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Detection of altered *N*-glycan profiles in whole serum from rheumatoid arthritis patients

Hiroaki Nakagawa [a,*], Megumi Hato [a], Yasuhiro Takegawa [a], Kisaburo Deguchi [a], Hiroki Ito [b], Masahiko Takahata [c], Norimasa Iwasaki [c], Akio Minami [c], Shin-Ichiro Nishimura [a,*]

[a] *Graduate School of Advanced Life Science, Frontier Research Center for Post-Genomic Science and Technology, Hokkaido University, W11 N21, Sapporo 001-0021, Japan*
[b] *Hitachi High-Technologies Company, Hitachinaka 312-8504, Japan*
[c] *Department of Orthopaedic Surgery, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan*

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Abstract

Altered *N*-glycosylation occurs in many diseases. In rheumatoid arthritis (RA), for example, reduction in galactose residues in IgG and an increase in fucose residues in α1-acid glycoprotein have been observed. To further analyse *N*-glycans in disease, we show *N*-glycan profiling from whole serum employing reversed phase high performance liquid chromatography/negative-ion mode by sonic spray ionization ion trap mass spectrometry with pyridylamination. Profiles from female 15 RA patients and 18 aged-matched healthy women were compared. The most significant change seen in RA was decreased levels of mono-galactosyl bi-antennary *N*-glycans, in agreement with the previous reports regarding IgG. We also show previously unreported differences between isomers and increased tri-antennary oligosaccharides. These results indicate that LC–MS analysis of whole serum *N*-glycans can identify *N*-glycan alterations in RA and that this is a promising method both for studies of RA mechanisms and diagnosis.
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Keywords: *N*-glycan; Serum; Rheumatoid arthritis; LC–MS

1. Introduction

N-Glycans are attached to nascent polypeptides in the endoplasmic reticulum and modified in the Golgi apparatus by numerous glycosidases and glycosyltransferases. *N*-glycans function as tags for protein localization and clearance, and their profiles, structures, and ratios are affected by several physiological conditions.

One particularly dramatic oligosaccharide change associated with disease is reduced galactose in IgG *N*-glycans seen in patients with rheumatoid arthritis (RA) [1]. Fucosylation of IgG [2] and *N*-glycan microheterogeneities in α1-acid glycoproteins (AGP) [3,4], transferrin [5], haptoglobin [6], α2-macrogloblin [7], and other plasma proteins [8] have also been reported in RA, although how alterations are associated with symptoms is not known [9]. Several methods have been applied to the analysis of IgG *N*-glycans, such as reversed phase-high performance liquid chromatography (RP-HPLC), liquid chromatography–mass spectrometry (LC–MS), lectin analysis, and high pH anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD) [10].

Here we compared profiles of whole serum *N*-glycans from RA patients and healthy women. RP-HPLC using fluorescence-tagged *N*-glycans is a useful method to detect alteration of core-fucosylated bi-antennary oligosaccharides including isomers in IgG. However, tri-antennary *N*-glycans which bind other serum glycoproteins overlap with core-fucosyl and mono-galactosyl bi-antennary glycans in RP-HPLC analysis [11]. Analysis of *N*-glycans by mass spectrometry (MS) is often complicated by low quantitative reliability. Sonic spray ionization ion trap mass spectrometry (SSI-IT/MS) ionizes mildly with nitrogen gas flow [12] and is applicable to quantitative analysis of neutral and sialic oligosaccharides in the negative ion mode [13,14]. Thus we

* Corresponding authors. Tel.: +81 11 706 9031; fax: +81 706 9032.
E-mail addresses: nakagawa@glyco.sci.hokudai.ac.jp (H. Nakagawa), shin@glyco.sci.hokudai.ac.jp (S.-I. Nishimura).

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combined SSI-IT and MS in order to compare *N*-glycan profiles from whole sera of healthy women and female RA patients.

