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Short communication

Altered glycosylation and expression of plasma alpha-1-acid glycoprotein and haptoglobin in rheumatoid arthritis

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ABSTRACT

Altered glycosylation patterns in plasma proteins are found to be associated with the pathogenesis of various malignancies and autoimmune disorders. Our previous studies demonstrated the occurrence of some differentially glycosylated plasma proteins in rheumatoid arthritis (RA) patients. The current study was conducted to evaluate the alterations in expression and glycosylation of major acute phase proteins from wheat germ agglutinin enriched RA patients' plasma. Immunoblotting studies revealed a significant enhancement in the plasma levels of alpha-1 acid glycoprotein (AGP) and haptoglobin (Hp) in RA patients with respect to healthy controls. Monosaccharide analysis by high performance anion exchange-chromatography with pulse amperometric detection showed significant variations in the relative percentage of galactose, glucosamine and mannose in AGP and of mannose in Hp in RA patients. Altered patterns of mannosylation in AGP and Hp were also established by enzyme linked immunosorbent assay and Western blotting using Concanavalin-A lectin. These results could give information for understanding the disease pathogenesis and may provide an insight into the development and progression of the disease.

1. Introduction

Glycosylation is the most important event of post translation modification observed in proteins. The clinical relevance of glycosylation in various physiological processes and diseases has been understood by sugar profiling. Aberrant glycosylation patterns have been recognised and well studied in several chronic diseases [1–6]. Advancements of glycan analysis tools have helped in deciphering the sugar code which is proved to be indispensible in identifying the disease mechanism as well as for the development of novel diagnostic or prognostic markers [7,8]. Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease characterized by inflammation of the synovial membrane of diarthrodial joints which causes swelling and pain of the proximal inter-phalangeal joints [9–11]. Alteration in glycosylation of serum IgG has been widely studied and is directly implicated in the pathogenesis of RA [12]. The concentration of agalactosylated IgG (IgG0) has been found to be increased in the sera of RA patients. The level of IgG0 correlates with the disease severity in patients of RA thus showing its role in disease pathogenesis [13]. Due to increased level of IgG0 the characteristic auto-antibody against the Fc region of IgG0 known as rheumatoid factor, is found to be increased in the sera of patients with RA [14].

Blood plasma is an imperative specimen to study disease pathogenesis and its progression. During inflammation and other related disorders aberrant glycosylation patterns are observed in several acute phase plasma proteins besides their alteration in expressions. The elevated level of alpha1, 3-fucosylation in tri- and tetra-antennary glycans in AGP in chronic inflammation, while increase in bi-antennary and reduction in tri- and tetra-antennary glycans in AGP in acute inflammation are reported [15]. There are also reports on increased fucosylation of glycans in Hp in RA [16] and prostate cancers [17].

This study demonstrates the potential of lectin affinity based methods in combination with other chromatographic techniques to identify differential expression and glycosylation patterns of plasma glycoproteins in RA. Plasma glycoproteins enrichment by WGA (Wheat germ agglutinin) lectin followed by 2DE (two dimensional gel electrophoresis), resulted in the identification of acute phase glycoproteins. Monosaccharide analysis of AGP and Hp using high performance anion exchange-chromatography with pulse amperometric detection (HPAEC-PAD) suggested their differential composition in RA as compared to control. These alterations were further confirmed by Western blotting and lectin ELISA (enzyme linked immunosorbent assay).

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2. Materials and methods

2.1. Sample collection and enrichment of plasma glycoproteins

Blood samples from 30 patients of average age 35 ± 6 years fulfilling the criteria of American College of Rheumatology for RA, were collected from the Department of Rheumatology, Army Hospital, Research and Referral, New Delhi, India. Blood samples from 30 healthy individuals of average age 30 ± 5 years served as controls. The sex and ethnicity were matched and informed consent was obtained from each patient and healthy individual. The institute and hospital ethical committees approved the study. Blood samples were centrifuged at 2000 rpm for 15 min at 4 °C to collect the plasma. The plasma samples were aliquoted and stored at −70 °C. For glycoprotein enrichment pooled plasma (500 μl) from five patients and five healthy individuals (separately) were diluted with TBS pH 7.2 (10 mM Tris containing 0.9% NaCl) and loaded on spin column (Sigma Chemicals) containing 500 μl of WGA-agarose beads (Vector Laboratories, USA) and were equilibrated with TBS. After incubating for 1 h at room temperature, unbound proteins were removed by washing with TBS till the O.D. value attended 0.001 at 280 nm. The bound proteins were eluted with the elution buffer (TBS containing 0.5 M GlcNAc). Eluted proteins were dialyzed with TBS (10 times diluted) and concentrated using 5 kDa protein concentrator (Amicon Inc., USA).The protein was estimated by Bradford method [18].

