GLYCOPROTEOMIC STUDIES IN RHEUMATOID ARTHRITIS

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Rheumatoid arthritis: An overview

Rheumatoid arthritis (RA) is associated with physical disability affecting 1-2% of the population worldwide. It is a chronic, systemic and autoimmune disease which has great socio and economic consequences as it affects patient’s efficiency and cost huge amount of money to manage the disease (Aggarwal et al., 2006). RA is characterized by inflammation of the synovial membrane (SM) of diarthrodial joints. Early indications of RA are swelling and pain of the proximal inter-phalangeal and later, the larger joints become affected, especially those of the knee, elbow and ankle. Hyperplasia or thickening of the SM is promoted by cytokines and growth factors released from migrating cells. The SM becomes revascularised making it redder than normal (Fig 1). The cytokine enriched environment produced by pro-inflammatory cytokines (IL-1α, IL-6 and TNF-α) results in the aberrant growth of complex vessels known as pannus which invades the cartilage resulting in the irreparable degradation of the articular surfaces.

Fig 1. Picture showing deformities in small joints of rheumatoid arthritis patient (Top). Schematic diagram showing the comparison among normal and the joints affected with osteoarthritis and rheumatoid arthritis (below).
The precise etiology of the disease is unknown however; genetic and environmental factors seem to be involved in its pathogenesis. Certain infections or factors in the environment (Edwards and Cooper, 2006) as well as smoking tobacco have been reported to increase the risk of developing RA (Criswell et al., 2006). The description of the human leukocyte antigen (HLA) associations with RA, has been a major source of support for the hypothesis that genetic factors are important for susceptibility to RA due to a closely related set of polymorphic sequences (the ‘shared epitope’) on several different DRB1 alleles such as DRB1*0401 and DRB1*0101 (Weyand and Goronzy, 2000). In addition, homozygosity for particular combinations of haplotypes, such as DRB1*0401/0404, appear to confer especially high risk or influence disease severity as well as risk (Suzuki et al., 2003).

There is no single test which clearly diagnoses early RA. Disease activity score-28 (DAS) score, erythrocyte sedimentation rate (ESR) and visual analogue score (VAS) are calculated, besides examining the joint inflammation and deformity. In RA, the small joints of the hands, wrists, feet, and knees are typically inflamed in a symmetrical distribution (affecting both sides of the body). Blood test for IgM and IgG RF, anti-CCP (ACPA) antibodies may suggest the presence of RA. ACPA against various citrullinated autoantigens like keratin (antiperinuclear antibodies, APA), filaggrin (antifilaggarin antibodies, AFA), vimentin (antivimentin antibodies, anti SA) can be detected very early even before the onset of clinical symptoms (Van Boekel et al., 2002). Presence of antinuclear antibody (ANA) and anti-MBL-antibodies have also been reported in patients of RA (Gupta et al., 2006).

Protein glycosylation is the most common post translational modification of proteins. Besides stability, some other important functions performed by glycans in the proteins are protection from proteolytic enzymes and viruses, host pathogen interaction, immune system, protein folding etc. During disease conditions, the intracellular environment of the cell is under stress, affecting the glycosylation machinery of the cell which consists of various glycosidases and glycosyltransferases present in endoplasmic reticulum (ER) and golgi bodies. This results in the altered glycosylation pattern of the protein which often leads to malfunction of the protein e.g. agalactosylation of human IgG (IgG0) has been found to be increased and correlated with the disease severity in RA patients. Rheumatoid factor (RF), the characteristic auto-antibody against the Fc region of IgG0, is also increased in the sera of patients with RA (Carson et al., 1987). The altered glycosylation of IgG activates the complement system via mannose binding lectin (MBL) through the interaction with penultimate GlcNAc residue exposed after degalactosylation (Malhotra et al., 1995). Besides IgG, other serum proteins also undergo glycosylation changes in RA patients (Raghav et al., 2006).
Aims and objectives

The present work aimed to study the disease specific glycosylation changes in plasma proteins for diagnosis and understanding disease mechanism in RA. To fulfill the above aim, the study was divided into following objectives.

1. Glycosylation alteration studies in acute phase plasma alpha 1 acid glycoprotein (AGP) and haptoglobin (Hp) in plasma of rheumatoid arthritis (RA) patients.
2. Jacalin bound O-linked plasma glycoprotein profiling in RA patients.
3. Glycoproteomic studies in collagen induced arthritis (CIA) in rats, the rat model of rheumatoid arthritis.

