Mass spectrometry in glycomics research: Application to IgA nephropathy

Part I

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IgA nephropathy

Immune complex glomerulonephritis

Diagnosis of glomerulonephritis - one of the following:

- History of macroscopic hematuria
- Microscopic hematuria: >5 RBC/high-power field
- Proteinuria: dipstick ≥1+ or UP/Cr ratio ≥0.2

In the absence of menstrual bleeding, known urologic source or nephrolithiasis
IgA nephropathy: Diagnosis

IgA1 mesangial deposits by immunofluorescence

- IgA1 (but not IgA2)
- Mostly polymeric IgA1 (but not secretory IgA1)
- C3 (but not C1q) co-deposits
- Often IgG (IgM) co-deposits
- Expansion of extracellular matrix
- Proliferation of mesangial cells

IgAN: Histology

Expansion of Extracellular Matrix
Proliferation of Mesangial Cells
Prognosis

• Usually slow progression towards glomerular and interstitial sclerosis (no disease-specific treatment of IgAN).
• 30-40% patients develop end-stage renal disease within 20 years.
• Dialysis, transplantation.
• IgN cause is extrarenal:
  – IgAN recurrent >50% after transplantation.
  – IgAN kidney transplanted to non-IgAN recipient cleared IC.

Circulating Immune Complexes (CIC) in IgA nephropathy

• IgA1-containing CIC present in most IgAN patients.
• IgA1-CIC levels correlate with the disease activity.

IgA1 deposits originate from CIC

Immune complex glomerulonephritis (GN)

Initial events in immune deposit formation:
  • deposition of CIC
    - pre-formation of CIC
    - only certain complexes are "nephritogenic".
    - host factors promoting glomerular IC deposition
    - reduced clearance or complement-mediated solubilization
  • in-situ formation
    - Ab recognizes glomerular antigens
    - Ab binds to planted Ag (models vs. naturally-occurring diseases)

Secondary events:
  • formation of aggregates detectable by IF and EM
    (redistribution of IC; addition of Ab, IC, other reactants)
**Human IgA1**

Structure and glycosylation

- Monomeric or polymeric (with J-chain) forms
- O-linked glycans
- N-linked glycans

*(JBC 273, 2260, 1998)*

Monomeric or polymeric (with J-chain) forms

**Hinge regions of human IgA subclasses:**

**IgA1 and IgA2**

- IgA1: hinge sequence similar to mucins, recognized by IgA proteases of bacterial pathogens
- **IgA1**
  - Ca1: Pro-Val-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Thr-Pro-Pro-Thr-Pro-Ser-Pro-Ser-Cys-(CHO)
  - Ca2: Pro-Val-Pro-Pro-Pro-Pro-Cys

- **IgA2m(1)**
  - Ca2: Pro-Val-Pro-Pro-Pro-Pro-Cys

**IgA1 glycosylation in IgAN: Initial analyses**

- Monosaccharide composition: Gas-liquid chromatography
- Terminal saccharides: Lectin ELISA, Western blot
- Gal-deficient O-linked glycans in the hinge-region

- (SA)
  - [2,6]
- GalNAc
  - Ser/Thr
- **HSPN**
- IgAN

- **HSPN**
- **Con**
- **HSP**

Gal-deficient IgA1 forms CIC with IgG
Size-exclusion chromatography of serum

Pooled CIC
Dissociated (pH 3)
Fractionated (HPLC)
pIgA and IgG detected by ELISA

Inhibition of reformation of IgAN IgG-IgA1 CIC

IgA1-CIC isolated
Dissociated (pH 3)
Immobilized inhibitors added
Sample neutralized
After o/n incubation
Free IgG measured

Localization of glycan-dependent antigenic determinants of Gal-deficient IgA1

- Gal-deficient IgA1 is present in sera in IgG-IgA1 immune complexes (IC)
- Free and IC-bound IgG and IgA1 anti-IgA1 antibodies are specific for the hinge region O-linked glycans (cross-reactive antibodies specific for mucosal pathogens or viruses?)
- The antigenic determinant(s) comprises GalNAc and/or GalNAc-α2,6 SA glycans
**In vitro model to study IgA1-CIC biological activity**