2.2. SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and 2-DE of WGA bound plasma proteins

Twenty microgram of protein from both control and RA patients was subjected to 12% SDS-PAGE. For 2DE, 300 μg of protein was used for isoelectric focusing (IEF) and second dimensional separation. The gels were stained by mass spectrometry compatible silver staining [19] and scanned as 16 bit grey scale tif-images with Alpha Digidoc 1201 scanner (Alpha Innotech Corp., USA) and analyzed for differential expression.

2.3. Separation of Hp and AGP from 2DE gel, MALDI-TOF MS (matrix assisted laser desorption ionization time of flight mass spectrometry) analysis and data base search

2D gels were compared with the standard plasma 2D gels available at Swiss-prot database (www.expasy.ch). Hp and AGP spots were cut from the 2D gels and were subjected to MALDI-TOF MS analysis [4].

2.4. Validation of MALDI-TOF MS data

The spots identified by MALDI-TOF were further validated by 2D immuno-Western blotting using HRP (horseradish peroxidase) labeled anti-AGP and anti-Hp [20]. Protein bands were visualized by ECL Western blotting detection system (Biological Industries, Israel).

2.5. In gel deglycosylation and HPAEC-PAD analysis

Haptogloin and AGP spots from 2D gels were deglycosylated using PNGase F (peptide-N-Glycosidase F, Roche diagnostics,) and released oligosaccharides were hydrolysed using 4 M HCl. Quantitative and qualitative estimation of monosaccharides was done by HPAEC-PAD [3]. Relative percentage of each sugar was calculated.

2.6. Lectin ELISA and statistical analysis

The binding of Con-A with AGP and Hp was studied using lectin ELISA [2]. Statistical analysis was made by two tail *t*-test and Mann–Whitney test. Results were expressed as mean ± S.D. for *n* not less than 25. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Glycoproteins enrichment from plasma and gel electrophoresis

Lectin (WGA) affinity column was used to enrich glycoproteins from plasma. WGA has an affinity for N-acetyl glucosamine (GlcNAc) chitooligosaccharides and sialic acid residues present in glycoproteins. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of WGA bound plasma glycoprotein showed number of bands of different molecular weights. Of them three protein bands of mol wt ∼14, ∼21 and ∼45 kDa appeared as major bands (Fig. 1A). The protein band of ∼14 kDa was found to be over expressed in control while of ∼21 kDa band was found to be under expressed in control. The WGA bound fraction was further resolved by 2DE which showed that ∼14 kDa band was consisting of four spots (region 4 and 5) appeared in the basic region of the gel. Similarly, ∼21 kDa band (region 3) resolved into three spots in the slightly acidic region. Region 1 and region 2 as seen in 2-DE gel were also found to be over expressed in RA patients (Fig. 1B).

3.2. MALDI-TOF MS identification and immuno-validation of protein spots

These protein spots were further identified by MALDI-TOF MS analysis. The mascot data base search of peptide mass fingerprinting (PMF) spectra of spots in region 4 and 5 were identified as haemoglobin beta and alpha chain respectively. This was further confirmed by tandem mass spectrometry (MS/MS sequencing) of the peptides and database search showed haemoglobin beta and alpha chains as a significant hits. This is non-specific binding of haemoglobin to WGA. Similarly the spots in region 3 and region 2 were identified as Hp-α2 and Hp-β chain isoforms respectively by database search of PMF and MS–MS spectra. MALDI-TOF MS analysis of region 1 identified the protein as AGP or orsomucoid (Table 1). Due to the preferential action of trypsin [21] and less ionization efficiency of glycopeptides [22], the sequence coverage of some of the proteins were found to be less. In the present study it was observed that potential N-linked glycopeptides were not covered by PMF spectra and this may be reflected in low sequence coverage. The mass spectrometry data was further validated by 2-D Western blotting using anti-AGP and anti-Hp antibodies. The Western blotting also confirmed the over expression of Hp and AGP with 30 RA patients and 30 healthy individuals (Fig. 1S A). The expression level of AGP and Hp was checked in 30 patients and 30 healthy individuals. Densitometric analysis of AGP and Hp-β Western blots showed nearly 2.5 times and 1.6 times higher expression of AGP ($p = 0$) and Hp ($p = 0.006$) respectively in the plasma of RA patients with respect to control (Fig. 1S B).

