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Changes in Glycosylation of Alpha-1-Protease Inhibitor in Inflammation (Rheumatoid Arthritis and Crohn's Disease)

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ABSTRACT

Alpha-1-proteinase inhibitor (API) is one of the acute phase proteins. Following an inflammatory stimuli the concentration of API increased up to four folds. Accompanying these quantitative changes, there is qualitative alterations in the structure of carbohydrate moiety (glycosylation). To determine the alterations in the glycosylation of API in inflammation, API was isolated from the sera of healthy individuals and from patients with rheumatoid arthritis and Crohn's disease. The isolated proteins were hydrolyzed to release the monosaccharides. Monosaccharide analysis of isolated API was carried out using high-performance anion-exchange chromatography with pulsed amperometric detection system (HPAE/PAD). Using a lectin binding assay (LBA) which was reported recently, the glycosylation of API was further studied. The results of monosaccharide analysis and LBA showed that in inflammation the fucose content of API is increased. Observation from both methods indicated the increase branching of API in inflammation. These findings may help to develop more precise markers for monitoring pathological progression in these diseases. *Iran. Biomed. J. 3 (1 & 2): 23-29 1999*

Keywords: Alpha-1-proteinase inhibitor , Crohn's disease, glycosylation, inflammation rheumatoid arthritis

INTRODUCTION

Alpha-1-anti-trypsin or alpha-1-proteinase inhibitor (API) is a serum glycoprotein that inhibits the enzyme activity of serine proteases such as elastase and cathepsin G [1]. The predominant site of synthesis of plasma API is liver. It is also synthesized and secreted by the human blood monocytes and breast milk macrophages [1]. Many studies have shown that there is an elevation in API concentration during the acute phase response. Using different cell culture systems (human hepatoma cell line HepG2, hepatocyte, etc.) it has been shown that interleukin-6 (IL-6) and other cytokines mediate the synthesis of API [2]. Serum concentration of API is 0.7-2.7 g/l but in infection, inflammation or malignancy it can be increased three to four folds [3]. API is a single chain about 54 kDa protein with 394 amino acids and three asparagine-linked complex carbohydrate side chains. These oligosaccharide side chains are in the bi-antennary or tri-antennary forms [3].

Several studies have shown that the structure of the carbohydrate moiety (glycosylation) of serum glycoproteins changes in different diseases [4]. There are evidences that API undergoes glycosylation alterations in breast and ovarian cancer [5], hepatocellular carcinoma [6], liver disease [7], burn patients [8], and in smokers [9]. Changes in fucosylation of API have also been reported in ovarian cancer [10]. The abnormally fucosylated form was found to be related to unresponsiveness to chemotherapy. However, later studies reported that these species were only found if the specimens were subjected to multiple cycles of freezing and thawing [11].

There is very limited number of studies in glycosylation of serum glycoproteins in Crohn's disease (CD). In patients with active CD it was shown that API is secreted into the bowel in a glycosylated form, whereas in normal and quiescent CD it is secreted in a deglycosylated form [12]. The presence of the deglycosylation form is closely related to the degree of inflammation. In a preliminary study Lundy and Wisdom indicated that the asialoform of API decreased in inflammation [13].

While the glycosylation of API has not been studied widely in rheumatoid arthritis (RA), there are

Abbreviations: Man: mannose , Fuc:fucose , GlcNAc: N-acetyl glucosamine, Gal: galactose , Neu5Ac: N-acetyl neuraminic acid.

some reports about alteration of the glycosylation of the other acute phase proteins, e.i., alpha-1-acid glycoprotein and haptoglobin in RA. Thompson *et al*. [14] using a carbohydrate analysis system (Dionex) have shown that the galactose and fucose content of Hp increased in active RA but not in inactive RA. Moreover, a correlation was reported between fucosylated form of Hp and disease activity indices which was similar to that for CRP.

To further study the glycosylation of API in chronic inflammation, API was extracted from sera of patients with RA and Crohn's disease patients. This was carried out by analysis of the monosaccharide composition of API using high pH anion exchange chromatography with pulsed amperometric detection system. Further structural information was obtained using a lectin binding assay which was recently reported [15]. In this method a panel of lectins including Concanavalin A (Con A), *Sambucus nigra* agglutinin (SNA), *Macckia amurensis* agglutinin (MAA), and fucose specific lectins i.e. *Lens culinaris* agglutinin (LCA) and *Lotus tetragonolobus* (LTA) were used. The major specificity of these lectins [16] is shown in Table 1.

MATERIALS AND METHODS

Patients and control group: Blood specimens were obtained by venipuncture and sera were separated by low speed centrifugation (600 × g) for 10 minutes and stored at -20°C until required for analysis. The patients with Crohn's disease consisted of 9 women and 3 men (median age 50, range 26-66). Disease activity of this group assessed between 3 and 8 according to Harvey and Bradshow scale [17]. They had the disease between 1 and 25 years at the time of collecting the samples. Some patients were receiving prednisolon, however the others did not receive any medication when the samples were collected.

The RA group consisted of 12 patients (11 female and 1 male) (median age 58, range 19-72). The disease activity was assessed as moderate in 10 patients and severe in two patients. The disease activity was based on the physician's subjective opinion of the patients and this was according to the degree of morning stiffness, the articular index and the joint score, in combination with erythrocyte sedimentation rate (ESR), hemoglobin (Hb) and C-reactive protein (CRP). The CRP and ESR were (median/range) 29 mg/l (7-59), and 64 mm/h (31-96) respectively.

A control study group consisted of healthy women or men with no known disease (median age 40 years. range 19-64) who were attending a blood donor session. They were non-smokers, their alcohol intake was very low and none of them were taking oral contraceptive or any other form of medication.

Isolation of API : API was isolated from sera of patients and healthy subjects using affinity chromatography method [5]. Briefly rabbit anti-human API (Sigma Ltd.) was coupled to CNBr-activated Sepharose-4B beads (Pharmacia Ltd.) at a concentration of 3.75 mg antibody (Ab) per ml beads. A 250 µl aliquot of serum was mixed with 500 µl of Ab coupled beads, incubated for 1.5 h at room temperature and unbound proteins were removed by washing with Tris-HCl (pH 8.0). Bound API was eluted from the beads with 1 ml of 0.1 mol/l trifluoroacetic acid (TFA). The concentration of purified API was determined by rocket immunoelectrophoresis. The purity of the extracted API was analyzed by SDS-PAGE using silver staining [18, 19].

Monosaccharide analysis: The monosaccharide content of the purified API was measured in a high-pressure anion-exchange chromatography system with a pulsed amperometric detector (Dionex carbohydrate system). The details of the method were described before [5]. Briefly a 380 µl aliquot of the TFA-extracted API was hydrolyzed at 80° C for 1 h and 80 µl were removed for N-acetylneuraminic acid analysis. To the reminder, 300 µl of 4 mol/l TFA were added and hydrolyzed for further 4 h at 100°C. After hydrolysis, 0.5 mg of N-acetylgalactosamine was added as an internal standard and the whole was lyophilized overnight to remove the TFA. The resultant material was resuspended in 150 µl deionised water, a 20 µl aliquot was injected onto a Carbopac PA1 column and eluted isocratically with 18 mmol/l NaOH. Data were collected from the system with an Advanced Computer Interface and analyzed by AI 450 software. The monosaccharide composition was calculated by comparing the profile with a mixture of monosaccharide standards (100 ng each of fucose, galactose, glucose, galactosamine, glucosamine and mannose). Neu5Ac analysis was done by injecting a 25 µl aliquot onto the same column and eluting isocratically with 50 mmol/l sodium acetate in 100 mmol/l NaOH. The Neu5Ac content was determined by comparison of the elution profile with that of a 100 ng Neu5Ac standard.

Interaction of API and lectins: The interaction of purified API with lectins was studied by a lectin-binding assay (LBA) that we have recently developed and published [5]. The lectins obtained from Boehringer Mannheim (Gmbh, Germany) conjugated either by digoxigenin or biotin. Briefly, API was dissolved in 100 µl of 25 mmol/l Tris /100 mol/l NaCl, pH 7.5 (TBS), added to a microtiter plate (Immulon 4, Dynatech Laboratories) and incubated at 37 °C for 2 h. Unbound protein was removed by washing three times with TBS containing 0.1% (v/v) Tween-20 (T-TBS). The same buffer was added to each well and incubated for 1 h at 37°C and then overnight at 4°C. After washing twice with 1 mmol/l Tris-HCl (pH 7.5) containing 0.1% Tween-20 and 1 mol/l $CaCl_2$, $MgCl_2$, and $MnCl_2$, 200 µl of digoxigenin (DIG) or Biotin labelled lectin in this buffer were added and incubated with 150 µl of anti-DIG labelled with horseradish peroxidase or streptavidin labelled with alkaline phosphatase 1 h in a sealed container at room temperature. After washing 4 times with T-TBS, the plate was tapped onto tissue paper to remove residual liquid. Adding the substrate of peroxidase or alkaline phosphatase a color reaction was developed and the reactivity of the lectins with API was assessed by reading the absorbance in a plate reader (Titertek Multiskan, MCC/340).

RESULTS

Table 2 shows the results of monosaccharide analysis of API in healthy individuals, patients with CD and RA. The fucose content of API in both diseases was significantly higher than that of healthy group. N-acetylglucosamine (GlcNAc) and galactose (Gal) content of API were also higher than that of healthy group. Although there was no significant difference between mannose (Man) content of API in the RA and healthy groups, in Crohn's patients it was slightly higher than that of control. The data also indicated that the N-acetylneuraminic acid (Neu5Ac) content of API was increased in CD.

Tables 3 & 4 show the results from analysis of API in the LBA for CD and RA patients. The data are also included from the statistical comparisons with the corresponding healthy group. These data indicated that in RA and CD , Con A and SNA reactivity of API were significantly less than those of healthy group. In contrast to this changes, there was a significant increase in MAA reactivity of API in both groups compared with healthy individuals.

Treatment of purified API from inflammatory sera with the fucose specific lectins (LCA & LTA) indicated that there was a significant increase in the reactivity of LCA and LTA with API compared with the healthy group (Table 4). Similar results were obtained for CD patients.

DISCUSSION

The data obtained in this study indicate that the carbohydrate structure of API is altered in serum of RA and CD patients. Complex N-glycan chains on proteins such as API have a common core structure of three mannose and two GlcNAc, galactose, fucose, and Neu5Ac [4]. The composition of the latter is very variable and within any single population there exist many molecules with different types of carbohydrate extension (glycoforms). Thus any changes in carbohydrate composition or reactivity of a glycoprotein indicates a shift in the average weight of the population. As API is known to contain complex chains with either two or three branches, the increase in GlcNAc content in inflammation suggests that the bi-antennary chains are being replaced by those of tri-antennary type. This means increased branching of API in inflammation. This finding was further supported by the results from LBA. Con A binds to the mannose core of glycan chains whereas SNA and MAA bind to alpha 2-6 and alpha 2-3 NeuAc respectively [16]. The API from inflammatory patients bound less Con A, less SNA and more MAA. Previous studies have shown that decrease in binding to Con A indicates increase branching [20]. Furthermore other studies have shown that with increase branching there is a shift from alpha 2-6 to alpha 2-3 Neu5Ac [21]. Increased branching or type II major microheterogeniety has also been reported for API in inflammation, in septicemia and in isolated liver cells after cytokines treatment. This type of alteration in glycosylation was observed in HepG2 cells after cytokine treatment. Thompson *et al.* [14] reported similar changes for Hp in RA.

The altered glycan chains in inflammatory API were also shown to contain more fucose. This was shown by both methods i.e. monosaccharide analysis and LBA. An elevation in fucose appears to be a

Table 1. A summary of carbohydrate specificity of some lectins used for the analysis of serum glycoproteins [taken from reference 16].

Lectin	Abbreviation	Major carbohydrate specificity
Concanavalin A	Con A	Man α1,3 or Man α1,6
Lens culinaris agglutinin	LCA	Man α1,3 or Man α1,6 (Fuc α1,6 GlcNAc is also required)
Lotus tetragonolobus	LTA	Fuc α1,2 Gal β1,4 [Fuc α1,3] GlcNAc
Maackia amurensis agglutinin	MAA	Neu5Ac α2,3 Gal
Sambucus nigra agglutinin	SNA	Neu5Ac α2,6 Gal

Table 2. Monosaccharide composition of API in patients with Crohn's disease, RA patients, and healthy individuals. Data are given as moles of monosaccharide per mole of API. The data was analyzed statistically using the Mann-Whitney U test.

Group		**Fucose**	**GlcNAc**	**Gal**	**Man**	**Neu5Ac**
Healthy n = 14	Median	0.86	13.7	7.7	9.6	7.2
	Range	0.66-1.4	11.1-17.8	6.6-9.0	7.4-10.5	6.1-8.7
Crohn's Disease n= 12	Median	1.35	14.9	9.95	10.2	7.95
	Range	1.1-1.7	14.2-16.7	8.9-11.0	9.0-11.4	7.0-8.7
	p value	0.0002	0.005	<0.0001	0.03	0.02
R A n= 11	Median	1.0	14.3	8.4	9.5	7.4
	Range	0.61-1.5	13.0-16.1	7.5-10.4	8.8-10.7	6.0-8.9
	p value	0.02	0.02	0.007	NS	0.38

Table 3. Lectins reactivity of API isolated from healthy individuals and patients with Crohn's disease. The data was analyzed statistically using the Mann-Whitney U test

		Absorbance	
Lectin		Healthy group (n = 8)	Crohn's disease (n = 8)
Con A	Median	1.08	0.76
	Range	0.80 - 1.47	0.49 - 1.05
	p value		0.007
SNA	Median	1.27	0.84
	Range	1.06 - 1.44	0.54 - 1.09
	p value		0.001
MAA	Median	0.37	0.62
	Range	0.33 - 0.51	0.51 - 0.77
	p value		0.001
LCA	Median	0.47	0.67
	Range	0.33 - 0.53	0.48 - 0.85
	p value		0.002
LTA	Median	0.35	0.48
	Range	0.27 - 0.45	0.34 - 0.67
	p value		0.001

Table 4. Lectin reactivity of API isolated from healthy individuals and patients with RA. The data was analyzed statistically using the Mann-Whitney U test.

	Absorbance	
Lectin	Healthy group (n = 8)	RA (n = 11)

	Median	1.76	0.98
Con A	Range	0.92 - 1.96	0.41-1.32
	p value		0.006
	Median	1.26	0.88
SNA	Range	1.03 - 1.92	0.57 - 1.17
	p value		0.001
	Median	0.26	0.51
MAA	Range	0.18 - 0.33	0.29 - 0.58
	p value		0.0008
	Median	0.51	0.80
LCA	Range	0.33 - 0.61	0.53 - 0.92
	p value		0.0006
	Median	0.44	0.79
LTA	Range	0.43 - 0.68	0.59 - 1.07
	p value		0.0004

common finding in other serum glycoproteins in cancer, inflammation and other diseases [21]. We have previously shown increase in the fucose content of Hp and API in ovarian and breast cancer [22]. It has been suggested that fucosylation index of alpha-feto protein can be useful for the prediction of hepatocellular carcinoma especially in patient with liver cirrhosis during long term follow up of disease [23]. Very few studies of glycosylation of API in inflammation have been reported, and there are discrepancies between the results.

The cell surface glycoproteins are also undergo glycosylation changes in disease and play an important role in the inflammatory process. Cell adhesion molecules recognizing carbohydrate ligands have been described for platelets (P-selectin), endothelial cells (E-selectin), leukocytes (L-selectins), and other cell types [24]. It has been shown that P-selectin on platelets or endothelial cells appear within minutes after inactivation by thrombin, phorbol myristate acetate, histamine, etc. and bind to sialylated Lewis structures on neutrophils and monocytes. L-selectins present on lymphocytes, neutrophils, and monocytes may be homing receptors that bind to endothelial cells, lymph nodes, and high endothelial venules and appear to be down regulated in inflammation. Different cell types of the immune system have not only their characteristic carbohydrate structures but also their characteristic sets of glycosyl transferase activity, implying different functions of carbohydrates [24]. Cell surface carbohydrate interactions are responsible for cell-cell recognition or binding to extracellular matrix, for example lymphoids, myeloid, and tumor cells migrate and settle in another organ via these interactions. Changes in sialylation and fucosylation of acute phase proteins can result in the increased expression of sialyl Lewis X structure that have been shown to be involved in the selectin-mediated interaction of leukocytes and endothelial cells in homing and inflammatory processes [21]. It has been postulated that the inflammation-induced changes in the expression of SLEX on acute phase proteins may have an inhibitory effect on the selectin-mediated influx of leukocytes into inflamed areas and may represent a humoral feedback response of the hepatic acute phase reaction to diminish the cellular inflammatory reaction [21].

Most of the carbohydrate changes in the inflammatory API were completely different from those previously reported for cancer API [5]. API isolated from ovarian and breast cancer patients showed decrease branching. This may suggest different mechanisms in inflammation and cancer.

Since synthesis of API has also been reported in leukocytes, monocytes and alveolar macrophages [3], the contribution of extrahepatic cells to glycosylation changes cannot be excluded. Because proliferation of leukocytes occurs in inflammation, it is thought that these cells could contribute in the observed changes in glycosylation of acute phase proteins in inflammation [20].

Studies of the carbohydrate structure of IgG in RA patients have shown that this disease is associated with an increase in glycoforms (G0) that lack galactose [25]. It has been shown that the degree of degalactosylation reflects disease activity. Since RA is a difficult disease to assess in the clinic, study of glycosylation alteration could provide a good indicator of disease activity. For example, RA patients with the higher G0% value have greater joint erosion, greater number of swollen joint and less functional ability [26]. Thompson *et al.* [14] reported that glycosylation changes of Hp in active RA pa-

tients were different from inactive group. In the active group there was a larger and very significant increase in the fucose content of Hp.

However ESR and CRP have been reported to correlate with disease activity, it should be mentioned that these tests are non-specific and can only be interpreted accompanied by other clinical information. In order to estimate the inflammatory activity of CD, to exclude infections disease and to evaluate the efficacy of drug treatment, several laboratory tests have been employed. Macckiewicz et al [27] suggested that AGP glycoforms appear to be a valuable biochemical indicator of RA activity. This also supports that the carbohydrate analysis of serum glycoproteins can provide a better indicator for assessing the disease activity.

API is commonly elevated in inflammation as part of the acute phase response, this study has demonstrated that there are also carbohydrate changes occurring in the structure of the molecule. Since there is evidence showing that cytokines involved in these changes, it may be speculated that in response to inflammatory disease mixture of cytokines are released that alter the glycosylation of secreted molecules [28].

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