2. Experimental

2.1. Chemicals

Trypsin and chymotrypsin were purchased from Sigma (Sigma–Aldrich, St Louis, MO). Peptide-*N*-glycosidase F (PNGase F, recombinant) was purchased from Roche Diagnosis (Basel, Switzerland). The following materials were purchased from the sources indicated: Pronase, Calbiochem (Merck KGaA, Darmstadt, Germany); Sephadex G-15, Amersham Biosciences (Piscataway, NJ); Bio-Gel P-4 (200–400 mesh), Bio-Rad (Hercules, CA); sodium cyanoborohydride, Sigma–Aldrich (St Louis, MO); and 2-aminopyridine, Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of serum N-glycans

Human serum samples were obtained from the Hospital of Hokkaido University with written informed consent of all subjects following ethical standards of Hokkaido University. Groups of 15 female RA patients and 18 female age-matched healthy controls were median aged 55 and 53 years, ranging from 50–58 and 45–64 years, respectively. One patient was stage 3 and the rest were stage 4, according to staging of Steinbrocker et al. [15].

Sera were treated as described [11,16] with minor modifications. Each 50 μl aliquot of sera was heated to 90 °C for 15 min and incubated with 35 μg each of trypsin and chymotrypsin for 37 °C for 16 h. Proteases were inactivated by heating, *N*-glycans were released by PNGase F, and peptides were then digested by pronase. *N*-Glycans were purified by gel-filtration using Bio-Gel P-4 (1 cm × 38 cm, H_2O) and tagged fluorescently by pyridylamination (PA) [17,18]. PA-glycans were purified by another gel filtration on a Sephadex G-15 column with 10 mM ammonium bicarbonate. To release sialic acid, collected PA-glycan fractions were heated to 90 °C for 1 h at pH 2.0 with HCl. Neutral PA-glycans were further purified using HPLC (Hitachi L-7000 HPLC system, Hitachi High-Technologies Co., Tokyo, Japan) on an amide column (TSKgel Amide-80, 4.6 × 250 mm, TOSOH, Tokyo, Japan) at a flow rate of 1.0 ml per minute at 40 °C with solvent A (3% (v/v) acetic acid - triethylamine (pH 7.3)/acetonitrile 35:65) and solvent B (3% (v/v) acetic acid - triethylamine (pH 7.3)/acetonitrile 65:35). The column was initially equilibrated with solvent A only, and the elution was changed 7 min after injection to solvent B only. The PA-oligosaccharide mixture eluted as one peak was detected by fluorescence (excitation and emission wavelengths of 320 nm and 400 nm, respectively). Separated *N*-glycans were then analyzed by LC–MS.

2.3. RP-HPLC/SSI-IT/MS analysis

Oligosaccharides were separated and their mass chromatograms monitored using the L-7000 HPLC system combined with an M-8000 3DQ ion trap-equipped SSI interface (Hitachi High-Technologies). Separations were on a reversed phase column, Develosil C30-UG (2 mm × 150 mm, Nomura Chem., Seto, Japan), at 40 °C and in buffer containing 0.2 ml/min of 1 mM ammonium acetate, pH 4.3, with a linear gradient of acetonitrile (from 1.3% at 0 min to 6.0% at 70 min). MS conditions were as follows: desolvation temperature, 260 °C; nitrogen gas pressure, 400 kPa; capillary voltage, 0 V; drift voltage, 80 V; scan range, *m/z* 200–2000; and scan time, 500 ms.

Data are presented as means ± SD. Statistical significance was determined using the two-tailed Student's *t* test.

3. Results

Chromatograms of serum *N*-glycans were monitored by fluorescence and base ion chromatography by mass spectrometry (Fig. 1). *N*-Glycan structures and linkages are shown in Fig. 1(C). The oligosaccharide numbering system is according to Takahashi et al. [16], which is a system suitable to distinguish isomers. As an example of numbering, numbers in the range of one hundred indicate the number of branches, the second number indicates the presence of core fucose, and the number before the dot bisecting GlcNAc and