3.3. HPAEC-PAD analysis

The monosaccharide analysis of AGP and Hp-β chain showed the presence of galactose (Gal), glucosamine (GlcN), glucose (Glc) and mannose (Man). The relative percentage of sugars was calculated and the amount of Gal ($p = 0.01$), GlcN ($p = 0.02$) and Man ($p = 0.0$) was found to be higher in AGP of RA patients' plasma compared to control. The amount of Man ($p = 0.0$) was found to be lesser in Hp-β chain of patients' plasma (Fig. 2A). The level of Fuc, GlcN and Gal

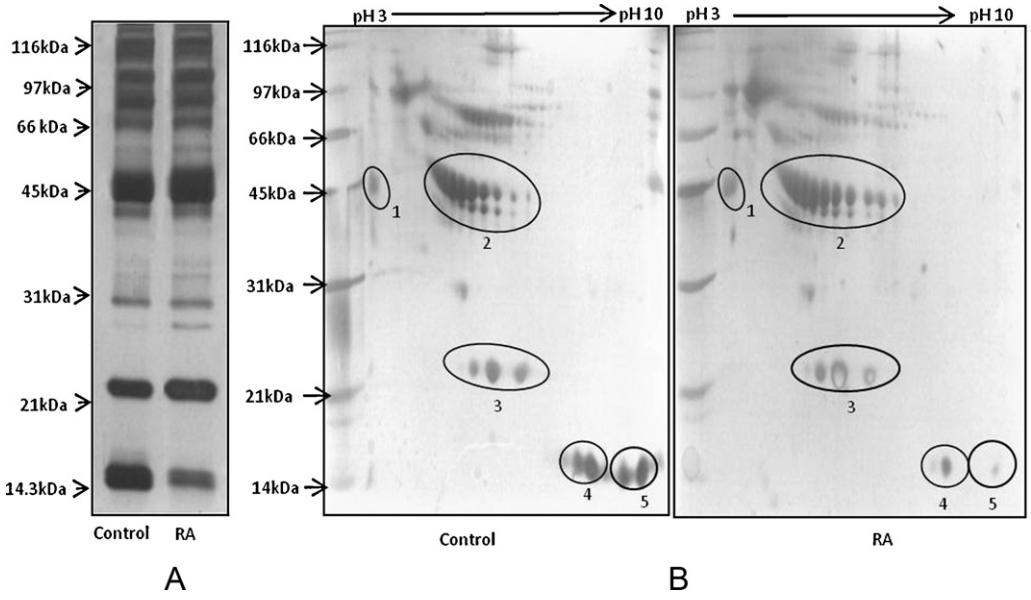

Fig. 1. Differential expression of WGA bound plasma proteins in RA and control. (A) SDS-PAGE and (B) 2DE profiling shows the increased expression of proteins in the region 1 and 2 in RA. Region 1 and region 2 were identified as AGP and Hp-β chains by MALDI-TOF MS respectively.

was found to be less in Hp-β chain of RA patients' plasma without statistical significance.

3.4. Lectin ELISA

The lectin sandwich ELISA showed the higher reactivity of Con-A to AGP while less reactivity to Hp (Fig. 2B). Con-A lectin blotting of AGP and Hp also confirmed the above results (data not shown).

4. Discussion

This study has addressed the alteration of glycosylation in two most common acute phase glycoproteins, AGP and Hp in the plasma of RA patients. The expression of AGP and Hp were observed to be 2.5 and 1.6 times higher respectively than that in control samples. This is in agreement with the previous studies where the levels of these two acute phase proteins have been shown to be increased in inflammatory diseases including RA [23].