- IgA1-CIC fractionated -> added to cultured MC
  - Binding, proliferation, activation markers,…
- **Proteomics** (ID proteins up- or down-regulated or with altered post-translational modifications)
- **High-density DNA arrays** (ID genes up- or down-regulated)

**IgA1 binding to mesangial cells *in vitro***

- Putative receptor (R) binds the Fc portion of IgA1
- Asialo-agalacto-IgA1 > normally glycosylated IgA1
- CIC from IgAN patients >>> asialo-agalacto-IgA1
- CIC from IgAN patients >> CIC from healthy controls
- Binding of CIC inhibited by IgA1 but not by IgG
- Fco R (CD71, Fcα/µ possible candidates but not CD89)

Novak et al., Kidney Int. 2002

**In-vitro assay of biological activity of CIC: proliferation of MC**

- CIC from serum fractionated by size-exclusion chromatography (Superose 6 column, calibrated)
  - Collected CIC \( V_0 \sim 700 \text{ kDa} \)
  - Filter-sterilize and add to quiescent MC
  - Incubate for 20 h
  - Add \(^3\text{H}\)-thymidine for 4 h
  - Harvest, measure \(^3\text{H}\)-thymidine incorporation

- Additional experiments: depletion of IgA, IgG
- Controls: PDGF, negative control (no CIC)

Novak et al., Kidney Int. 2002
IgAN-CIC differentially stimulate or inhibit MC proliferation
Size-exclusion chromatography

- Removal of IgA1-CIC
- Loss of stimulating / inhibiting CIC

Background levels

Convalescent
Gross hematuria

Primary IgAN: IgA1-CIC activate MC

IgA1-supplementation of serum increased stimulating activity

Novak KI. 2005

Novak KI. 2005
Proteome analysis of IgAN-CIC-stimulated MC

Pick a spot on 2-D gel, digest with trypsin
MALDI peptide mass map
Protein identification: vimentin → Q-TOF (AA sequence)

Protein expression in mesangial cells
Western blot
Phosphorylation variants
2-D gel

Protein identification:
vimentin

HSP onset
4 weeks before nephritis manifestation
HSP onset no nephritis developed

Control
V0 700 kDa

MC proliferation after stimulation with CIC from patients with HSP
Pre-existing “nephritogenic” CIC at onset

8
Vimentin over-expressed in IgAN renal biopsies

Normal glomerulus  IgAN glomerulus

Photo: Dr. Lea Novak

Overproduction of extracellular matrix proteins
IgAN-CIC induced laminin expression in MC in vitro

Control CIC  IgAN-CIC  Control CIC  IgAN-CIC

Cell lysates  Medium supernatants

- 216 kDa

IgAN-CIC induce cytokine/chemokine mRNA in MC

<table>
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<tr>
<th></th>
<th>IL-6</th>
<th>IL-8</th>
<th>MCP-1</th>
<th>PDGF B/ PDGF BB</th>
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<tbody>
<tr>
<td>Control  (No CIC added)</td>
<td>+</td>
<td>±</td>
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<tr>
<td>Large CIC (800-900 kDa)</td>
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<td>Small CIC (&lt;800 kDa)</td>
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Elevated IgA-CIC in IgAN patients

Total CIC isolated with IC-specific affinity matrix

Analysis of IgAN CIC by proteomic approaches
**Hypothesis for pathogenesis of IgAN**

**Formation of IgA1-CIC**
- Gal-deficient IgA1 bound by anti-glycan ab (IgG, IgA1)
- Mesangial deposition
- Activation of MC (Proliferation, ECM expansion)

**IgAN is an autoimmune disease**

- **Antigen** - galactose-deficient O-glycan-containing pIgA1  
  possibly induced by mucosal pathogens or their products
- **Antibody** - glycan-specific IgG, IgA1  
  possibly induced by mucosal pathogens bearing O-glycans (viruses, bacteria)

Ratio of Ag:Ab determines **size** (and thus **biological activity**).  
(Serum sickness may be a prototype of this kind of IC-disease)

Mesangial cells have **IgA receptor(s)** bind IgA1-CIC with high affinity  
- differential cellular activation by IgA1-CIC of different sizes