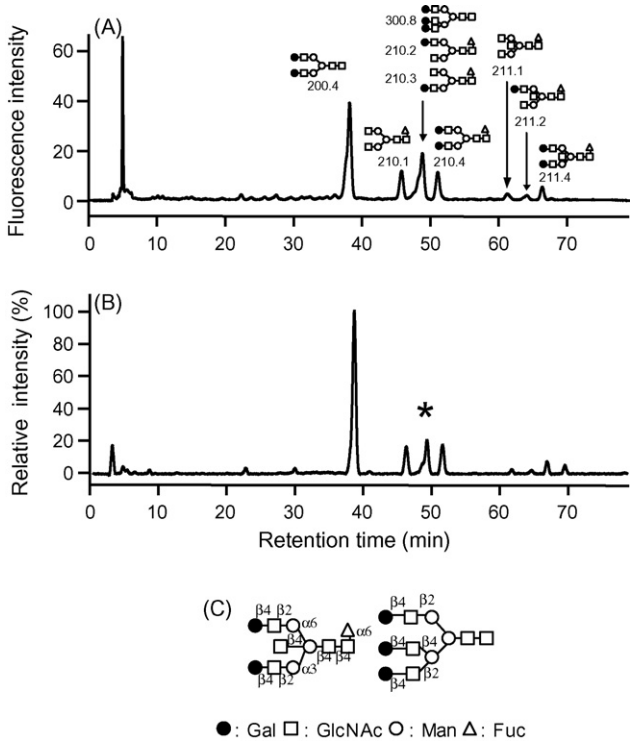

Fig. 1. Chromatograms of serum *N*-glycans on a reversed phase column using HPLC. A, fluorescence detection: Ex = 320 nm and Em = 400 nm. Elution conditions were as follows: column, Develosil C30-UG 2 × 150 mm; temperature, 40 °C; solvent, 1 mM ammonium acetate pH4.3 with a linear gradient of acetonitrile 1.3 to 6.0% in 70 min. B, base ion chromatogram of *m/z* 200–2000 in negative mode by SSI-IT/MS. MS is connected after the fluorescence detector. The asterisk indicates a peak including three major oligosaccharides: 210.2, 200.3, and 300.8. C, *N*-glycan linkages shown in Fig. 1A. Details of this numbering system and list are presented in reference [16] and at http://www.glycoanalysis.info/index.html.

Table 1
Oligosaccharides with mass numbers and ion types analyzed in this study

Oligosaccharide	*m/z*	Ion type	Elution time (min)
200.4	859	$[M-2H]^{2-}$	36.8–39.2
	878	$[M-H+Cl]^{2-}$	36.8–39.3
	1718	$[M-H]^{-}$	37.1–39.2
210.1	770	$[M-2H]^{2-}$	45.2–47.0
	1540	$[M-H]^{-}$	45.1–47.1
210.2	851	$[M-2H]^{2-}$	47.4–48.7
	870	$[M-H+Cl]^{2-}$	47.6–48.7
	1702	$[M-H]^{-}$	47.5–48.7
210.3	851	$[M-2H]^{2-}$	48.7–50.0
	870	$[M-H+Cl]^{2-}$	48.7–50.1
	1702	$[M-H]^{-}$	48.7–49.9
300.8	1042	$[M-2H]^{2-}$	47.7–49.8
	1061	$[M-H+Cl]^{2-}$	47.7–49.9
210.4	932	$[M-2H]^{2-}$	50.4–52.2
	951	$[M-H+Cl]^{2-}$	50.4–52.5
	1864	$[M-H]^{-}$	50.4–51.9
211.1	871	$[M-2H]^{2-}$	60.4–62.8
	1743	$[M-H]^{-}$	60.6–62.2
211.2	952	$[M-2H]^{2-}$	63.0–65.7
211.4	1034	$[M-2H]^{2-}$	65.6–67.6
	1052	$[M-H+Cl]^{2-}$	65.5–67.8

For example, 300.8 was detected at *m/z*1042 and corresponds to $[M-2H]^{2-}$ ion, and 1060 corresponds to $[H-H+Cl]^{2-}$ ions. The mass chromatogram of 1042 (±1) such as that seen in Fig. 2B-2 was recognized between 47.7–49.8 min.

to the right of the period represents a serial number. A list of numbers and structures is presented in reference [16] and on the website http://www.glycoanalysis.info/index.html. Each oligosaccharide was detected with several ions; *N*-glycans identified and types of ions are shown in Table 1. Amounts of *N*-glycans were calculated based on areas of their mass chromatograms. Three *N*-glycans overlapped at the point marked by an asterisk in Fig. 1 [13]. Mass spectra of this peak included the following signals: 851, 870 and 1702 were from 210.2 or 210.3, which are isomers of fucosylated mono-galactosyl bi-antennary oligosaccharides, and 1042 and 1061 were from the tri-antennary oligosaccharide 300.8 (Fig. 2A). These *N*-glycans could not be distinguished by fluorescence analysis. On the other hand, mass chromatograms separated these species, and areas of these *N*-glycans were determined quantitatively (Fig. 2B). In Fig. 2B-1, the mass chromatogram of 851 *m/z* showed separation of isomers 210.2 and 210.3 on RP-HPLC, and only 300.8 appeared in mass chromatograms of 1042 (Fig. 2B-2).

