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Increased α3-fucosylation of α_1-acid glycoprotein in Type I diabetic patients is related to vascular function

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Diabetic mellitus is attended by the development of endothelial dysfunction which is suggested to be accompanied with a chronic low-degree of inflammation. During a chronic hepatic inflammatory response, specific changes in glycosylation of the acute phase protein α_1-acid glycoprotein (AGP) can be detected. In this report we studied the changes in glycosylation of AGP in more detail and evaluated the relation between a change in glycosylation of AGP and urinary albumin secretion in Type I diabetic patients. The glycosylation of AGP, studied by crossed affinity immunoelectrophoresis (CAIE) and high pH anion exchange chromatography with pulse amperometric detection (HPAEC-PAD), showed an increase in α3-fucosylation. Staining with an antibody against sialyl Lewis^x (sLe^x) implied that part of the α3-fucosylation was present in a sLe^x-conformation. In the group of Type I diabetic patients with increased urinary albumin excretion, a significant increase in α3-fucosylation of AGP ($p < 0.0005$) could be detected. Therefore, the increased α3-fucosylation of AGP can be used as an additional marker for the development of vascular complications in Type I diabetic patients.

Keywords: Type I diabetic, α_1-acid glycoprotein, α3-fucosylation, (s)Le^x, albuminuria

Abbreviations: AAL, Aleuria aurantia lectin; AGP, α_1-acid glycoprotein; CAIE, crossed affinity immunoelectrophoresis; Con A, concanavalin A; HPAEC-PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; (s)Le^x, (sialyl) Lewis^x.

Introduction

Patients with Type I diabetes mellitus have an increased risk for the development of micro- and macrovascular complications [1–3]. We observed a correlation of the acute phase protein CRP with markers of endothelial dysfunction, which suggested the involvement of an inflammatory response in the development of vascular complications [4].

A feature of the hepatic inflammatory response is a change in the fucosylation of α_1-acid glycoprotein (AGP). AGP is a heavily glycosylated acute phase protein containing five N-linked glycans of the complex type. Heterogenicity in the glycosylation of AGP, such as differences in degree of branching and extent of fucosylation, results in the presence of different glycoforms of AGP in human plasma that are detectable by their reactivity with concanavalin A (Con A) and Aleuria aurantia lectin (AAL), respectively. Specific changes in lectin reactivity of AGP occur during different phases of inflammatory reactions. Acute inflammatory reactions appear to induce a long-lasting increase in AAL reactivity in combination with a temporal increase in Con A reactivity [5–7]. Under chronic inflammatory conditions an increased AAL reactivity is also apparent, but the Con A reactivity is decreased or remains unchanged [5,7–9]. Therefore, determination of both the Con A reactivity and AAL reactivity of AGP can be used to discriminate between acute and chronic inflammation [8,10]. Increased CRP levels and the specific changes in the state of glycosylation of AGP suggested the existence of a chronic inflammatory response in Type I diabetic patients [4].

In the present study we have studied the increased AAL reactivity in Type I diabetic patients ($n = 39$) in more detail.

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To study whether the increased AAL reactivity was of the type Lewis^x (Le^x) and may have led to an increase in sialyl Lewis^x (sLe^x) groups, we have applied high-pH-anion-exchange chromatography (HPAEC) of the PNGase-F released glycans of AGP. In addition we have assayed the activity of plasma α3-fucosyltransferase in order to assess whether a hepatic inflammatory reaction was responsible for the increased fucosylation of AGP. This activity has previously been shown to be a marker for an increased hepatic fucosyltransferase VI activity, the enzyme responsible for the α3-fucosylation of acute phase proteins [11,12].

The second aim of this study was to evaluate the relation between a change in the state of glycosylation of AGP and endothelial dysfunction. Since the development of urinary albumin excretion is believed to be accompanied by a generalized impairment of vascular function we studied the change in fucosylation of AGP in Type I diabetic patients with normo-, micro- or macroalbuminuria.

