Laboratory Testing for TTP: ADAMTS-13 Activity and Antigen Assays

Marlies Ledford-Kraemer, MBA, BS, MT(ASCP)SH

Topics for Discussion

- Introduction to ADAMTS-13
  - ADAMTS-13 Activity Assays
  - ADAMTS-13 Inhibitory Assays
  - ADAMTS-13 Antigen Assays

Introduction to ADAMTS-13

The 13th member of the ADAMTS (A Disintegrin And Metalloprotease with Thrombospondin type 1 motifs) family of metalloproteases

Previously termed VWF cleaving protease

ADAMTS-13 Protease

- ADAMTS-13 is specific for von Willebrand Factor (VWF) and appears to be resistant to "all" plasma protease inhibitors
- ADAMTS-13 gene is mapped to chromosome 9q34
  - Gene spans 37kb and contains 29 exons
- Precursor polypeptide composed of 1427 amino acid residues
- Synthesized in liver and endothelial cells as a zymogen and becomes activated upon propeptide cleavage
- Highly glycosylated (apparent mass of 180 kDa)
- Plasma concentration is ~ 1 μg/mL
- Plasma half-life is ~ 2-3 days
- Protease activity is dependent on both Zn^{2+} & Ca^{2+} ions

ADAMTS-13 Function

- Cleaves its substrate, von Willebrand Factor (VWF), in the A2 domain at Tyr^{1605}—Met^{1606}
- Responsible for normal proteolysis of VWF multimers
  - On VWF multimer gels, seen as satellite bands (consisting of dimers either with or without N-terminal fragment attached)
  - On denatured gels, seen as 176 kDa & 140 kDa fragments resulting from digestion of 225 kDa protomer
- VWF substrate, in vivo, regulates its own cleavage
  - Shear stress makes VWF A2 domain accessible to protease
- Denaturing agents or low salt concentrations unfold normal VWF in vitro
- Mutations within VWF A2 domain (VWD Type 2A), impair folding of VWF thereby exposing Tyr-Met bond resulting in exaggerated proteolysis

Disclosure

- I have no relevant financial relationship(s) that are in any way associated with the content of this presentation
- I will not be discussing "off label usage" of any pharmacologic agent/product/device
ADAMTS-13 Assays
CLOT-ED 2007

Marlies Ledford-Kraemer
MBA, BS, MT(ASCP)SH

ADAMTS-13 Assays

CLOT-ED 2007

Thrombotic Thrombocytopenic Purpura
TTP is a life-threatening disseminated thrombotic microangiopathy of which the pathologic hallmark is platelet-rich microthrombi in the small vessels of multiple organs

ADAMTS-13 Assays

CLOT-ED 2007

Overview of Assay Types

Gel electrophoresis
- Based on VWF multimer analysis (Furlan, 1997) using SDS-agarose gels & western blotting to measure VWF multimer size (extent of VWF multimer degradation)
- Detection of dimeric VWF fragments on denatured SDS-PAGE gels & visualized by western blotting (Tsai, 1998)

ELISA (enzyme-linked immunosorbent assay)
- Based on collagen binding assay (Gerritsen, 1999)
- Measure residual substrate which is inversely proportional to ADAMTS-13 activity (Whitelock, 2004 & Zhou, 2004)
- Measure cleaved products; amount of color released is directly proportional to ADAMTS-13 activity (Wu, 2006)

Techniques

ELISA
- Measure residual substrate which is inversely proportional to ADAMTS-13 activity (Whitelock, 2004 & Zhou, 2004)

Approximate Multimers

Dimer
Multimer
Very Large Multimers

VWF Proteolytic Fragments

Slower Triplet
2050 + 2050 + 842 = 4942 aa
MW=545 kDa

Intermediate Triplet
2050 + 2050 = 4100 aa and/or
1208 + 2050 + 842 = 4100 aa
MW=450 kDa

Faster Triplet
1208 + 2050 = 3258 aa
MW=360 kDa

ADAMTS-13 and TTP

Congenital TTP (Upshaw-Schülman Syndrome)
- Caused by homozygous or compound heterozygous mutations in the ADAMTS-13 gene
- ADAMTS-13 is non-functional (activity <5-10%) or absent

