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Fucosylation in synovial fluid as a novel clinical marker for differentiating joint diseases – a preliminary study

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ABSTRACT

Objective. *To investigate fucosylation of synovial fluid glycoproteins in patients with rheumatoid arthritis (RA), juvenile arthritis (JIA), gonarthrosis (GA) and reactive arthritis (ReA), referred to traumatized knee (TK).*

Methods. *Synovial fluid glycoproteins were separated by SDS-PAGE and either silver stained or blotted onto nitrocellulose and probed with the fucose-specific* Aleuria aurantia *lectin. Five bands were chosen for densitometric analysis. Total fucose content and density of fucosylated epitopes were analyzed.*

Results. *Fucose content was elevated in all patient groups and almost all bands, comparing to TK. The density of fucosylated epitopes was increased in the 42-kDa band of RA and JIA cases, and lowered in the 26-kDa band of RA and JIA, but not in GA. In all RA cases F_R 42-kDa > F_R 26-kDa. The relation was opposite in 8 out of 9 GA cases.*

Conclusion. *The density of fucosylated epitopes differs significantly in particular glycoproteins of synovial fluid in joint diseases and may be of potential diagnostic value in differentiating diseases of inflammatory and degenerative origin.*

Introduction

Altered glycosylation is connected with the progress of rheumatic diseases (1). Plasma immunoglobulin G bears trimmed oligosaccharides, lacking sialic acid and galactose, and sugar printing of IgG glycans enables differentiation between joint inflammation (rheumatic arthritis) and cartilage damage (osteoarthrosis) (2, 3). A drawback of such analysis is the necessity of IgG purification and isolation of glycans.

The expression of fucose residues is another important element of glycosylation status. This monosaccharide directly takes part in intra- and extracellular recognition (4, 5). Fucosylation was extensively studied in serum α_1-acid glycoprotein (AGP) of RA patients (6-8).

Although serum reflects to some extend the disease status, more information can be obtained by investigating pathological events directly in intra-articular milieu (8). It is still unclear if glycosylation plays a signaling or recognition-mediating role inside the joint. Data concerning synovial fluid glycoproteins may be of an importance in this field.

Other glycoproteins can also carry oligosaccharides of altered structure (9), which may result from altered activity of enzymes involved in glycan processing (10, 11). This was an incentive to analyze different glycoproteins simultaneously. The current study describes preliminary investigation of the expression of *Aleuria aurantia* lectin-reacting fucosylated epitopes on synovial fluid glycoproteins. We focused our attention on reciprocal relations of fucose content in five chosen glycoprotein bands and in different pathological conditions of the knee joint.

Materials and methods

Clinical subjects

Samples of synovial fluid (SF) were obtained from patients treated in Medical University of Bialystok. There were eleven cases of juvenile idiopathic arthritis (JIA, 7 girls and 4 boys, age range 6-18 years), five of reactive arthritis (ReA, 1 girl and 4 boys, age range 12-18 years), ten cases of rheumatoid arthritis (RA, 6 women and 4 men, age range 23-74), nine patients with gonarthrosis (GA, 4 women and 5 men, age range 56-74 years), and seven cases with injured anterior ligament or meniscus meditis latientis of the knee (TK, 1 woman and 6 men, age range 17-21 years).

Arthrocentesis of the knee joint of JIA, ReA, RA and GA patient were performed because of prolonged exudation or intra-articular injections of steroids. Samples of SF of patients with traumatized knee were obtained during routine diagnostic arthroscopy. The study design was approved by Ethical Committee of Medical University of Bialystok, Poland.

SDS-PAGE and Western Blotting

A constant amount of 2.5μg of protein was loaded on the gel lane. This assures linearity in densitometric analysis in silver-stained gels as well as in lectin-probed blots, as we have previously

demonstrated (12). The samples were denatured for 5 min with 1% SDS and 2.5% of β-mercaptoethanol. Electrophoresis was performed in 10% polyacrylamide gels containing 1% of SDS (13). Two identical gels were developed simultaneously, one of them was further silver stained and the other was blotted to the nitrocellulose according to standard procedure. Blots were blocked overnight with 1% Tween-20 in 10 mM Tris-buffered saline, pH 7.4 (TBS) before lectin staining.

As a control lane, in every gel the sample of human reference serum (hRS), containing 2.5 μg of protein, was developed. The reference serum was prepared as described earlier (12), and showed no significant difference towards DAKO serum calibrator concerning electrophoretical pattern as well as the protein, haptoglobin (Hp) and AGP concentration. Reciprocal OD values in hRS bands provide a control for reproducibility between experiments.

Fucosylation analysis and densitometry

Fucose content in glycoproteins of synovial fluid was determined as their reactivity with fucose-specific *Aleuria aurantia* lectin (AAL, Vector). Blots were incubated with the biotin-labeled lectin (4μg/ml TBS-Tween 0.1%) for 1 hour at 37°C and next with extravidin-alkaline phosphatase conjugate (Sigma, 1:10 000) in the same conditions. Bands were visualized with nitroblue tetrazolium (0.5 mg/ml) and 5-bromo-4-chloro-3 indolyl phosphate (0.2 mg/ml Tris-HCl buffer, pH 9.5, containing 0.05 mol/l of $MgCl_2$).

