Glycoproteomic analysis of abnormal N-glycosylation on the kappa chain of cryocrystalglobulin in a patient of multiple myeloma

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SUMMARY

Crystalglobulinemia (cryocrystalglobulinemia) is a rare complication of multiple myeloma. Crystallization of immunoglobulin in blood circulation causes systemic vasculopathy especially in skin and kidney. We found a rare case of crystalglobulinemia in which the light chain was N-glycosylated. The abnormal N-glycosylation was primarily detected as the molecular mass shift on SDS-PAGE by PNGase F treatment. The cryocrystalglobulin was shown to be composed of 55-kDa heavy and 32-kDa light chains on SDS-PAGE. However, the apparent molecular masses of them shifted to 51 kDa and 28 kDa, respectively by PNGase-F treatment. The cryocrystalglobulin was identified as an IgG κ type by peptide mass fingerprinting. The N-glycans on the κ light chain were assigned to non-fucosylated biantennary oligosaccharides and their bisected forms by MALDI-TOF MS/MS analysis of glycopeptides. Sialylation of the abnormal N-glycans was suggested by linear-mode MS and confirmed by HPLC analysis. The N-glycosylation consensus Asn (Asn-Xxx-Ser/Thr) was found in the glycopeptide at the N-glycosylation site determined as “EIVMTQSPANLSV-LPGER” by MALDI-TOF MS/MS, in which the consensus Asn (N) was converted to Asp (D) in the enzymatically deglycosylated peptide.

Key words: cryocrystalglobulin, glycoproteomics, immunoglobulin, multiple myeloma, N-glycan.

INTRODUCTION

We found a rare case of multiple myeloma complicated with crystalglobulinemia in which the light chain was N-glycosylated¹. Crystalglobulinemia, characterized by spontaneous crystallization of monoclonal immunoglobulin, has been occasionally observed as a rare complication of multiple myeloma patients²–⁴. In most cases, crystallized immunoglobulin causes vasculopathy especially in skin and kidney¹,⁵,⁶. However, the structural basis underlying the abnormal immunoglobulin crystallization remains to be defined.

The similar protein fibrilization has been observed in AL amyloidosis⁷–¹⁰, in which immunoglobulin light chain (Bence Jones protein) solely forms amyloid fibrils without association of heavy chain. In AL amyloidosis, involvement of glycosylation of immunoglobulin light chain has been suggested. Stevens et al.¹¹,¹² have reported that more than 80% of amyloidogenic κ1 variable domains are identifiable by the presence of substitutions acquiring a consensus Asn for N-glycosylation. Omtvedt et al.¹³ have also independently obtained the data suggesting that there is a

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Abbreviations: DHBA, 2,5-dihydroxybenzoic acid; CHCA, α-cyano-4 hydroxycinnamic acid; DTT, dithiothreitol; TFA, trifluoroacetic acid; GlcNAc, N-acetylgalactosamine; HexNAc, N-acetylhexosamine; PNGase F, peptide N-glycosidase F; PA, pyridyl-2-amine; PMF, peptide mass fingerprinting.
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preponderance of consensus glycosylation sequon in amyloidogenic light chains, to which sialylated and core-fucosylated biantennary oligosaccharides mostly with bisecting GlcNAc were preferentially linked.

Crystalglobulinemia has been thought to be a different syndrome from AL amyloidosis, however we found an interesting case of crystalglobulinemia, in which the κ chain was glycosylated, suggesting a common molecular mechanism underlying both AL amyloidosis and crystalglobulinemia. In this paper, we report the results of our comprehensive glycoproteomic analysis performed for characterizing the abnormal N-glycosylation on the IgG κ chain of cryocrystalglobulin observed in a rare case of multiple myeloma patient.

MATERIALS AND METHODS

Chemicals and equipments
PNGase F (Code No. 4450, EC 3.5.1.52) was purchased from Takara Bio Inc. Sequencing grade modified trypsin was from Promega. SYPRO Ruby Protein Gel Stain was from Invitrogen. Quick CBB Protein Stain Reagent was from Wako Pure Chemicals. DHBA was from Shimadzu GLC Ltd. Hydrazine anhydride was from Tokyo Kasei. CHCA, Trizma Base, tricine, glycine, SDS, Nonidet P-40, DTT and other chemicals were from Sigma. AXIMA-CFR plus and AXIMA-QIT were products of Shimadzu. EXQuest spot cutter and PDQuest image analyzing system were of Bio-Rad Laboratories.