Acquired idiopathic TTP
- Occurs in patients with no apparent pre-existing or concurrent illness
- Caused by autoantibodies (either inhibitory or non-neutralizing) to ADAMTS-13

Acquired non-idiopathic TTP
- Associated with pregnancy, autoimmune disease, HIV infection, bone marrow stem cell transplantation, cancer, or particular drugs
- Autoantibodies to ADAMTS-13 may or may not be present

Overview of Assay Types

Techniques
- Gel electrophoresis, ELISA, FRET, flow chamber
- Static versus non-static test conditions
  - Static (conducted under motionless conditions)
  - Non-static (conducted under conditions of flow, which potentially mimic high shear environments)
- Substrates used for ADAMTS-13 digestion
- Assessment of ADAMTS-13 digestion
  - Direct assays assess cleavage products of "VWF" substrate
  - Indirect assays measure residual "VWF" substrate

ADAMTS-13 and TTP

- Thrombocytopenia
- Fever
- Renal Dysfunction
- Neurologic Symptoms
- Hemolytic Anemia

ADAMTS-13 Activity Assays

UL MWM
TTP Normal
TTP = Thrombotic Thrombocytopenic Purpura; UL MWM = Unusually Large Molecular Weight (VWF) Multimers

In the high shear stress environment of the microcirculation, presence of UL MWM (due to decrease in ADAMTS-13) leads to platelet clumping

Thrombocytopenia, and ischemic neurological, renal & other organ dysfunction as well as red blood cell fragmentation in partially occluded arteries and capillaries

Overview of Assay Types

Techniques
- Gel electrophoresis
  - Based on VWF multimer analysis (Furlan, 1997) using SDS-agarose gels & western blotting to measure VWF multimer size (extent of VWF multimer degradation)
  - Detection of dimeric VWF fragments on denatured SDS-PAGE gels & visualized by western blotting (Tsai, 1998)
- ELISA (enzyme-linked immunosorbent assay)
  - Based on collagen binding assay (Gerritsen, 1999)
  - Measure residual substrate which is inversely proportional to ADAMTS-13 activity (Whitelock, 2004 & Zhou, 2004)
  - Measure cleaved products; amount of color released is directly proportional to ADAMTS-13 activity (Wu, 2006)
Techniques continued:

- **FRET** (fluorescence [Förster] resonance energy transfer)
  - An energy transfer mechanism between two fluorescent molecules: a fluorescent donor (emitter) and a fluorescent acceptor (absorbs or quenches the signal)
  - If ADAMTS-13 cleaves the Tyr-Met bond, then the excited donor energy, which is transferred when the substrate is intact and quenched by the acceptor, is no longer quenched thereby allowing fluorescent emission by the donor molecule (Kokame, 2005)

- **Flow chamber** (high shear, non-static environments)
  - Parallel plate perfusion system using human umbilical vein endothelial cells (HUVEC)
    - HUVEC release UL VWF multimers that form very long platelet decorated string-like structures; in the presence of ADAMTS-13 these structures disappear rapidly (Dong, 2002)
  - Cone and Plate(let) Analyzer (Shenkman, 2006)
    - Shear stress unfolds VWF allowing for subsequent platelet-platelet interactions and deposition onto a polystyrene surface

Substrates:

- **VWF**
  - Purified, full length VWF (Furlan, 1997), (Tsai, 1998), and (Gerritsen, 1999)
  - Recombinant VWF [rVWF] (Obert, 1999) & (Shenkman, 2006), VWF from high purity VWF concentrate (Bohm, 2002)
  - Fusion Proteins (peptidyld substrates fused with various detectors)
    - rVWF-A2 domain with N-terminal 6xHis-tag (Cruz, 2003)
    - rVWF-A2 domain tagged with N-terminal 6xHis and C-terminal Tag-100 (Whitelock, 2004)
    - Recombinant 73 amino acid fragment of VWF-A2 domain [rVWF73] tagged with N-terminal GST and C-terminal 6xHis (Zhou, 2004), (Kokame, 2004)
    - Fluorogenic peptide, FRETS-VWF73, in which Q1599 is replaced with emitter A2pr Nma and N1610 is replaced with quencher A2pr Dnp (Kokame, 2005)