Materials and methods

Materials

Concanavalin A (Con A) (Type V) and Coomassie Brillant Blue R250 were from Sigma (St. Louis, MO). *Aleuria Aurantia* lectin (AAL) and the AAL-HiTrap column were obtained from Biomed Labs (Newcastle Upon Tyne, UK). Both the FPLC system and the HiTrap columns were obtained from Pharmacia (Uppsala, Sweden), the Dionex system and the Carbopac PA-100 column from Dionex Corporation (Sunnyvale, CA), and the Carbograph SPE columns from Alltech Corporation (Deerfield, IL). GDP-[^{14}C]Fucose, specific activity: 5.15 Ci/mol was purchased from NEN (Boston, MA), polyacrylamide and agarose M from BioRad (Richmond, CA), human plasma protein calibrator (HSPC) from Dakopatts (Glostrup, Denmark) and PNGase-F from New England BioLabs Inc. (Beverly, MA). Calf fetuin was obtained from Gibco BRL (Breda, The Netherlands) and desialylated calf fetuin was prepared by mild acid hydrolysis (0.1 M trifluoroacetic acid, 1 h at 80°C). Mouse anti-sialyl Lewis^x IgM CSLEX-1 was obtained from ATCC (HB 8580), alkaline phosphatase-conjugated goat anti-mouse IgM from Zymed (San Francisco, CA) and monospecific goat anti-human AGP polyclonal antibodies from Dr. A. Mackiewicz (Poznan, Poland).

Source of plasma

Study A

After obtaining informed consent, 39 non-smoking Type I diabetic patients (21 men/18 women) without clinical symptoms of macrovascular complications were recruited from the out-patient clinic of the VU Medical Center. The mean age of the Type I diabetic group was 40 ± 15 years (range: 17–79) for the men and 37 ± 10 (range: 23–56) for the women. For the control group 24 non-smoking non-diabetic subjects (12 males/12 females) of comparable age and sex distribution were recruited.

Study B

60 non-smoking Type I diabetic patients were recruited from the outpatient clinic of De Weezenlanden Hospital, Zwolle, the Netherlands. Patients were categorized into three groups, of 20 patients each, having normoalbuminuria, defined as an urinary albumin excretion (AER) less than 30 mg/24 h, microalbuminuria (AER: 30–300 mg/24 h) and macroalbuminuria (AER: >300 mg/24 h).

Detection of glycoforms of AGP differing in degree of branching and fucosylation of the glycans

Serum AGP concentrations were measured by nephelometry with the Beckman Array® 360 system. The state of glycosylation of AGP was determined by crossed affinity immunoelectrophoresis (CAIE) as described earlier [7]. Briefly, in this two-dimensional immunoelectrophoretic method 1–4 µl of total plasma (approximately 0.8 µg AGP) were subjected to electrophoresis in the first dimension through a lectin-containing polyacrylamide gel. This resulted in the fractionation of AGP into a non-retarded glycoform (C0 and A0) and various retarded glycoforms (C1 and C2, A1 to A5) differing in degree of branching or extent of fucosylation when Con A, respectively AAL was used as the lectin. The glycoforms were detected by immunoelectrophoresis in the second dimension gel against precipitating monospecific goat anti-human AGP polyclonal antiserum and subsequent staining of the resulting precipitation lines by Coomassie Brillant Blue. The relative occurrence of lectin retarded (C1 and C2, A1 to A5) and non-retarded glycoforms (C0 and A0) was calculated from the areas under the curves as determined by Summagraph (ACECAD D-9000) analysis.

The ratio of the peak heights of the weakly retarded glycoform A1 and the strongly retarded glycoform A4 was used as a qualitative index for the extent of fucosylation of the retarded glycoforms by AAL. Control values were obtained by determining the relative occurrence and peak height ratios of AGP glycoforms present in standard serum (HSPC) which was coelectrophoresed in each first dimension run of 14 samples.