Results

Glycosylation alteration studies in acute phase plasma alpha 1 acid glycoprotein (AGP) and haptoglobin (Hp) in plasma of rheumatoid arthritis (RA) patients.

![Fig. 2. 2DE profiling of WGA bound plasma of control and RA patients using pH 3-10 NL IPG strips. The marked proteins spots/regions were analyzed by alpha digidoc software for differential expression and were identified as AGP (region 1), Hp-β (region 2), Hp-α2 (region 3) and hemoglobin alpha (region 4) and beta (region 5) chains by MALDI-TOF.](image)

Comparative analysis of albumin and IgG deleted plasma of RA patients and healthy controls showed the differential expression of many acute phase proteins like alpha 1 acid glycoprotein (AGP), haptoglobin (Hp), alpha 1 antitrypsin
(AAT) in RA patients. The expression level of two important acute phase proteins *i.e.* AGP and Hp was validated in more number of control and RA patients using Western blotting which showed the statistically significant (*p*<0.05) increase in plasma level of AGP (~2.5 fold) and Hp (~1.6 fold) in RA patients. Plasma glycoproteins from RA patients and controls were enriched using wheat germ agglutinin (WGA)-agarose affinity column followed by 2DE (*Fig.* 2). N-linked glycans were separated by in gel digestion of WGA enriched AGP and Hp using peptide N-glycosidase F (PNGase F). Monosaccharide analysis of glycans by high pH anion exchange chromatography using pulse amperometric detection (HPAEC-PAD) showed the presence of many neutral sugars like fucose, galactose, glucose and mannose and basic sugar like glucosamine in AGP and Hp. HPAEC-PAD analysis revealed the statistically significant increase and decrease in the level of mannose in AGP and Hp respectively in RA patients compared to controls (*Fig.* 3). The differential glycosylation was further validated using concanavalin A (ConA) lectin binding to these proteins by enzyme linked lectinosorbent assay (ELLA). Con-A showed higher and lower

![Histograms showing the relative percentages of monosaccharides from AGP and Hp-β chain from plasma of healthy controls and RA patients (*n*=30) as analyzed by HPAEC-PAD. The relative percentage level of GlcN, Gal and Man was found to be higher in AGP while the percentage of Man was found to be less in Hp-β chain in plasma of RA patients. *p* value less than 0.05 is considered as statistically significant.](image)

binding to AGP and Hp respectively in RA patients, confirming the HPAEC-PAD results. Serum AGP has high 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 have lewis^x (Le^x) or sialyl lewis^x (SLex) structures. Serum AGP fucosylation and sialylation have been shown to be significantly increased in RA resulting in the increased expression of the SLex (Ryden et al., 2002). It has been shown that the bi-antennary glycan structures of AGP are responsible for ConA binding which are rich in Man contents (Shiyan SD and Bovin NV, 1997). ELLA studies using ConA lectin also showed the higher binding of ConA with AGP in RA plasma indicating a possible increased expression of bi-antennary 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 (Jorgensen et al., 1998).

**Jacalin bound O-linked plasma glycoprotein profiling in RA patients**

![Fig. 4. 2D DIGE images of jacalin bound Cy3 labeled control plasma (A) Cy5 labeled patients plasma (B) and overlay of A and B (C).](image)

Here, comparative analysis of O-linked glycoprotein profiling of plasma proteins was done using jacalin affinity agarose column chromatography in RA patients. Glycoprotein profiling using difference in gel electrophoresis (DIGE) and 2DE showed the differential expression of many jacalin bound O-linked plasma proteins in RA patients. The PD-Quest analysis of these gels revealed the differential expression of 18 protein spots with statistical significance. The
expression level of two O-linked glycan containing proteins *i.e.* alpha 2 HS-glycoprotein (A2HSG) and inter alpha trypsin inhibitor 4 (ITIH4) which were found to be differentially expressed in plasma of RA patients were validated in more number of controls and patients by Western blotting. The later showed the statistically significant increase (~2 fold) in the expression of ITIH4 and decrease (~2 fold) in the expression of A2HSG in RA patients. The increased level of ITIH4 was found to be in good agreement with previous studies where its level has been found to be increased in various inflammatory conditions (Choi-Miura *et al.*, 2000; Pineiro *et al.*, 2004). ITIH4 is a 120 kDa protein which is cleaved into N-terminal 85 kDa and C-terminal 35 kDa fragment by plasma kallikarein system. The 85 kDa fragment is further cleaved into 57 kDa and 28 kDa. In our study, the level of 85 kDa fragment was found to be increased by ~2 fold in plasma of RA patients. ITIH4 contains potential actin and calcium binding sites suggesting a role in inhibition of actin polymerization and calcium metabolism.