**Miniaturizing the Substrate**

**Why Miniaturization?**

- Disadvantages of full-length VWF as substrate
  - Requires denaturing agents to unfold VWF in order to expose cleavage sites in VWF-A2 domains
  - Long incubation times required for protease to act upon the substrate
  - Full-length VWF derived from plasma contains ADAMTS-13, which requires removal prior to use
- Advantages of monomeric fragments
  - Contain only one cleavage site
  - No denaturing reagents required since the fragment is not bulky and hence cleavage site is not hidden
  - Greater susceptibility to proteolysis therefore no need for divalent cations, in the assay, to activate ADAMTS-13
  - Provide opportunity to incorporate specific detector tags to enhance visualization/quantification

**Direct Assays**

- Detection of dimeric 176 & 140 kDa fragments on denatured SDS-PAGE gels (Tsai, 1998)
- Quantify colorimetric changes due to appearance of cleavage products (Wu, 2006)
- Quantify fluorometric changes attributable to appearance of cleavage products (Kokame, 2005)

**Indirect Assays**

- Measure change in VWF multimer size (Furlan, 1997)
  - On multimer analysis, disappearance of high molecular weight VWF multimers after digestion with ADAMTS-13
- Low molecular weight VWF multimer forms resulting from digestion show reduced collagen binding activity (Gerritsen, 1999) or ristocetin cofactor activity (Bohm, 2002)
- Residual “VWF” substrate quantified by the binding of tagged monoclonal antibodies to the substrate (Whitelock, 2004 & Zhou, 2004); there is an inverse relationship between ADAMTS-13 activity and colorimetric changes
- Platelet VWF string formation (Dong, 2002) or platelet deposition onto a plate (Shenkman, 2006) is inversely related to ADAMTS-13 activity
ADAMTS-13 Assays
CLOT-ED 2007

Multicenter Studies

- Study in 2002 (Studt, et al) compared 4 assays available in 5 research centers
  - 30 samples distributed with 14 having activity levels of <10% (these were also assessed for inhibitory antibodies)
  - Methods: electrophoresis, indirect assays based on collagen binding or ristocetin cofactor activity, and an immunoradiometric assay measuring residual VWF antigen
    - Collagen binding assay was discrepant with 3 samples
- 2003 study (Tripodi, et al) compared 7 methods from 11 laboratories
  - 60 samples (10 vials each from 6 plasmas)
  - Expected ADAMTS-13 activity: 0%, 10%, 20%, 40%, 80%, & 100%
  - Best concordance between observed and expected levels was seen with assays based on collagen binding and ristocetin cofactor
    - Both methods also had lowest coefficients of variation and best discriminatory power

Commercial Availability

- FRET methodologies
  - Measure change in fluorescence over time, quantify that change against a standard curve, and can be performed in ~1 hour
  - American Diagnostica: Actifluor ADAMTS13™ (RUO)
    - FRET assay using a recombinant VWF86 peptidyl substrate (Q1599C/P1611C) in which the 2 substituted cysteine residues are coupled to Alexa488 fluorochromes (Zhang, et al)
    - Kinetic reading for 10 minutes
  - GTI: ATS-13™ (RUO)
    - Peptidyl substrate is FRETS-VWF73 (Kokame, et al)
    - Non-kinetic reading after 30 minutes
  - Fluorogenic substrate, FRETS-VWF73, can be purchased directly from Peptides International (Kentucky)

Commercial Availability....Continued

- ELISA/FRET assay
  - Technoclone: TECHNOZYM® ADAMTS-13 (RUO)
    - Distributed in US through diaPharma
    - Determines both ADAMTS-13 activity and antigen in the same microtiter plate well
  - Activity assay
    - Wells coated with monoclonal anti-ADAMTS-13 directed against the CUB domain
    - Patient ADAMTS-13 is captured by monoclonal antibody (2 hours)
    - Patient ADAMTS-13 acts on a FRETS-VWF73 fluorogenic substrate (Kokame, et al)
    - Kinetic measurement for 15 minutes
    - Activity interpolated from a standard curve

Quality Concerns

- Hemolyzed specimens cannot be used for testing as hemoglobin concentrations exceeding 2g/L will inhibit ADAMTS-13
- EDTA collection tubes cannot be used because the anticoagulant inhibits ADAMTS-13
- Lower detection limits as some assays (direct or indirect) cannot detect ADAMTS-13 levels below 5-10%
- Reliability of some assays (direct or indirect) cannot detect differences between moderately reduced and normal activity
- Technical expertise required to perform some assays
- Turn-around-times for some assays