A comparison of RA patients and healthy women is shown in Fig. 3. A previous study showed that the mass chromatogram area was correlated with the chromatography area by fluorescence [14]. The ratio of a specific oligosaccharide is calculated on the mass chromatogram from the area of the peak of that glycan relative to the total area of 9 glycans, which is defined as 100%. In RA patients, 210.3, an isomer of fucosylated mono-galactosyl bi-antennary oligosaccharide, was significantly decreased ($P<0.001$), as was the 210.2 isomer ($P<0.01$). On the other hand, the tri-antennary *N*-glycan 300.8 ($P<0.01$) was increased in RA relative to control subjects.

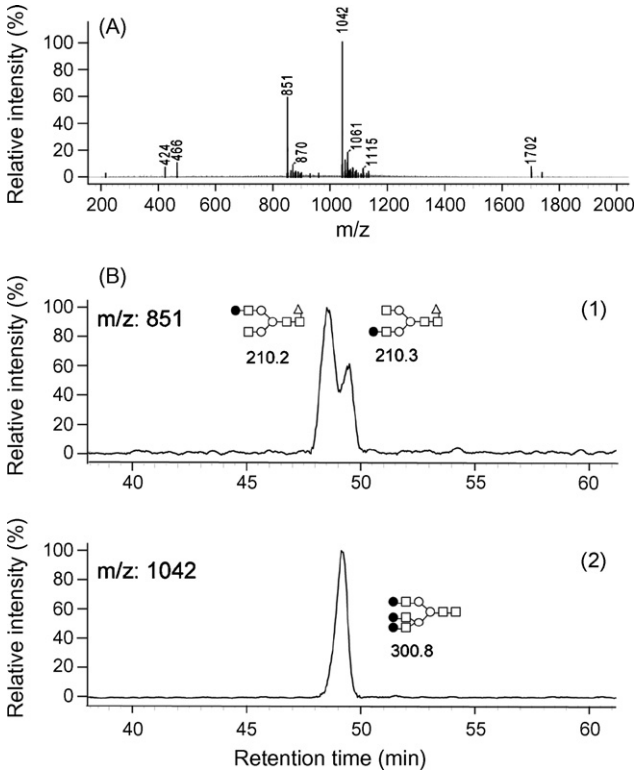

Fig. 2. Analysis of the peak marked by an asterisk in Fig. 1B using mass spectrometry. A, mass spectrum of the peaks. Signals *m/z* 1042 and 1061 were from 300.8, and 851, 870 and 1702 were ions of 210.2 and 210.3. B-1; separated isomers 210.2 and 210.3 detected by mass chromatogram of *m/z* 851 ±1 correspond to $[M-2H]^{2-}$ ions of these isomers. B-2; Mass chromatogram of *m/z* = 1042 ±1 corresponding to the $[M-2H]^{2-}$ ion of tri-antennary oligosaccharide, 300.8.