Preparation of cryocrystalglobulin
The myeloma patient with recurrent cutaneous ulcers in fingers of hands and feet was admitted to the clinical department of hematology at the Tokyo Metropolitan Geriatric Hospital. In the procedure of clinical investigation, precipitation of opaque crystals was observed in the serum at room temperature, and the precipitate was resistant to resolubilization. In the procedure of clinical investigation, precipitate of opaque crystals was observed in the serum at room temperature, and the precipitate was resistant to resolubilization. In the procedure of clinical investigation, precipitate of opaque crystals was observed in the serum at room temperature, and the precipitate was resistant to resolubilization.

PNGase F treatment of cryocrystalglobulin
Precipitated cryocrystalglobulin was dissolved in denaturing buffer (0.5 M Tris-HCl, pH 8.6, 0.5% SDS, 0.1 M 2-mercaptoethanol) by incubation at 100°C for 3 min. After chilling to room temperature, a 10-μl aliquot of the denatured glycoprotein preparation was combined with 10 μl of 5% Nonidet P-40, 26 μl of Milli-Q water and 4 μl of 0.5 units/ml PNGase F. The reaction mixture was then incubated at 37°C overnight.

SDS-PAGE and in-gel digestion
SDS-PAGE was performed using 7.5%T, 3%C polyacrylamide gel in the Tris-tricine buffer system. After electrophoresis, protein bands were visualized by CBB staining, excised from the gel using EXQuest spot cutter, and subjected to tryptic digestion in our original protocol shown in our Web site (http://proteome.tmig.or.jp/2D/2DE_method.html). In brief, gel pieces were first incubated with 1.5 mg/ml DTT and then 10 mg/ml iodoacetamide in 50 mM ammonium bicarbonate for 30 min each at room temperature. The CBB dye was completely removed by two-step rinsing in 50% methanol, 50 mM ammonium bicarbonate first, and then in 50% acetonitrile, 50 mM ammonium bicarbonate. The gel pieces were dried, and re-swelled in 25 μl of 50 mM ammonium bicarbonate buffer containing 30% acetonitrile and 5 μg/ml trypsin. In-gel digestion was performed by overnight incubation at 30°C.

MALDI-TOF MS and MS/MS analyses
After overnight incubation, the tryptic digests were combined with matrix solution (10 mg/ml CHCA, 0.1% TFA, 50% acetonitrile, 40% methanol) and applied to MALDI sample plate. MALDI-TOF MS analysis was performed using AXIMA-CFR plus (Shimadzu). The PMF database search for protein identification was carried out using our in-house Mascot Server (Matrix Science). Determination of peptide sequences and glycan structures were performed by MALDI-TOF MS/MS analysis using AXIMA-QIT.

HPLC analysis of PA-glycans
Glycans were isolated from the glycoprotein by in-gel hydrazinolysis, and pyridylaminated according to the procedures previously reported by Tanabe and Ikenaka14). The PA derivatives of glycans were subjected to HPLC on Mono-Q HR5/5 HPLC column (5×50 mm) to separate asialo, monosialo and disialo forms. Further analysis of oligosaccharide isoforms was performed by 2-D HPLC after removing sialic acid according to the procedure previously reported by Fujimoto et al.15).

RESULTS

N-Glycosylation on the light chain of cryocrystalglobulin
N-Glycosylation on the light chain was primarily found as the molecular mass shift on SDS-PAGE by PNGase F treatment. The cryocrystalglobulin before PNGase F treatment was comprised of 55-kDa heavy chain and 32-kDa light chain in the same molar ratio (Fig. 1 (A) lane 2). The apparent molecular masses of them shifted to 51 kDa and 28 kDa, respectively, by PNGase F treatment (Fig. 1 (A) lane 3 and Fig. 1 (B)), indicating both chains had almost similar size of oligosaccharides.

Proteins in bands (a) and (b) were assigned to immunoglobulin γ and κ chains, respectively, by PMF. The identification of the κ chain was confirmed by MS/MS using m/z 1946 precursor ion (data not shown). In an ordinary case of myeloma patient without complication of cryocrystalglobulinemia, the molecular mass shift by PNGase F treatment...
was observed in the heavy chain (bands e and g) but not in the light chain (bands f and h).