Antibodies to ADAMTS-13

- Main epitope for ADAMTS-13 IgG autoantibodies is cysteine-rich / spacer domains
- Acquired idiopathic TTP
  - Patients may have either type of autoantibody or no autoantibody
  - Inhibitory autoantibodies
    - Act by either inhibiting ADAMTS-13 proteolytic activity or accelerating the clearance of ADAMTS-13
      - Very low ADAMTS-13 activity (~5-10%)
    - Anti-ADAMTS-13 IgG found in 97% of untreated patients
      - High titer correlate with more relapses
  - Non-inhibitory autoantibodies
    - Do not neutralize ADAMTS-13 activity in an inhibitory assay
    - Moderate ADAMTS-13 activity and antigen levels may be explained by decrease in ADAMTS-13 clearance
- Acquired non-idiopathic TTP
  - Patients may have non-inhibitory autoantibodies or have no autoantibody
  - Normal to moderately reduced levels of ADAMTS-13 activity/antigen
ADAMTS-13 Assays
CLOT-ED 2007

Assay Types
- Both direct and indirect activity assays can be modified to detect inhibitors of ADAMTS-13 activity
  - Mixing study is used to determine effect that antibody in patient plasma has on ADAMTS-13 and VWF substrate supplied by normal pooled plasma (NPP)
    - If inhibitory activity is present in patient plasma, then residual ADAMTS-13 in NPP is low and can not digest VWF
      - Expected VWF cleavage products are not produced and high molecular weight VWF multimers (active forms) are retained
    - If no antibody is present, then residual ADAMTS-13 activity in NPP is high and digests the VWF substrate
      - Expected loss of high molecular weight multimers occurs with concomitant loss of VWF activity
- ELISA assays to directly quantify the inhibitor

Mixing Study Method
- Patient plasma heated at 56°C for 30-60 minutes to eliminate endogenous ADAMTS-13 activity
  - NPP treated in same manner to serve as control
- Serial dilutions of treated plasma can be made in TBS or PBS buffers, with or without bovine serum albumin
- Heat treated plasma (either neat or serial dilutions as noted above) mixed 1:1 with NPP
- 1:1 mixture(s) incubated at 37°C for 30-120 minutes
  - Time for antibody to act against ADAMTS-13
- Measure residual ADAMTS-13 activity by any direct or indirect assay
  - Direct assays show less end-product
  - Indirect assays detect more residual substrate

Mixing Study Interpretation
- Qualitative
  - General (definite inhibitor, uncertain/low titer inhibitor, or no inhibitor)
  - Percent inhibition
    - Patient sample considered positive for ADAMTS-13 inhibitor if >30% of proteolytic cleavage of NPP substrate is blocked
  - Cut-off percentages should be set by individual laboratories
- Semi-quantitative
  - Residual ADAMTS-13 activity seen in 1:1 patient plasma/NPP mixtures is reported in Bethesda units
  - Calibration curve: Mix NPP with 1:1 heat-inactivated NPP (ADAMTS-13 inactivated) or buffer and incubate at 37°C for 30-120 minutes
  - Dilute mixture serially with buffer
  - 1:1 mixture(s) incubated at 37°C for 30-120 minutes
    - Time for antibody to act against ADAMTS-13
  - Measure residual ADAMTS-13 activity by any direct or indirect assay
    - Direct assays show less end-product
    - Indirect assays detect more residual substrate

Commercial Availability
- IgG autoantibodies to ADAMTS-13 can be measured directly using ELISA assays
  - ELISA plate coated with recombinant human full-length ADAMTS-13
  - Anti-ADAMTS-13 IgG autoantibodies in diluted patient plasma bind to capture antigen
  - Colorimetric detection is by the action of a secondary conjugated anti-human IgG labeled with horseradish peroxidase upon the substrate TMB (perborate-3,3',5,5'-tetramethylbenzidine)
  - A reference curve is based on five dilutions of a plasma with known concentration of anti-ADAMTS-13 IgG
  - Inhibitor levels are reported in anti-ADAMTS-13 units/mL
- American Diagnostica: IMUBIND® ADAMTS13 Autoantibody (RUO)
- Technoclone: TECHNOZYME ADAMTS-13 INH (RUO)
  - Distributed in the US through diaPharma