Lectin affinity chromatography is widely used as a tool for enrichment of glycosylated proteins. Here lectin affinity column and 2DE were used to enrich and separate the plasma glycoproteins respectively. Spot 1 identified as AGP by MALDI-TOF MS, is an acute phase glycoprotein but its physiological function is not well understood. Serum AGP has great heterogeneity in glycan structures, mainly composed of bi-, tri- and tetra-antennary structures. Most of the tri- and tetra-antennary forms are sialylated of which few are terminated by lewis^x (Le^x) or sialyl lewis^x (sLe^x) structures [24]. This structural heterogeneity in glycans of AGP is known to be altered in various diseases. In stomach cancer patients, bi-antennary structures of AGP are increased in ascitic fluid [25]. Serum AGP fucosylation and sialylation have been shown to be significantly increased in RA resulting in the increased expression of the (sLe^x) [4]. sLe^x may function as a competitive inhibitor of the blood cell borne endogenous counter ligand of endothelial E-selectin at the inflammation site, i.e. arthritic joints in RA [26]. Increased fucosylation was found to have a negative correlation with collagenease activity [matrix metalloproteinase enzymes (MMPs)] in RA joints [27]. In the present study we have found significantly higher levels of GlcN, Gal and Man in plasma AGP of RA patients. It has been shown that the bi-antennary glycan structures of AGP are responsible for Con-A binding which are rich in Gal and Man contents [24]. ELISA studies using Con-A lectin also showed the higher binding of Con-A with AGP in RA plasma indicating a possible increased expression of biantennary glycans. Higher content of Gal on AGP may facilitate more binding of sialic acid residues which might help in binding of AGP to sialic acid receptors (E-selectin) present on neutrophils [26].

The glycosylation alteration in Hp has been studied in various diseases and revealed abnormally elevated level of fucosylation in the sera of alcoholic cirrhosis and RA patients [16,28]. Various types of cancers are also found to be associated with glycosylation alteration in Hp. A study by Fujimura et al. has shown that besides enhanced expression of Hp, the sialylated bi-antennary and fucosylated N-linked tri-antennary glycans were the dominant structures in Hp β-chain in prostate cancers [17]. In our study we have also found the altered expression and glycosylation in Hp-β chain. The monosaccharide composition of Hp-β chain has revealed a decrease in the amount of Man ($p < 0.05$) in plasma of RA patients which was further validated by ELISA using Con-A lectin. This reduction in mannosylation may be due to reduction

Table 1
MS identification of 2DE spots from WGA enriched plasma. Spots were digested with trypsin and peptide mass finger printing (PMF) spectra of spots were taken using MALDI-TOF and searched against NCBInr database.

Spot no.	Protein identified	Accession number	Peptides matched	Mowse score	Sequence coverage (%)
1	Alpha-1acid glycoprotein (AGP)	gi|998943	12	130	42.5
2	Haptoglobin β chain	gi|1212947	11	108	34.6
3	Haptoglobin α-2 chain	gi|296653	10	106	25.7
4	Haemoglobin α chain	gi|63080988	3	49.5	33.6
5	Haemoglobin β chain	gi|61679604	10	163	72.4