Protein sialylation plays a very important role in structural and functional establishment of synaptic pathways, nutrition, leukocytes rolling and extravastation during inflammation. The reduced sialylation of A2HSG may have a major effect on the protein structure and its functions *i.e.* bone calcification, ossification and cell adhesion because the interaction of A2HSG with other molecules may be mediated via sialylation (Kundranda *et al.*, 2004).

![Fig 5. 2D Western blot analysis shows the more acidic pl of A2HSG in control as compared to RA which was shifted more in control compared to RA towards basic pl after sialidase treatment. PNGase F treatment resulted in equal shift in pl of A2HSG in both control and RA patients.](image-url)
Generation and glycoproteomic studies in collagen-induced arthritis (CIA)

Fig 6. (A) Representative photographs of hind paws of control and CIA rats. (B) Percent change in body weight of control and CIA rats with reference to day 12 (appearance of inflammation) till the day of euthanization (day 42th). (C) Representative radiographs of the hind limbs (showing the tibiotarsal and tibiofemoral joints) of control and CIA rats. Arrows indicate the bone damage in both tibiotarsal as well as tibiofemoral joints. (D) Representative histological figures (hematoxylin and eosin stained slides) of knee joints showing smooth and monolayer synovial linings and uniform synovial space of ‘control’ rats. Hyperplastic synovial cells indicated by circle, erosion and disruption of synovial linings as indicated by arrows was observed in CIA rats. m=meniscus, f=fibula, js=joint space, t=tibila.

Collagen-induced arthritis (CIA) is an experimental model of arthritis induced in susceptible strains of mice or rats by immunization with heterologous collagen type II, a joint-specific protein, in complete Freund’s adjuvant (Trentham et al., 1977). CIA has been extensively studied to elucidate the pathological mechanisms relevant to human RA and to identify potential therapeutic targets. In this study, CIA rat model of RA was developed in Wistar rats using porcine type II collagen. Visual analysis like inflammation and swelling in hind paws, loss of body weight, increased arthritic score and arthritis
index in starts appearing within 12-16 days of period. Histochemical and radiological analysis also showed the joints deformities and narrowing of space between joints in CIA rats as compared to control rats, confirming the successful development of rat model of RA (Fig. 6).

![Fig 7. A representative 2DE image of WGA bound plasma of pooled control and CIA rats from each group. Three hundred microgram of protein was focused in pH 3-10 IPG strips followed by second dimensional separation in 12% polyacrylamide gel.](image)

Proteomic analysis of plasma proteins using both WGA bound and unbound plasma fractions showed the differential expression of many acute phase proteins like AGP, hemopexin, kininogen, clusterin, alpha-1 major acute phase protein (T-kininogen 1) etc in CIA rats (Fig 7). The level of T-Kininogen 1 was found to be highly increased (~6 fold) in CIA rats which was further validated using Western blotting. Kininogens are important precursor molecules for vasodilator peptide called bradykinin via plasma kallikrein-kinin system (KKS) which participates in the pathogenesis of various inflammatory reactions like involved in cellular injury, coagulation, complement activation, cytokine secretion, release of proteases etc. The RT-PCR analysis of two genes namely clusterin and kininogen from liver tissues also showed the change in the expression of these two genes in CIA rats as compared to control rats. However, the expression level of these two genes did not corroborate with the corresponding protein level in the plasma.
**Significant achievements**

- The comparative plasma glycoprotein profiling of patients and healthy controls, using different lectins affinity columns based glycoprotein enrichment, has identified the number of glycoproteins underwent changes in the expression level.

- Glycan analysis of plasma AGP, Hp and A2HSG has shown the disease specific glycosylation changes in RA patients. These aberrant glycosylation patterns, along with other clinical parameters, can be used for precise diagnosis of RA.

- The glycoproteomic studies in collagen induced arthritis (CIA), the rat model of RA, have identified the differential expression of many plasma glycoproteins similar to RA, suggesting the similarity of CIA to RA at proteomic level.

**Publications:**


**Ashish Saroha**, Saravanan K, Bishnu P Chatterjee, Hasi R Das. (Manuscript under revision in Journal of chromatography B)

**References**