Analysis of the glycan structures

AGP was isolated from 0.5 ml plasma of three representative individuals from the control and the Type I diabetic group, by the method of Chan and Yu [13]. The purity of the preparations was controlled by SDS-PAGE as well as Superose 12 HR chromatography. The glycans of 100 µg AGP were released by PNGase-F treatment. Incubations were carried out under reducing conditions in 50 mM sodium phosphate buffer, containing 1% NP-40 and 1000 U PNGase-F (total volume of 50 µl) for 24 h at 37°C. After increasing the volume to 1 ml

with demineralized and filtered water (milliQ water), the released glycans were bound to Carbograph SPE columns and washed with 5 ml milliQ water, which was followed by elution with 3 ml 25% CH_3CN + 0.05% TFA [14]. The glycan fraction was lyophilized, dissolved in 200 µl of phosphate buffered saline (PBS) (pH 8.6) and subsequently subjected to affinity chromatography on a 5-ml AAL-HiTrap affinity column with a flow rate of 1 ml/min. Non-retarded glycans (V0) were collected in the void volume of the column and bound glycans (Vf) were eluted with 10 mM fucose in the running buffer. The fractions were concentrated by lyophylization and desalted by passing over a Bio-Gel P-2 column (1.2 × 45 cm) in milliQ water followed by lyophylization. The fractions were dissolved in 100 µl milliQ water and analyzed by HPAEC-PAD, using a Dionex Carbopack PA-100 column (0.4 × 25 cm) which was run in 0.1 M NaOH at 1 ml/min. Prior to sample injections the column was washed for 5 min. with 0.1 M NaOH/0.5 M sodium acetate followed by 15 min with 0.1 M NaOH. Elution was isocratic with 0.1 M NaOH for 10 min. after which a gradient of sodium acetate was applied by increasing the concentration from 0–0.25 M in 100 min.

Characterization of the Carbopac PA-100 column

Different complex type oligosaccharide structures were used to characterize the Carbopac PA-100 column for the analysis of sialylated and fucosylated complex-type glycans. The structures were kind gifts of Dr. D. Joziasse and Dr. Th. de Vries (Dept. Molecular Cell Biology) and are detailed in Table 1 together with their observed retention times under the conditions employed.

Detection of expression of sLe^x on AGP

Qualitative analysis of the expression of sLe^x epitopes on AGP was performed as described previously on nitrocellulose blots of SDS-PAGE gels using the mouse monoclonal CSLEX-1 [12]. Purified non-fucosylated (A0) and strongly fucosylated AGP-A4 glycoforms, isolated from the plasma of a trauma patient, were used as a negative and a positive control, respectively [15].

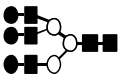
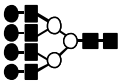
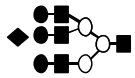
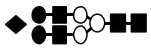
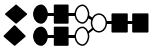
Assay of α3-fucosyltransferase in plasma

The activity of plasma α3-fucosyltransferase was determined directly by using desialylated calf fetuin as an acceptor [16]. All assays were performed in duplicate for 16 h in 10 µl of plasma.

Statistics

All values were tested for statistical significance using the two-sided Student's *t* test. Correlations were estimated according to Pearson. Differences among normoalbuminuric, microalbuminuric and macroalbuminuric subjects were analysed using ANOVA. A two-tailed *p*-value of less than 0.05 was considered significant. All calculations were done using SPSS 9.0.