**IgA nephropathy: a disease of abnormal post-translational modification?**

- Abnormal O-glycosylation of IgA1 as etiopathogenic factor in IgAN (Mestecky 1993)
- Gal-deficient IgA1 complexed in CIC with anti-glycan IgG/IgA1 (Tomana 1997, 1999)

- What is the **heterogeneity of O-glycosylation** of IgA1, and what are the **sites of O-glycan attachment**?
- Does Gal deficiency in IgAN occur **randomly or preferentially at specific sites**?
Human IgA1
Structure and glycosylation

O-linked glycans

IgA glycosylation: Analytical approaches

Monosaccharide composition (Gas-liquid chromatography)
Terminal saccharides (Lectin analyses: ELISA, Western blots)
N-linked glycans profile -> Composition & heterogeneity
(N-glycanase release -> MALDI-TOF MS)
-> Localization: predicted (Asn-X-Ser/Thr)
verification (NMR, MS)
O-linked glycans: Monosaccharide composition (GalNAc)
Terminal saccharides (lectin analyses)
Heterogeneity & Localization (NMR, FT-MS?)

O-glycan biosynthesis of circulatory IgA1

Most common forms in normal serum IgA1
Stepwise addition of monosaccharides in ER (GalNAc) and
Golgi (GalNAc, Gal, SA)

SA
α2,3
Gal
β1,3
GalNAc
Ser/Thr

SA
α2,6
GalNAc
Ser/Thr

SA
α2,3
Gal
β1,3
GalNAc
Ser/Thr

SA
α2,6
GalNAc
Ser/Thr
Methods

- Naturally Gal-deficient pIgA1 myeloma protein mimicking IgA1 from IgAN patients (Tomana 1999) analyzed after enzymatic removal of sialic acid
- Isolated trypsin-pepsin-thermolysin fragments
- IgA1 protease-generated fragments (single and double digests: Fc and Fd or released hinge region)
- Analyses: Gas-liquid chromatography
  Mass spectrometry
  Western blots with lectins
IgA1 Mce fragments in SDS PAGE (reducing 4-20% gradient)

1 - no protease
2 - IgA1 protease from C. ramose AK183
3 - IgA1 protease from S. pneumoniae TIGR4
4 - IgA1 protease from N. influenzae
5 - IgA1 protease from N. gonorrhoeae HF13
6 - IgA1 protease from N. gonorrhoeae HF48

Western blots with O-glycan-specific lectins of Gal-deficient IgA1 myeloma protein digested with IgA proteases

1 - no protease
2 - C. ramose
3 - S. pneumoniae
4 - H. influenzae
5 - N. gonorrhoeae HF13
6 - N. gonorrhoeae HF48

Reactivity of IgG antibodies with desialylated IgA1 and its fragments generated by IgA proteases

1 - uncleaved
2 - S. pneumoniae IgA1 protease
3 - N. influenzae IgA1 protease
4 - N. gonorrhoeae HF13 IgA1 protease
5 - N. gonorrhoeae HF48 IgA1 protease
Hinge region can be released using two IgA proteases

MALDI-TOF MS of IgA1 HR glycopeptides (trypsin-pepsin) (multiple O-glycans of variable composition and number)

Summary
- IgA1 HR showed heterogeneity in number and composition of glycans
- Sites of glycan attachment were determined by MS and WB with lectins using proteolytically generated HR fragments.
- Sites that are aberrantly glycosylated may be specific and not random and may thus serve as biomarkers.

Major glycosylation forms:

Asialo- and Sialo- carbohydrates with various oligosaccharides and their structures.
Analysis of glycan attachment sites by mass spectrometry

Example of hinge variant with one glycan:

[peptide sequence]

-Val-Pro-Val-Pro-Pro-Thr-Pro-Pro-Thr-Pro-Pro-Thr-Pro-Pro-

| CHO | CHO | CHO | CHO |

? One specific site of attachment or mixture of variants?

Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry

- Fragmentation of peptide
- Electron capture dissociation (ECD)
- Electron transfer dissociation (ETD)
- Fragmentation of glycosidic bond
- Infrared multiphoton dissociation (IRMPD)