Both silver-stained gels and AAL-probed blots were scanned with Plustec OptiPro scanner. Scion Image (Mackintosh Package) software was applied for the measurement of optical density (OD) of the bands. The fucose unit was defined as the amount of this monosaccharide carried by 42-kDa acute phase proteins in hRS. Total fucose content in every analysed band was therefore calculated as $F = OD_x/OD_{42\text{-}kDa}$ hRS. The protein content was calculated similarly in each band, to enable calculation of the fucosylation coefficient, defined as $F_R = F/P$. This value describes a density of fucosylated glycans on the surface of analysed glycoprotein.

Statistics and cluster analysis

Results were analyzed with Statistica 5.0 software. The nonparametric Mann-Whitney U-test was used and p-value < 0.05 was considered significant.

In the clustering procedure, each of the subjects was represented by a vector of 10 features in ten-dimensional vector space. These were the relative amounts of protein and fucose in five bands: 77-, 42-, 37-, 29- and 26-kDa. The distance between the points was calculated using Euclidan metrication on the scaled data points. The mean values for each point in the cluster were calculated as the weighted mean average (14).

Table I. Fucose content in five glycoprotein bands in joint diseases.

	77 kDa	42 kDa	37 kDa	29 kDa	26 kDa
TK	0.76 ± 0.25	0.64 ± 0.21	0.55 ± 0.16	0.58 ± 0.30	0.70 ± 0.29
RA	0.78 ± 0.11	1.08 ± 0.21**	0.81 ± 0.19*	1.11 ± 0.20**	1.15 ± 0.22**
JIA	0.91 ± 0.23	1.01 ± 0.33*	0.89 ± 0.17**	1.01 ± 0.30*	0.96 ± 0.24
GA	0.85 ± 0.27	1.09 ± 0.46*	0.71 ± 0.22	0.71 ± 0.26	1.03 ± 0.40
ReA	0.87 ± 0.23	0.64 ± 0.23	0.87 ± 0.09	0.83 ± 0.19	0.87 ± 0.08

Statistical significance towards TK group *$p < 0.05$, **$p < 0.005$.

Results

Fucose content

Fucose content measured as AAL-reactivity was significantly elevated in all except 77-kDa bands in RA patients, when compared to TK (Table I). In JIA the increase was observed in 42-, 37-, and 29-kDa bands, and in GA patients only in 42-kDa band. The amount of fucose in ReA glycoproteins was similar to this present in TK ($p > 0.05$).

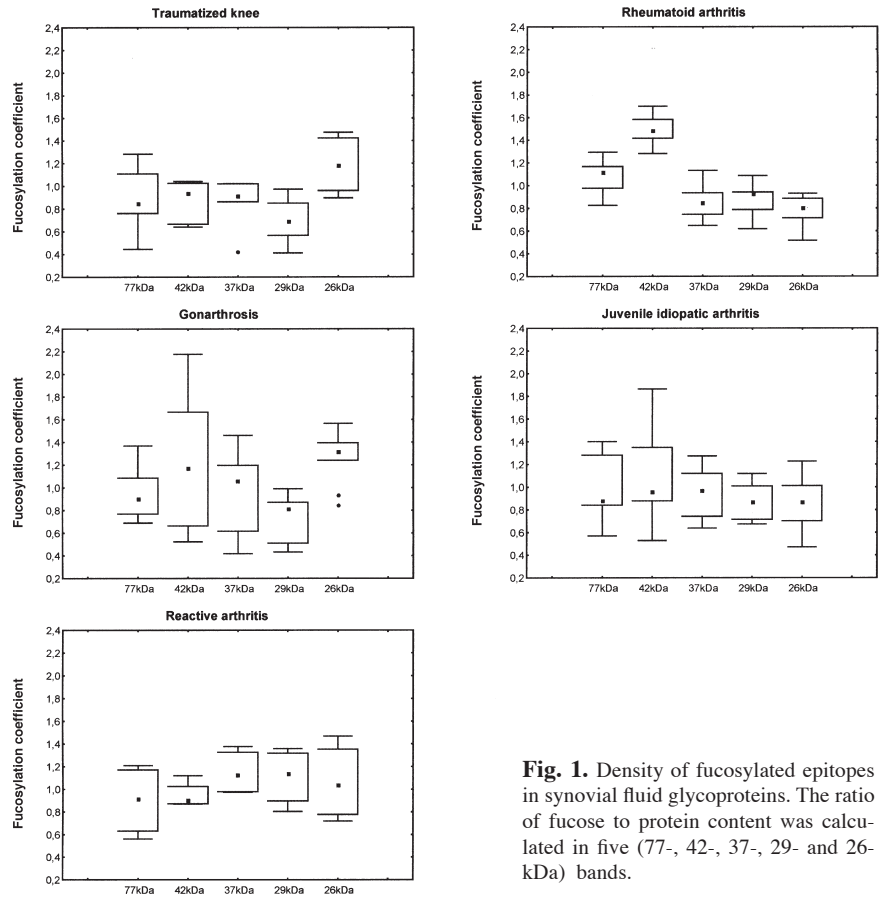

Fig. 1. Density of fucosylated epitopes in synovial fluid glycoproteins. The ratio of fucose to protein content was calculated in five (77-, 42-, 37-, 29- and 26-kDa) bands.