Table 1. Summary of the different glycan standards used to characterize the Dionex Carbopac PA-100 column. ■ = N-acetylglucosamine, ○ = mannose, ● = galactose, ◆ = sialic acid. First column: in case of a sialic acid addition, the different linkages α2→3 and α2→6 are shown. Second column: in case of a fucose addition, a fucose residue is α1→3-linked to one of the external GlcNAc residues or α1→6-linked to the core GlcNAc

Glycan structure	*Addition*	*Retention time (min)*
	No Fuc	30.3
	+ 1 α3-Fuc	25.1
	No Fuc	31.9
	+ 1 α3-Fuc	27.0
	No Fuc	42.7
	+ 1 α3-Fuc	37.2
	+ 2 α3-Fuc	32.7
	+ 3 α3-Fuc	28.6
	+ 4 α3-Fuc	24.9
 α2 → 3	No Fuc	40.6
 α2 → 3	No Fuc	40.5
 α2 → 6	No Fuc	38.9
	+ 1 core α6-Fuc	37.7
 α2 → 6	No Fuc	50.9
	+ 1 core α6-Fuc	49.7

Results

Characterization of the glycosylation of AGP in Type I diabetic patients (study A)

Concentration and lectin reactivity of AGP in plasma

No significant differences ($p = 0.94$) were found between the plasma AGP concentrations (mean ± SD) of the control group (618 ± 126 mg/ml) and the Type I diabetic subjects (615 ± 208 mg/ml). Analysis of the degree of branching of AGP by CAIE with Con A (Figure 1) showed no significant differences ($p = 0.55$) between the Type I diabetic patients (C0: 49 ± 10, C1: 42 ± 7, C2: 9 ± 6%) and the control group (C0: 48 ± 12, C1: 41 ± 6, C2: 11 ± 8%). In contrast, the total AAL-reactive fraction of AGP was significantly increased in the Type I diabetic group (Figure 1 and Table 2), particularly the strongly AAL-reactive fractions as

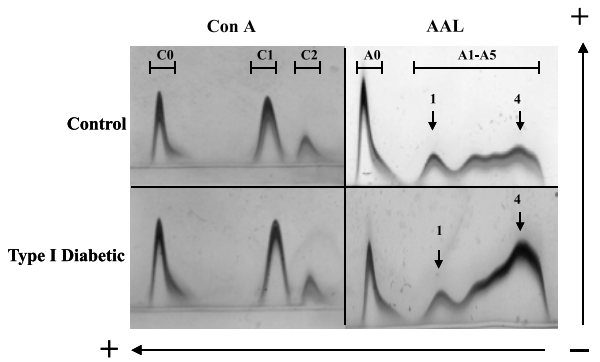


Figure 1. CAIE patterns of AGP of a representative control and a Type I diabetic patient with Con A (left) and AAL (right). The first dimension gel, containing the lectin, was electrophoresed from right to left, the second dimension gel from bottom to top; see Materials and methods for experimental details. C0, AGP glycoforms lacking diantennary glycans; C1, AGP glycoforms with one diantennary glycan; C2, AGP glycoforms with two diantennary glycans(10). A0, non-fucosylated AGP glycoforms, A1–A5, AGP glycoforms reactive to increasing extent with AAL from A1 to A5. The arrows 1 and 4 indicate the positions at which the peak heights were determined for the weakly AAL-reactive A1 and the strongly AAL-reactive A4 glycoform.

Table 2. Reactivity of α_1-acid glycoprotein (AGP) with Aleuria Aurantia Lectin in plasma from Type I diabetic patients and healthy subjects

	Percentage of retarded AGP molecules (A1–A5)	*Peak height ratio A4/A1*
Controls (n = 24)	69 ± 14	0.9 ± 0.4
Type I diabetic patients (n = 39)	79 ± 12	1.7 ± 1.1
p-value	0.005	0.002

Data are expressed as mean ± S.D., see Materials and methods for experimental details and Figure 1 for explanation.

indicated by a significant increase in the A4/A1 peak height ratio relative to the control group (Table 2).

