: 29 X 10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP 5 uM</td>
<td>00-100</td>
<td>00-100</td>
</tr>
<tr>
<td>Collagen 3 mg/mL</td>
<td>00-180</td>
<td>00-180</td>
</tr>
<tr>
<td>Collagen 1.25 mg/mL</td>
<td>00-120</td>
<td>00-100</td>
</tr>
<tr>
<td>Epinephrine 6 uM</td>
<td>00-120</td>
<td>00-100</td>
</tr>
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<td>Arachidonic Acid 1.6 mM</td>
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</tr>
<tr>
<td>Thromboxane analog 1 uM</td>
<td>00-120</td>
<td>00-100</td>
</tr>
<tr>
<td>Ristocetin 0.5 mg/mL</td>
<td>00-70</td>
<td>00-70</td>
</tr>
<tr>
<td>Ristocetin 1.25 mg/mL</td>
<td>00-70</td>
<td>00-70</td>
</tr>
</tbody>
</table>

Special Diagnostic Evaluations
Illustration of Glycoprotein Analysis for Glanzmann Thrombasthenia
Testing for Rare Disorders - Quebec Platelet Disorder

classic family history, delayed bleeding responsive only to thrombolytic inhibitors, absent epinephrine aggregation, reduced to low normal platelet counts

<table>
<thead>
<tr>
<th>Platelet u-PA Western Blot</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1200</td>
</tr>
<tr>
<td>QPD</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1200</td>
</tr>
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</table>

Clots prepared with 0 or 1200 X 10^9 platelets/L

Mystery Case – VWD screen
55 year old male, severe bleeding after renal biopsy

- First sample (referred in)
  - FVIII: 2.43 U/ml (243 U/dL)
  - VWF:Ag: 1.31 U/ml (131 U/dL)
  - VWF:RCo 0.29 U/ml (29 U/dL)

Interpretative comment: The von Willebrand factor ristocetin cofactor activity is significantly reduced. The discrepancy between this value and the normal VWF:Ag suggests a form of type 2 von Willebrand disease. An analysis of von Willebrand factor multimers would be helpful to further evaluate. Is there a family history of von Willebrand disease or a bleeding history that suggests acquired von Willebrand factor abnormalities?

Further Investigations

<table>
<thead>
<tr>
<th>Sample size</th>
<th>% aggregation</th>
<th>Patient</th>
<th>Control turned same day at same platelet count</th>
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</thead>
<tbody>
<tr>
<td>250 X 10^9 platelets/L</td>
<td>93</td>
<td>93</td>
<td>0</td>
</tr>
<tr>
<td>1000 X 10^9 platelets/L</td>
<td>20</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

Additional Investigations

- VWD screen done on day of aggregation testing
  - FVIII: 1.34 U/ml
  - VWF:Ag: 2.39 U/ml
  - VWF:RCo: 0.29 U/ml (29 U/dL)
  - Multimers Normal

- Further RIPA testing (1.25 mg/mL) done after a 30 minute incubation of patient or control PPP, with control PRP (PPP added to adjust platelets from 440 down to 250 X 10^9/L)
  - Patient Mixture: 2% aggregation
  - Control Mixture: 86% aggregation

Diagnosis? Further tests that you would do?

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