Table II. Statistical relevancy of fucosylation coefficient variation in different synovial fluid glycoproteins in joint diseases.

		42 kDa	29 kDa	26 kDa				
TK	vs. RA	0.0001	0.033	0.00041	vs. JIA	×	×	0.015
GA	vs. RA	×	×	0.00026	vs. JIA	×	×	0.0016

The table contains statistically relevant p values obtained in Mann-Whitney U-test; × - $p > 0.05$.

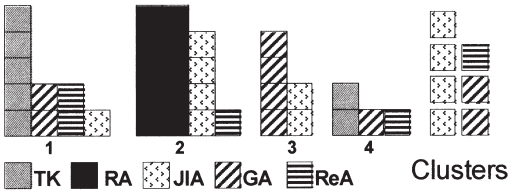

Fig. 2. Group arrangement in cluster analysis

The fucose content in low molecular weight bands of 29- and 26-kDa was as high as in acute phase 42-kDa band in both RA and JIA. In GA, glycoproteins of 37- and 29-kDa contained slightly less fucose units than 42- and 29-kDa bands, but the difference was not statistically significant.

Fucosylation coefficient

The density of fucosylated glycotopes was calculated as the fucose/protein ratio and expressed as fucosylation coefficient (F_R). Distribution of this feature in particular glycoprotein bands and patient groups is shown in Figure 1. In the reference TK group fucosylation coefficient was close to one in bands 77-, 42- and 37-kDa, slightly lower in 29- and higher in 26-kDa band.

In RA the significant increase in the density of fucosylated epitopes is accompanied by lowered F_R in 26-kDa band (Fig. 1). Both changes are of high statistical significance (Table II). Decrease of F_R value in 26-kDa band was observed also in JIA, though its statistical relevance was lower. The 26-kDa band clearly differentiated GA from RA and JIA, as there was slight increase in GA F_R, in a contrary to RA and JIA (Fig. 1). When particular cases were analysed, the F_R of 42-kDa band >26-kDa indicated for RA, and the opposite relation, F_R of 26-kDa band > 42-kDa indicated for GA. This was true for all RA and 8 out of 9 GA patients.

Cluster analysis

Cluster analysis was aimed on grouping samples of the highest similarity. An arrangement of groups is shown in Figure 2. Subjects were grouped into four clusters, with seven cases excluded out of them. Cluster 1 was separated from the other cases at 0.9 distance, indicating for its distinct character. This group contained mainly TK cases (5 out of 7), accompanied with two GA, two ReA and single JIA cases. All ten RA cases were gathered together (cluster 2), indicating for their close similarity. They were accompanied with one ReA and four JIA cases. About 35% of GA cases were gathered in cluster 3. The remaining GA cases were spread over other groups, except the "RA" cluster, and two of them were excluded from the formed clusters. JIA appeared the most variable group, as these cases were present in three clusters and 4 out of 11 cases were excluded out of the groups.

Discussion

Progress in understanding mechanisms of joint diseases still have not solved problems with their differential diagnostics, especially in their early, pre-radiological stages. Application of laboratory tests would bring some help. Reports on altered fucosylation of α_1-acid glycoprotein in the sera of RA patients (6-8) urged us to study this feature also in other glycoproteins and joint diseases of different background.

We have referred fucosylation of synovial fluid glycoproteins in RA, JIA, ReA and GA to synovitis caused by mechanical injury, not complicated with prolonged inflammation or degenerative process. Increase of total fucose content was observed in all groups of patients and almost all analyzed bands, but this feature has not differentiated particular diseases.

Comparison of the density of fucose epitopes was more informative. Both F and P values were referred to hRS, thus, $F_R \cong 1$ means that the density of fucosylated epitopes was similar to this observed in serum acute phase proteins (AGP and Hp) of healthy subjects. Respectively, $F_R > 1$ indicates for higher packing of fucosylated glycans, and $F_R < 1$ means that fucosylation is rather low. The highest F_R values were observed in 42-kDa acute phase proteins in RA. This elevation was statistically relevant towards TK, but not GA, so this single parameter still did not differentiate these diseases. However, F_R values in 26-kDa band and relationships between fucose density in analyzed bands is significantly different in GA and RA and may become a novel differentiating factor. These findings were confirmed by cluster analysis, which has separated GA from RA cases.

Diversity in GA and JIA groups, observed as higher range of F_R values, was confirmed in cluster analysis, which spread these cases over several clusters or excluded them out of groups. This may be related to different course of disease, its severity and duration, and indicates for the necessity of taking these factors under consideration in further studies.

Lectin-sensors were recently involved in structural glycomics (15). This technique is successfully applied in investigation of pathological mechanisms. It may be helpful also in further studies concerning identification of altered glycoproteins and may lead to improve diagnostics of joint diseases.

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