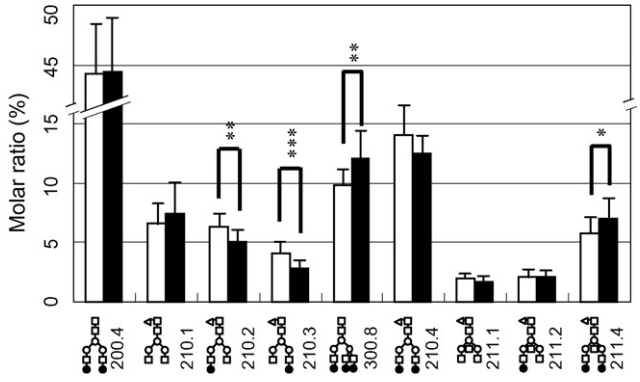

Fig. 3. Comparison of oligosaccharide ratios between 15 RA patients and 18 age-matched healthy women. A previous study showed that the mass chromatogram area was correlated with the chromatography area by fluorescence [14]. The ratio of a specific oligosaccharide is calculated on the mass chromatogram from the area of the peak of that glycan relative to the total area of 9 glycans, which is defined as 100%. Error bars show SD. Asterisk shows statistical significance using two-tailed Student's *t* test; $*P<0.05$; $**P<0.01$ and $***P<0.001$. *P* values of 210.2, 210.3, 300.8 and 211.4 are 0.00599, 0.00019, 0.00493 and 0.04637 respectively.

4. Discussion

Twenty years have passed since the finding that IgG *N*-glycans are altered in RA. Such *N*-glycan alterations have also been reported also other serum proteins; thus alterations in serum

N-glycans in RA are anticipated but have not yet been reported because of technical difficulties. Here, we undertook glycomic analysis of whole sera using RP-HPLC/SSI-IT MS and succeeded in showing such alterations in RA patients.

In previous comparisons of serum *N*-glycans from RA to age-matched healthy women, galactose residues were reportedly decreased [1,10]. Here we report two new findings. First, decreases in the mono-galactosyl oligosaccharide isomer 210.3 were more significant than those of another isomer, 210.2. These isomers differ in a branch that binds galactose, and it has been suggested that their ratio reflects a difference between IgG subclasses [19,20]. IgG subclasses have different properties and their activities depend on the type of antigen [21]. Thus oligosaccharide alterations associated with IgG subclass may be critical.

Another altered tri-antennary oligosaccharide may be derived from AGP, a major serum glycoprotein. AGP increases in RA, and alteration in its glycans detected by lectin binding has been reported [3]. Another major glycoprotein, transferrin, accounts for a small proportion of the tri-antennary oligosaccharides found in healthy adults [22], and IgG and haptoglobin reportedly exhibit bi-antennary [23,24] rather than tri-antennary oligosaccharides. However, alteration in AGP *N*-glycans in RA is reported to involve only fucosylation, and proteomic analysis of serum in RA using 2-D PAGE and lectins shows changes in IgG, haptoglobin, and two unknown glycoproteins, but not in AGP [8]. Thus, the observed increase in tri-antennary oligosaccharides may occur in glycoproteins other than AGP, supporting the importance of analyzing whole serum *N*-glycans rather than just oligosaccharides on purified glycoproteins. Further analysis, such as quantitative glyco-proteomics analysis, is required. Another reported alteration in RA, an increase in fucosylated tri-antennary species, was not analyzed in this study, possibly because it was a minor peak [11]. In Fig. 2A, the *m*/*z* 1115 signal coincides with fucosylated tri-antennary oligosaccharide, but signals from some samples were too weak to be detected in mass chromatograms. Following improvements in sensitivity or resolution, the method utilized here is applicable to other disease or oligosaccharide alterations. SSI-IT/MS can also be used to analyze sialyl oligosaccharides and distinguish isomers using MS^n [12,13]; thus a combination of SSI-IT/MS and RP-HPLC should be widely applicable to *N*-glycan analysis.

Weak acid lysis for sialic acids were used in this and the previous studies [19,20,23]. Conditions are different with each reference, because the result may differ by tube material and size, sample volume and heating unit using which affect heating. We confirm the conditions, at 90 °C, pH 2.0 with HCl, for 60 min using our tube and heater.

In conclusion, glycomics of whole serum using RP-HPLC/SSI-IT MS showed decreased mono-galactosyl bi-antennary oligosaccharides in RA patients, as reported previously. We also report two new findings: a difference in mono-galactosyl oligosaccharide isomers in RA patients and increased levels of tri-antennary *N*-glycans. Glycomics analyses can provide valuable information relevant to disease and should aid the progress of the research about disease mechanisms. Changes in IgG glycan profiles are useful for early stage diagnosis of RA [25]. This method does not require IgG purification and can provide information about other serum proteins. It also could facilitate diagnosis, because early stage diagnosis, which is not yet achievable, is critical for treatment [26,27]. Future studies should focus on well designed, large scale groups including subjects of various ages, sex, disease progression, and values of serum proteins including IgG subclass.

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