Analysis of glycan structures

The increased AAL reactivity of AGP of Type I diabetic patients was accompanied by an increased expression of sLe^x groups as indicated by the abundant staining with the anti-sLe^x monoclonal antibody CSLEX-1 as compared to AGP from a healthy control (Figure 2). To further characterize the increased fucosylation of AGP, the glycans of AGP isolated from three representatives of both groups were subjected to HPAEC-PAD analysis on a PA-100 column. The glycans were released by PNGase-F treatment and the efficiency of hydrolysis was checked by SDS-PAGE (not shown). Prior to analysis the glycans were subfractionated by AAL-HiTrap affinity chromatography into a non-retarded V0 fraction and a bound Vf fraction

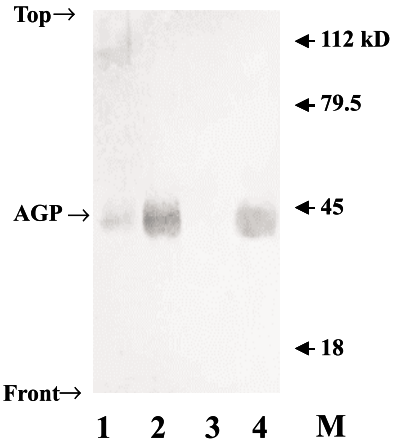


Figure 2. Expression of sLe^x on AGP from a representative control (1), a Type I diabetic patient (2), non-fucosylated AGP A0 glycoform (3) and a strongly AAL-reactive AGP A4 glycoform isolated from a trauma patient (4). Equal amounts of AGP were subjected to SDS-PAGE, subsequent blotting and detection of sLe^x with CSLEX-1. M = broad range molecular weight marker.

to reduce the occurrence of overlapping peaks in the PA-100 chromatograms. All glycans present in the non-fucosylated V0 fractions eluted at positions of the di- (A), tri- (B) or tetrasialylated (C) complex-type glycans (Figure 3, Table 3). The glycans in the Vf fraction did not co-elute with glycans present in the V0 fraction, but were recovered at positions appointed to fucosylated di- (D), tri- (D–F) and tetrasialylated (F–H) complex-type glycans with fucose in an α3-linkage to the N-acetyllactosamine unit(s) (Figure 3 and Table 3). All peaks in the elution patterns of V0 and Vf could be identified with respect to elution position and relative occurrence in the profile of normal pool AGP obtained by Rydén *et al.*, without prior subfractionation of PNGase-F released oligosaccharides on a AAL affinity column [17]. The patterns depicted in Figure 3 clearly show that large differences existed between the various glycans of AGP obtained from healthy controls or Type I diabetic patients. Quantification of the HPAEC-PAD analysis demonstrated a strong increase in fucosylated glycans in Type I AGP relative to control AGP, particularly regarding the tri- and tetrasialylated glycans. This is indicated by the impressive reduction of glycans eluting at position B and C relative to those at position A in the V0 fraction, as well as by the absolute increase in glycans present in the Vf fraction eluting at the positions of α3-fucosylated tri- and tetrasialylated glycans, E–H (Figure 3 and Table 3).

Correlation between AAL reactivity of AGP and plasma α3-fucosyltransferase activity

The activity of α3-fucosyltransferase was measured in plasma of 6 healthy individuals and 6 Type I diabetic patients, which were selected to cover the whole range of A4/A1 peak ratios. As shown in Figure 4, a strong

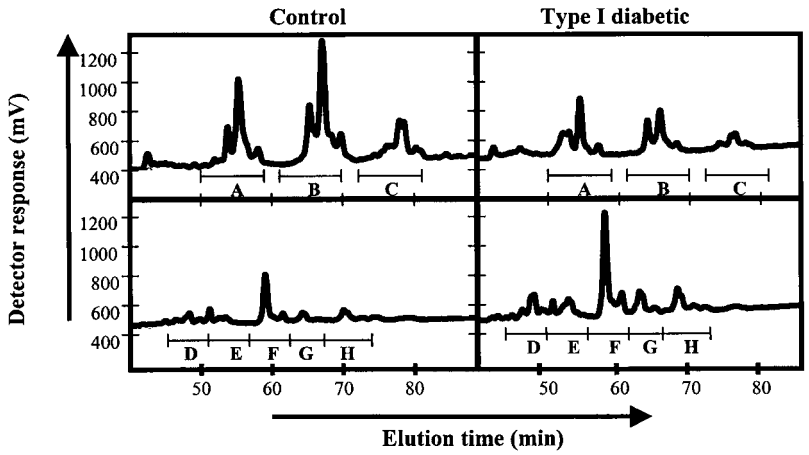


Figure 3. Representative HPAEC-PAD chromatograms of PNGase-F-released glycans of AGP from a healthy control and a Type I diabetic patient after subfractionation of the glycans into a non-retarded (V0) and a retarded fraction (Vf) by AAL-HiTrap chromatography. The patterns were obtained by injection of equal aliquots of the V0 and Vf fractions originating from 100 μg AGP (see materials and methods for details). Areas A–C represent the elution positions of di-(A), tri-(B) and tetrasialylated (C) complex type standard glycan structures. Area D–H represent the provisionally appointed elution positions of mono-(H), di-(G) and trifucosylated (F) tetrasialylated, mono- (F), di- (E) and trifucosylated (D) trisialylated and monofucosylated disialylated (D) complex type glycans (cf. Table 1).

Table 3. Quantification of HPAEC-PAD elution patterns of the V0 and Vf oligosaccharide fractions of control and Type I diabetic AGP

	Glycan distribution (% of total)									
	Glycan distribution (% of total)		*Vo*			*Vf*				
	V0 total	*Vf total*	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Control (n = 3)	76.5 [13.0]	17.3 [1.2]	22.2 [6.1]	42.2 [10.6]	15.3 [3.2]	3.1 [1.9]	3.1 [1.2]	7.6 [1.6]	1.3 [0.9]	2.4 [0.7]
Type I diabetic patients (n = 3)	53.1 [9.7]	45.6 [1.8]	23.1 [4.8]	21.6 [8.7]	7.9 [3.5]	2.0 [2.6]	10.4 [7.1]	21.8 [9.7]	5.2 [2.0]	5.9 [0.5]

See legend of Figure 3 for definition of subfractions A–H. The results are presented as median [range].

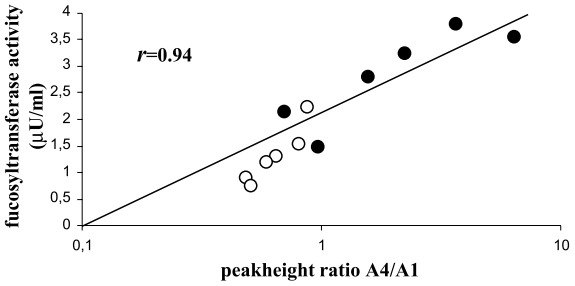


Figure 4. Correlation between the AAL reactivity of AGP and the plasma α3-fucosyltransferase activity. The extent of the AAL reactivity is expressed as the peak height ratios of A4/A1. ○ = control subjects, ● = Type I diabetic patients.

correlation ($r = 0.94$) was detected between the α3-fucosyltransferase activity and the stringency of the interaction of AGP with AAL.

Association between the fucosylation of AGP and albuminuria (study B)

We extended our study by analyzing the fucosylation of AGP in additional Type I diabetic patients categorized as normo-, micro- and macroalbuminuria. Increased albuminuria was associated with a significant increase in fucosylation (A1-5) of AGP across the three groups (72.9 ± 9.5, 76.4 ± 12.4 and $83.7 \pm 16.6\%$, respectively; $p < 0.0005$) (Figure 5 and Table 4). The increase in fucosylation of AGP across the three groups remained when adjusted for age.