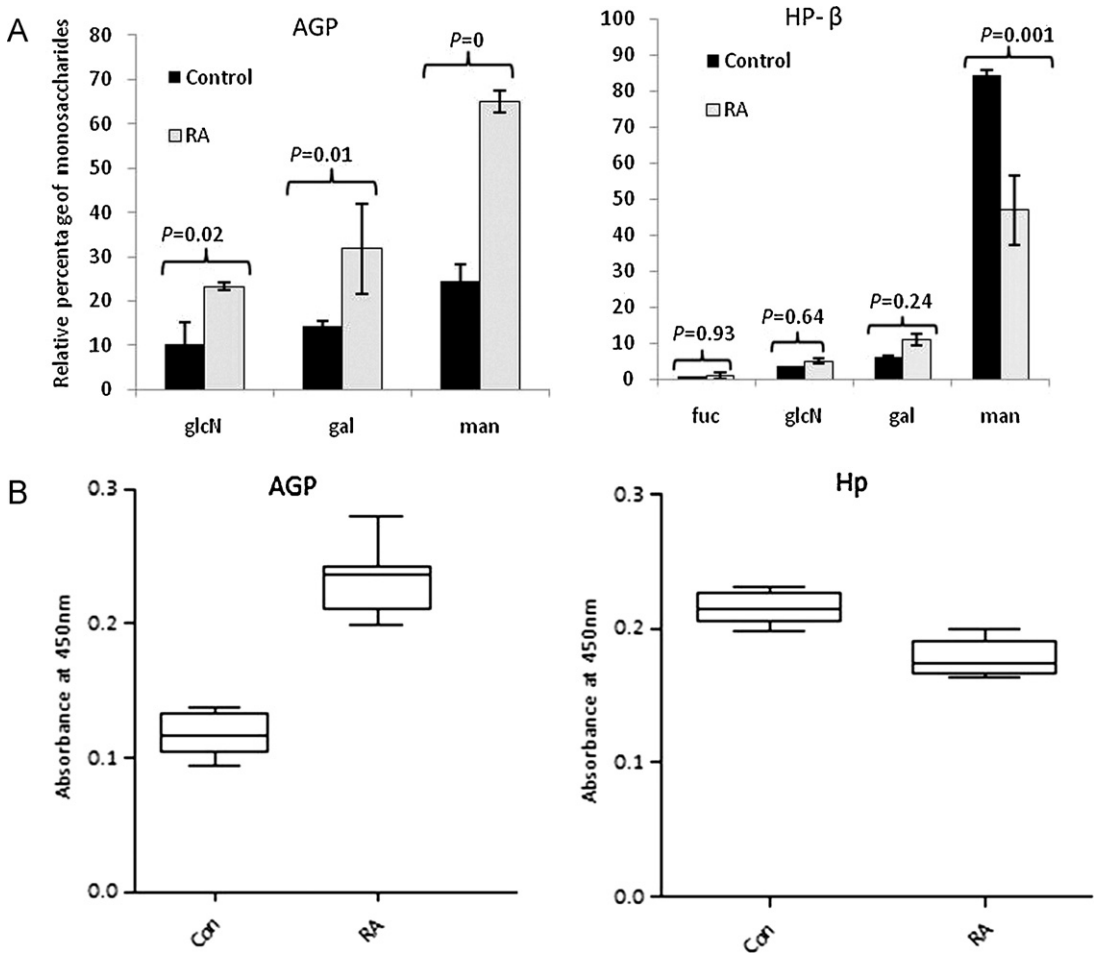

Fig. 2. (A) Histograms showing the relative percentages of monosaccharides from AGP and Hp-β chain in plasma of healthy controls and RA patients ($n = 30$) as analyzed by HPAEC-PAD (B) Con-A reactivity of plasma AGP and Hp from healthy controls and RA patients ($n = 30$) as measured by lectin ELISA. Statistical analysis was done using Mann–Whitney test and p value less than 0.05 is considered as statistically significant.

of oligomannosidic structures on Hp. Decrease in the amount of oligomannosidic structures in serum proteins has been shown in the case of acute pancreatitis and sepsis [29]. Whether these glycosylation changes are the results or causes of the disease is still a matter of debate. There might be possibilities that under the effect of altered cytokines patterns in RA, Hp undergoes aberrant glycosylation in the liver tissues. Another probability is that Hp with altered glycosylation pattern is produced locally (extra hepatic origin) at the inflammation site and is secreted into the plasma [30,31]. Further studies are required to understand the above possibilities.

In conclusion, our strategy offers an operative approach in predicting a preliminary altered glycosylation status of Hp and AGP in RA patients. The observed unique monosaccharide pattern of AGP and Hp was found to be specifically associated with RA and the combined use of these observations along with our recent diagnostic method using autoantibody against MBL [32] could improve the diagnostic accuracy. Many subtle changes in function of a glycoprotein are being found to be attributable to changes in composition or structure of the glycan moiety. Identification of clinically important protein biomarkers with possible glycosylation alterations is an expanding area of research that will extend diagnostic capabilities. Using a panel of lectins as enrichment tools or lectin based fractionation strategies can be an excellent initial step for glycoprotein isolation and a powerful approach towards biomarker discovery. Thus, glycoproteomics is a promising approach to discover more novel biomarkers for diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.04.024.

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