Abnormal N-glycan structures on the cryocrystalglobulin light chain

MALDI-TOF MS peaks of the tryptic peptide ions, derived from cryocrystalglobulin light chains before and after deglycosylation (band b and d in Fig. 1), were compared to find out glycopeptides and their deglycosylated form. The glycopeptide ions (m/z 3579.4 and 3782.4), which showed the mass difference of 203 Da corresponding to HexNAc, were detected in the tryptic digests of κ chain before PNGase F treatment (Fig. 2 (A)). The new MS signal corresponding to the deglycosylated form of the glycopeptides was appeared at m/z 1958 by PNGase F treatment.

Oligosaccharide on the glycopeptide at m/z 3579.4 was further analyzed by high-energy CID MALDI-TOF MS/MS. From the product ions of the glycopeptide, the oligosaccharide was assigned to non-fucosylated A2G2-type biantennary glycan (Fig. 3).

Since sialylation of the N-glycan was suggested by MALDI-TOF MS in linear mode, asialo, monosialo and disialo forms of PA-derivatives of isolated glycans were primarily subjected to separation by HPLC on a Mono-Q HR5/5 column. Further analysis of PA-glycan structures in these fractions was performed by 2-D HPLC after removing sialic acids by neuraminidase treatment. The results of the HPLC analysis of glycans isolated from the cryocrystalglobulin are summarized in Table 1.

N-Glycans on the γ chain of normal human IgG are known to be mostly asialo forms, however, nearly half of the oli-

Table 1. Sialylated oligosaccharides on the cryocrystalglobulin.

<table>
<thead>
<tr>
<th>Oligosaccharides</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disialo forms</td>
<td>27.4</td>
</tr>
<tr>
<td>Disialo-A2G2</td>
<td>18.2</td>
</tr>
<tr>
<td>Disialo-A2G2B</td>
<td>7.2</td>
</tr>
<tr>
<td>Disialo-A2G2F</td>
<td>2.0</td>
</tr>
<tr>
<td>Monosialo forms</td>
<td>16.9</td>
</tr>
<tr>
<td>Monosialo-A2G2</td>
<td>10.2</td>
</tr>
<tr>
<td>Monosialo-A2G2B</td>
<td>6.1</td>
</tr>
<tr>
<td>Monosialo-A2G2F</td>
<td>0.6</td>
</tr>
<tr>
<td>Asialo forms</td>
<td>37.8</td>
</tr>
<tr>
<td>Asialo-A2G2</td>
<td>13.6</td>
</tr>
<tr>
<td>Asialo-A2G2B</td>
<td>4.3</td>
</tr>
<tr>
<td>Asialo-A2G2F</td>
<td>0.5</td>
</tr>
<tr>
<td>Asialo-A2G1(6)F</td>
<td>15.5</td>
</tr>
<tr>
<td>Asialo-A2G1(3)F</td>
<td>3.9</td>
</tr>
<tr>
<td>Other types (not assigned)</td>
<td>17.9</td>
</tr>
</tbody>
</table>

○: mannose, ●: galactose, ■: N-acetylglucosamine, ◆: sialic acid, □: fucose

Fig. 1. (A) SDS-PAGE analysis of aberrant N-glycosylation on the light chain of cryocrystalglobulin.

Lane 1: marker proteins. Lane 2: cryocrystalglobulin. Lane 3: the cryocrystalglobulin after PNGase F treatment. Lane 4: control monoclonal IgG of another myeloma patient who had no complication of crystalglobulinemia. Lane 5: control monoclonal IgG after PNGase F treatment. (B) Calibration of molecular mass shift by PNGase F treatment. Bands (a), (c), (e) and (g) were assigned to immunoglobulin γ chain. Bands (b), (d), (f) and (h) were identified as κ chain.
gosaccharides on the cryocrystalglobulin were sialylated. Furthermore, the main frame of oligosaccharide on the cryocrystalglobulin was quite different from the ordinary core-fucosylated bi-antennary structure observed in normal IgG \( \gamma \) chain. The cryocrystalglobulin preferentially conjugated non-fucosylated bi-antennary oligosaccharides and those with bisecting GlcNAc.

Further abnormality of oligosaccharides on the cryocrystalglobulin \( \kappa \) and \( \gamma \) chains was detected by reversed-phase 2-D HPLC after desialylation. The results are summarized in Table 2. The data indicated that the abnormality in oligosaccharide structure on the \( \kappa \) chain was much more significant than that on the \( \gamma \) chain, though the oligosaccharide on the \( \gamma \) chain of cryocrystalglobulin was yet different from that on normal human IgG.