Quality Concerns
- ELISA inhibitor assays
  - Do not distinguish between inhibitory and non-inhibitory IgG antibodies
  - May lead to over-estimation of anti-ADAMTS-13 antibodies
  - High titers of non-ADAMTS-13 autoantibodies may cause false positives
- Activity-based inhibitor assays
  - Autoantibodies must be inhibitory in order to be detected and titrated by classical mixing studies
  - Non-neutralizing antibodies will not be detected
  - Lower detection limits of various ADAMTS-13 activity assays must be considered as these will impact modified inhibitor assays
  - Shelat, et al noted that a modified activity assay based on FRETS-VWF73 is sensitive and specific for detecting inhibitory autoantibodies to ADAMTS-13
  - Both activity-based and ELISA assays detect only free IgG inhibitors and not those already bound to ADAMTS-13 (antigen/antibody complex)
Overview of Assays

- Quantify level of ADAMTS-13 antigen present in plasma
- For assays using polyclonal antibodies, both free ADAMTS-13 and that complexed to antibody (bound) are detected in acquired TTP
  - Two kits available commercially
  - Rieger, et al developed a polyclonal antibody-based antigen assay and attempted to use ADAMTS-13 activity to antigen ratios to differentiate between various conditions
- Feys, et al developed a monoclonal antibody-based ELISA
  - Autoantibodies did not appear to interfere with the assay

American Diagnostica

- IMUBIND® ADAMTS13 ELISA (RUO)
- Standard for curve is a dilute normal human plasma calibrated against a recombinant ADAMTS-13
- Interpolated patient values are reported in ng/mL
- Quality concerns
  - Platelet contamination interferes with assay
  - Only 2 freeze-thaw cycles can be tolerated

Technoclone (diaPharma)

- TECHNOZYM® ADAMTS-13 (RUO)
  - Test system determines both ADAMTS-13 activity and antigen in the same microtiter plate well
  - After removal (washing step) of substrate used to assess ADAMTS-13 activity, ADAMTS-13 antigen is assayed by standard ELISA technology
  - Use a polyclonal anti-human ADAMTS-13 antibody conjugated with horseradish peroxidase (HRP)
  - HRP acts on a substrate tagged with a fluorophore
  - Fluorescence emission is read at 360/460nm

So What’s the Point?

- Differentiate between hereditary and acquired TTP
- Discriminate between inhibitory and non-inhibitory autoantibodies
- Aid in determining appropriate therapies

ADAMTS-13 Assays

<table>
<thead>
<tr>
<th>Healthy Controls (Reference)</th>
<th>Hereditary TTP</th>
<th>Acquired TTP (Hepatotropic vs Non-hepatotropic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (&gt; 60%)</td>
<td>Positive</td>
<td>(10% - 50%)</td>
</tr>
<tr>
<td>Low to Undetectable</td>
<td>Positive</td>
<td>(10% - 50%)</td>
</tr>
<tr>
<td>Undetectable</td>
<td>Negative</td>
<td>Non-ADAMTS-13 Negative</td>
</tr>
</tbody>
</table>

American Diagnostica

- ADAMTS-13 Antigen (ELISA)
  - Normal (1-10 ng/mL)
  - Low to undetectable
  - Undetectable

References

- References and articles of interest:

American Diagnostica

- Assay Design
  - ADAMTS-13 Activity
  - Detects ADAMTS-13 activity and ADAMTS-13 antigen
  - Using an antibody-based ELISA
  - Normal (> 12 U/mL) Negative Positive Positive Negative
  - Undetectable Negative Positive Negative Negative

IMUBIND® ADAMTS13 ELISA (RUO)

- Assay Design
  - Detects ADAMTS-13 activity and ADAMTS-13 antigen
  - Using an antibody-based ELISA
  - Normal (> 12 U/mL) Negative Positive Positive Negative
  - Undetectable Negative Positive Negative Negative

ADAMTS-13 Antigen (ELISA)

- Normal (1-10 ng/mL)
- Low to undetectable
- Undetectable

References

- References and articles of interest:

Reviews and articles of interest: