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Aberrant Glycosylation of Igg Heavy Chain in Multiple Myeloma

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A B S T R A C T

Although the majority of eukaryotic proteins are glycosylated, there is a dearth of knowledge regarding protein sugar moieties and their changes in disease. Most multiple myeloma cases are characterized by production of monoclonal immunoglobulins (Ig). We studied galactosylation and sialylation of IgG heavy chains in 16 patients with IgG myeloma using lectin blotting and densitometry. In comparison to age and sex matched controls, galactosylation was reduced in multiple myeloma (median 317 vs. 362, range 153–410 vs. 309–447 relative units, p=0.015, Student's t-test). Sialylation was stage dependent; samples from patients with stage IIA had lowest amounts of sialic acid, IIIA intermediate and IIIB highest (142.6 vs. 185.9 vs. 248.5 relative units, correlation coefficient r=0.55). Both galactosylation and sialylation levels were independent of age, sex, treatment type, response to treatment, disease duration and IgG and b2 microglobulin concentration. These data indicate that multiple myeloma is characterized by aberrant immunoglobulin glycosylation.

***Key words**: multiple myeloma, immunoglobulins, glycosylation, sialylation, galactosylation*

Introduction

Over half of all known proteins contain covalently linked oligosaccharides and are called glycoproteins[1]. Because of the difficulties associated with the study of the sugar chains of glycoproteins, elucidation of the functional aspects of sugar moieties of glycoproteins lagged very much behind those of proteins and nucleic acids. However, development of various sensitive methods for the analysis of carbohydrate structures has changed the situation dramatically, and modern science is focusing more and more on glycoconjugates known to be involved in many vital physiological processes of multicellular organisms, from fertilization and development, to modulation of immune functions and memory consolidation[2–4]. Their carbohydrate structures undergo specific changes in many diseases[5–8], and the investigation of the sugar chains formed under pathological states is expected to provide important data for better understanding of the underlying mechanisms.

Constant regions of IgG heavy chains are associated with a population of slightly different oligosaccharides attached to Asn^{297} (Figure 1). Specific changes in distribution of these glycoforms were found in several diseases like rheumatoid arthritis, juvenile rheumatoid arthritis and lupus erythematosus, but their significance is not completely understood[9–12].

Multiple myeloma is a malignant hemopoietic disease characterized by clonal proliferation of plasma cells13. In most cases plasma cells produce and secrete immunoglobulins and/or immunoglobulin light chains, detectable as monoclonal peaks on serum electrophoresis or as free immunoglobulin light chains in urine (Bence-Jones proteins). Because of the production of large quantities of pathological proteins, multiple myeloma is ideal for studying glycosylation abnormalities. Studies on very small numbers of patients indicated that multiple myeloma

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could be associated with changes in IgG glycosylation. Decreased galactosylation was reported, and initial results indicated that in some cases oligosaccharide profiles of polyclonal IgG reflected the profile of paraprotein from the same patient, and not that of normal polyclonal IgG[14,15].

We analyzed IgG heavy chain galactosylation and sialylation in 17 patients with IgG multiple myeloma.

Materials and Methods

Patients and healthy controls

Patients with multiple myeloma were diagnosed according to standard criteria[13,16], and treated at one of our institutions. Sixteen consecutively seen patients with IgG multiple myeloma in various disease phases were included in the study. All patients had active disease and were treated prior to sample collection with polychemotherapy. Patients' characteristics are presented in Table 1. The control group consisted of 19 apparently healthy volunteers, sex and age matched with the patient group, chosen from a larger cohort in whom normal galactosylation patterns were studied (Table 1).

The study was performed in accordance with the Declaration of Helsinki. All participants were informed of the purpose of the study by one of the investigators and gave oral informed consent.

Clinical and Laboratory Data

The following data were obtained for multiple myeloma patients: gender, age, disease type, stage and duration, IgG and β_2-microglobulin concentration. Disease stage was determined according to the standard Durie and Salmon criteria[15]. IgG concentration was measured using the immunonephelometry method[17]. β_2-microglobulin concentration was measured by a competitive enzyme immunoassay (Behring, Marburg, Germany)[18].

Preparation of Serum

During venepuncture approximately 9.5 mL of blood was drawn into Vacutainers (Beckton-Dickinson, Plymouth, UK) containing a gel and clot activator. Samples were left for 1 hour at room temperature to allow clotting, and then centrifuged at 6000 rpm for 20 minutes. Sera were removed and stored at –20 °C until analysis.

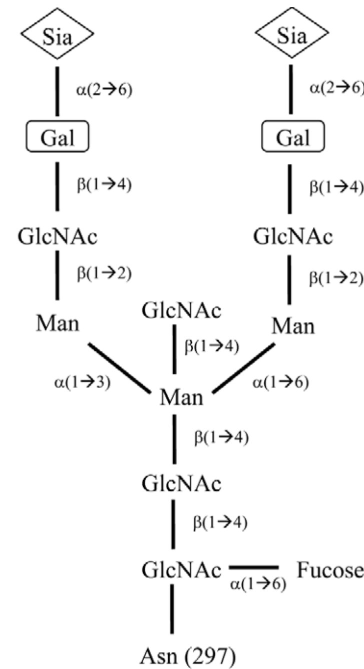

Fig. 1. The largest neutral biantennary complex oligosaccharide found at the Fc region of human serum IgG (attached to asparagine (Asn) 297). There is a common pentasaccharide core containing two mannose (Man) residues attached to a b-mannosyl-di-N-acetylchitobiose unit. Terminal galactose (Gal), sialic acid (Sia) and bissecting N-acetylglucosamine (GlcNAc) are not present on all molecules and their levels vary among individuals.

Materials

All chemicals were of analytical grade. Immobilon PVDF membrane was from Millipore Corp. (Bedford, MA, USA); bovine serum albumin from Roth (Karlsruhe, Germany); biotinylated RCA-I lectin from Vector laboratories (member of Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany); digoxingenin-labeled SNA lectin and anti-digoxigenin Fab fragments labeled with alkaline phosphatase from Boehringer Mannheim (Mannheim, Germany).

TABLE 1
CHARACTERISTICS OF PATIENTS AND CONTROLS

		Patients	Controls
Gender	M/F	9 / 7	10 / 9
Age (years)	median / range	53 / 43–68	56 / 50–78
Light chain	κ/λ	11 / 5	
Stage	IIA/IIIA/IIIB	3 / 9 / 4	
Disease duration (months)	median / range	17 / 1–68	
β_2 microglobulin (mg/L)	median / range	2.89 / 1.58–>44	1.0–2.6
IgG (g/L)	median / range	28.85 / 6.59–125	9.2–17.5

M – male, F – female

Purification of IgG by anion-exchange chromatography

Proteins from 0.7 mL of serum were precipitated with addition of aqueous 4 M ammonium sulfate to final concentration of 2,4 M, centrifuged for 5 minutes at 5000 rpm and resuspended in the original volume of 10 mM potassium phosphate buffer (pH 6.8). Following desalting on Sephadex (Amersham-Pharmacia, Uppsala, Sweden) G25 columns (1.5 × 5 cm), the samples were applied to the anion-exchange column (Fractogel® TMAE-650; 1.5 × 3.5 cm, Merck, Darmstadt, Germany), equilibrated in the 10 mM potassium phosphate buffer (pH 6.8). The flow rate was 0.2 mL/min. Fractions were analyzed spectrophotometricaly and collected until the absorbance at l=280 nm approached baseline. IgG, which eluted as a first fraction was collected and stored at –20 °C. Other proteins were eluted with 2 M NaCl, and were not collected. All procedures were performed at room temperature. Purity of IgG samples was assessed by SDS polyacrylamide gel electrophoresis.

Protein estimation

The concentrations of purified IgG fractions were determined spectrophotometricaly at l=280 nm using commercially obtained pure IgG as a standard.

Glycosylation analysis

Heavy and light chains of IgG were separated by denaturing polyacrylamide gel electrophoresis[19], and transferred onto PVDF membranes using a semi-dry technic[20]. Labeled lectins were used to analyze galactosyla- tion and sialylation of IgG[21]. After overnight blocking in 3% BSA, blots containing electrophoretically separated IgG chains were incubated with biotinylated galactose- specific RCA-I lectin, or digoxin-labeled, α(2,6)-sialic acid-specific SNA lectin. After washing, blots were incubated with streptavidin, or anti-digoxigenin antibodies labeled with alkaline phosphatase and developed with 0.02 mg/mL 5-bromo-4-chloro-3-indolyl phosphate and 0.04 mg/mL nitro-blue tetrazolium in 50 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2.

All samples were analyzed in duplicate and an internal IgG standard was included into each blot to enable comparison of samples on different blots. Optical density of the bands corresponding to IgG heavy-chains was measured using Ultroscan XL® laser densitometer (Pharmacia LKB). For maximal precision, two-dimensional scanning (0.4 mm resolution on the X axis, and 0.2 mm resolution on the Y axis) was performed. Scans were analyzed in Gel Scan™ software (Pharmacia LKB). All results were expressed in absorbance units per mg of IgG.

An example of lectin-blot analysis of IgG heavy chain sialylation (using SNA lectin from *Sambucus nigra*) is shown in Figure 2.

Statistical analysis

The two-tailed heteroscedastic Student's t-test was used to measure statistical significance between groups. A pavlue below 0.05 was considered significant. Trendlines were calculated as a least square fit for a linear equation (ax+b). Population correlation coefficients were calculated as covariance of two data sets divided by the product of their standard deviations.

Results

The extent of heavy chain IgG galactosylation was reduced in multiple myeloma patients in comparison to healthy controls (Table 2). The extent of galactosylation did not correlate with light-chain type, stage, disease duration, IgG or β_2-microglobulin concentration (data not shown).

Although the average level of sialic acid in the multiple myeloma group differed only slightly from the control group, the variation in levels of sialic acid among patients with multiple myeloma was higher than in the

TABLE 2
GALACTOSYLATION AND SIALYLATION OF IGG HEAVY CHAIN MEASURED IN RELATIVE UNITS

	Myeloma patients	Controls	p (Student's t-test)
Galactosylation (median / range)	317 (153–410)	362 (309–447)	0.015
Sialylation (median / range)	188.4 (99.7–338.4)	190.8 (169.8–237.6)	0.953

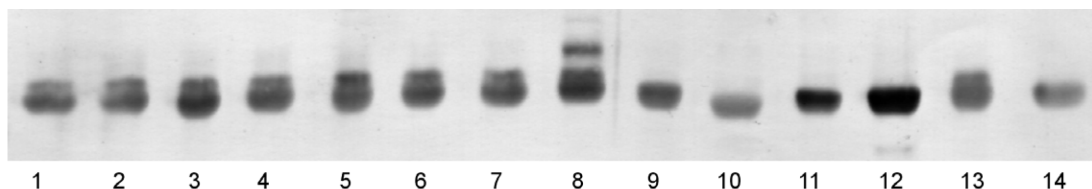

Fig. 2. Multiple myeloma is associated with increased variability in IgG sialylation. Lanes 1–7: IgG from control samples; lane 8: standard IgG (used to compare results from different blots); lanes 9–14: IgG from patients with multiple myeloma. IgG from control sera have similar levels of sialic acids, while IgG sialylation in patients with multiple myeloma is more variable.

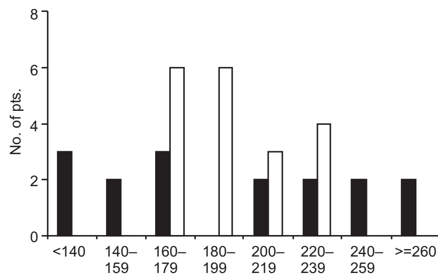

Fig. 3. Distribution of IgG heavy chain sialylation in control and multiple myeloma patients measured in relative units. Filled bars – multiple myeloma, Open bars – controls.

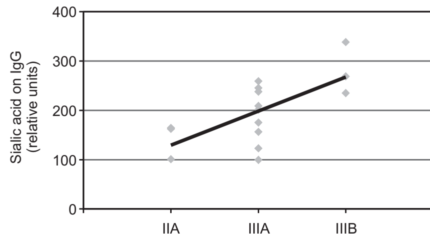

Fig. 4. Increase in stage of multiple myeloma is accompanied with increased sialylation of IgG heavy chains.

control group (Figure 3). We analyzed the correlation between sialylation of IgG heavy chain and gender and age of the patients, duration of the disease, disease stage, IgG concentration, and concentration of b2-microglobulin. Disease stage and renal insufficiency correlated with the increase in sialylation ($\rho=0.40$ and 0.48, respectively), while other analyzed parameters did not. The best parameter predicting ($\rho=0.55$) for increased sialylation was the combination of disease stage and renal insufficiency (Figure 4).

In multiple myeloma there was a significant correlation between galactosylation and sialylation of IgG heavy chains ($\rho=0.63$), that was not found in controls ($\rho=0.08$).

Discussion

We studied glycosylation of IgG heavy chains in sera of 16 patients with multiple myeloma and 19 healthy control individuals. Contrary to previous studies that used sophisticated glycosylation analysis techniques in small numbers of patients[14,15,22], we determined amounts of terminal galactose and sialic acids using simple lectin blotting that can be repeated in most clinical laboratories. In addition we focused on IgG heavy chains to evade influence of unknown glycosylation of light chains on the obtained results. Since relatively large amounts of IgG are needed for this method we were unable to analyze IgG glycosylation in patients with other types of myeloma (IgA, light chain or non-secretory), who typically have reduced amounts of IgG.

The small (approximately 15%), but statistically significant ($p<0.01$) decrease of galactose observed (Table 2) is in accordance with results published by Roy Jefferis' group[14,15]. This seems to be an inherent characteristic of multiple myeloma, independent of other, patient or disease related factors. The same group of authors reported that the abnormal glycosylation pattern is present also on polyclonal IgG in myeloma patients. Although these results were obtained by studying only three patients and require further confirmation, the hypothesis that abnormal physiological environment of the bone marrow caused by a disease may also affect normal plasma cells producing polyclonal IgG is very attractive.

In contrast to galactosylation, sialylation was dependent on disease stage at diagnosis and renal insufficiency. These findings are in line with those from a study indicating that the ratio of sialylated to neutral oligosaccharides in monoclonal gammopathy of unknown significance (MGUS) is decreased in comparison to multiple myeloma[22], MGUS being a »benign« plasma cell disorder[23]. The influence of renal function on glycosylation of plasma proteins is unknown. It would be interesting to see whether sialylation of heavy chain IgG is increased in persons with other types of renal disease and whether other protein types show a similar pattern.

The finding, that galactosylation and sialylation are correlated in samples from patients but not from healthy controls, suggests that aberrations of sialylation and galactosylation present in multiple myeloma have a similar pathogenetic mechanism.

The limitations of this study are obvious: the number of subjects involved was small, all were treated prior to sample collection, and disease status and treatment types varied considerably. However, since the data on protein glycosylation in various diseases are extremely limited, the results are at least interesting, if not definitive. It seems that patients with multiple myeloma have aberrant IgG heavy chain glycosylation. This could, by interfering with normal IgG metabolism and distribution, contribute to the immunodeficiency characteristic of this disease. Malignant plasma cells are known to produce structurally aberrant immunoglobulins[24]. This could adversely affect its glycosylation. Alternatively, it could be an effect of bone marrow microenvironment abnormalities caused by aberrant local production of cytokines and disturbances of cytokine networks. A number of different cytokines, including interleukin-6, are known to be abnormally produced by bone marrow stromal cells in multiple myeloma[25,26]. The fact that polyclonal IgG in multiple myeloma patients have a similar glycosylation pattern as monoclonal[14] seems to favor the latter explanation. Exact mechanisms involved should be the focus of additional studies including larger number of patients and using more precise methods for isolation of monoclonal paraproteins.

Acknowledgements

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POREMEĆAJ GLIKOZILACIJE IgG TEŠKOG LANCA U MULTIPLOM MIJELOMU

SAŽETAK

Iako je većina eukariotskih proteina glikozilirana, nema puno podataka o šećerima vezanima na proteine niti o njihovim promjenama u pojedinim bolestima. Multipli mijelom je većinom karakteriziran stvaranjem monoklonskih imunoglobulina (Ig). Istraživali smo galaktozilaciju i sijalilaciju IgG teškog lanca u 16 bolesnika s IgG mijelomom koristeći specifično vezivanje lektina i denzitometriju. U usporedbi s kontrolama podjednake dobi i spola je u multiplom mijelomu smanjena galaktozilacija (medijan 317 naprema 362, raspon 153–410 naprema 309–447 relativnih jedinica, $p=0.015$ koristeći Studentov t-test). Sijalilacija je ovisila o stadiju. Najmanje sijalinske kiseline je nađeno u uzorcima bolesnika sa stadijem IIA, nešto više u onih s IIIA, a najviše u IIIB stadiju (142,6 naprema 185,9 naprema 248,5 relativnih jedinica, koeficijent korelacije $\rho=0{,}55$). Ni razina galaktozilacije niti sijalilacije nisu ovisile o dobi, spolu, vrsti liječenja, odgovoru na liječenje, trajanju bolesti, koncentraciji IgG ili β_2 mikroglobulina. Ovi podaci ukazuju da je multipli mijelom karakteriziran poremećajem glikozilacije imunoglobulina.