**N-Glycosylation consensus Asn in variable region of \( \kappa \) light chain of cryocrystalglobulin**

The peptide sequence of N-glycosylation site in the cryocrystalglobulin \( \kappa \) light chain was determined by MALDI-TOF MS/MS analysis using the enzymatically deglycosylated peptide (m/z 1958.0 in Fig. 2 (B)) as precursor ion. The \( \gamma \) series of product ions (\( y_5 \) to \( y_{17} \)) were assigned to the peptide sequence “EIVMTQSPADLSV” in which the methionine (M) was oxidized (Fig. 4 (A)). The product ion \( y_8 \) (m/z 870.5) was subjected to MS/MS for further fragmentation. The contiguous sequence “LPGER” was confirmed by the MS/MS analysis (Fig. 4 (B)).
Fig. 4. Mass spectrometric determination of sequence of deglycosylated peptides.

(A) MALDI-TOF MS/MS spectrum of the precursor ion (at m/z 1958 in Fig. 2). The peptide sequence from y₅ to y₁₇ was assigned to EIV(oxM)TQSPADLSV. The intense signal detected at m/z 870.5 was then subjected to MS³ analysis for further fragmentation. (B) MALDI-TOF MS/MS spectrum of the precursor ion gated at m/z 870.5. The sequence “LSVLPGER” was confirmed with the spectrum.

Table 2. Difference in fucosylation of oligosaccharides on the cryocrystalglobulin κ and γ chains from normal human IgG γ chain.

<table>
<thead>
<tr>
<th>Oligosaccharide structures¹)</th>
<th>Relative abundance (%) on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cryocrystalglobulin κ chain</td>
</tr>
<tr>
<td>Fucosylated forms</td>
<td></td>
</tr>
<tr>
<td>A2G2F</td>
<td>3.3</td>
</tr>
<tr>
<td>A2G1(6)F</td>
<td>0.4</td>
</tr>
<tr>
<td>A2G1(3)F</td>
<td>0.6</td>
</tr>
<tr>
<td>A2G0F</td>
<td>0.7</td>
</tr>
<tr>
<td>Unfucosylated forms</td>
<td></td>
</tr>
<tr>
<td>A2G2B</td>
<td>50.4</td>
</tr>
<tr>
<td>A2G2</td>
<td>24.5</td>
</tr>
<tr>
<td>A2G1(3+6)</td>
<td>4.7</td>
</tr>
<tr>
<td>Others (not assigned)</td>
<td>46.3</td>
</tr>
</tbody>
</table>

Ο: mannose, ●: galactose, ■: N-acetylglucosamine, □: fucose
¹) The chromatographic determination of structural diversity in N-linked glycan was carried out after removing sialic acid by neuraminidase treatment.
²) The commercial sample purchased from Sigma.
Specifically existing in the tryptic digests of the sylated peptide ions were detected as unique MS signals by PNGase F treatment. The cryocrystalglobulin was identified as IgG chain and its light chain was primarily comprised non-fucosylated bi-antennary oligosaccharides with/without bisecting GlcNAc.

The abnormal N-glycosylation on the light chain of the cryocrystalglobulin was determined by anion exchange HPLC. N-Glycans on the \( \gamma \) chain of normal human IgG are mostly asialo forms, however, nearly half of the oligosaccharides on the cryocrystalglobulin were sialylated. The further abnormality in the backbone structures of PA derivatives of isolated oligosaccharides were sialylated.

The peptide sequence of the N-glycosylation site before glycosylation was determined as “EIVMTQSPANLSVLPGER” by MALDI-TOF MS/MS analysis. The results of our comprehensive glycoproteome analysis indicated that the abnormal N-glycosylation was occurred at the consensus Asn acquired in variable region of \( \kappa \) light chain of monoclonal IgG, and might be involved in complication of crystalglobulinemia of the rare case of multiple myeloma patient.

**ACKNOWLEDGMENTS**

We are grateful to Dr. Tamao Endo in TMIG for his helpful advice on our glycoproteomic analysis. We also thank Mr. Koich Tanaka in Shimadzu Corporation for his helpful suggestions on MALDI-TOF MS/MS analysis of glycopeptides.

**REFERENCES**