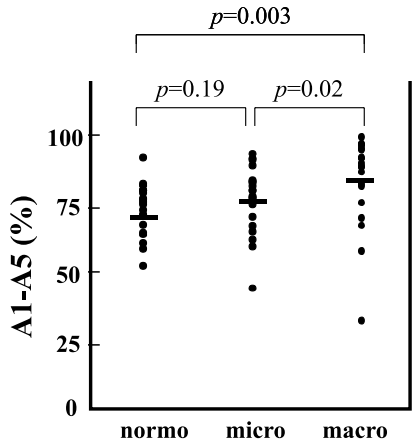


Figure 5. The distribution of A1-5 (%) in Type I diabetic patients categorized as normoalbuminuric (normo), microalbuminuric (micro) and macroalbuminuric (macro) in study B (Table 4). Horizontal lines denote means.

Discussion

In this report we demonstrated that the increased AAL reactivity of AGP in Type I diabetic patients is the result of elevated α3-fucosylation of AGP (Figure 1 and Table 2). Not only the fraction of fucosylated AGP glycoforms is increased, but also the number of fucose residues per glycoform. The fucose residues were present in a Le^x conformation, as could be concluded from HPAEC-PAD analysis of the PNGase-F-released glycans that were bound to an AAL-HiTrap column. These oligosaccharides were recovered at positions appointed to di-, tri- and tetrasialylated complex-type glycans with one, two or three fucose residues in a α3-linkage (Figure 3 and Table 3). This type of substitution of fucose is in accordance with earlier structural analyses of the glycans of AGP [7,18]. The analyses imply that some of the fucose residues are present in a sLe^x conformation. This was confirmed by the abundant staining of AGP from diabetic Type I patients with the anti-sLe^x antibody relative to AGP from healthy controls (Figure 2) [12]. sLe^x-containing human AGP glycoforms have been shown to be able to ameliorate both neutrophil- and complement-mediated injuries and to reduce albumin permeability in lung and intestine in a rat ischemia-reperfusion model [19,20]. Since sLe^x is the ligand for the cell adhesion molecules E- and P-selectin which are involved in the inflammation-dependent adhesion of neutrophils, inflammation induced increase in sLe^x-groups on AGP could function as a feedback inhibition for granulocyte extravasation [25]. Therefore, it is tempting to speculate that the increased fucosylation of AGP is part of a inflammatory reaction to counteract pathologically developing processes in the blood vessel walls occurring during diabetic mellitus. The results of this study extend the list of pathophysiological conditions in which the fucosylation of AGP is altered [21–23]. An increased fucosylation appears related to the disease activity of rheumatoid arthritis, as was shown during remission of the disease induced by pregnancy or methotrexate treatment of patients [24,25]. Furthermore, we have shown that a transiently increased fucosylation occurs during acute-phase responses, whereas oral estrogen treatment and pregnancy induce a transient decrease in the fucosylation of acute-phase proteins in healthy individuals [25,26].

In our previous report, we concluded that the increased plasma CRP concentration in Type I diabetic patients indicated a hepatic inflammatory reaction [4]. The reactivity of AGP with Con A and AAL suggested a chronic rather than an acute inflammatory response. Our present results support the idea that the increased fucosylation of AGP indeed resulted from an increase in the hepatic fucosylation capacity. This can be deduced from the high correlation between the increase in fucosylation of AGP and the plasma α3-fucosyltransferase activity (Figure 4). The activity of this enzyme has been shown to be a marker for the hepatic fucosyltransferase VI activity, the enzyme responsible for the α3-fucosylation of acute-phase proteins [12].

In this study we also showed a significant increase in α3-fucosylation of AGP in the group of Type I diabetic patients with macroalbuminuria compared to the groups with normo- ($p = 0.003$) or microalbuminuria ($p = 0.02$) (Figure 5 and Table 4). This further supports the idea that endothelial dysfunction induces a hepatic inflammatory response. As a

Table 4. Characteristics and fucosylation of AGP in Type I diabetic patients categorized according to albuminuria

	Normoalbuminuria (n = 20)	*Microalbuminuria (n = 20)*	*Macroalbuminuria (n = 20)*	*p-value*
Age (years)	37.4 ± 17.7	46.6 ± 20.5	55.8 ± 11.6	0.002
Diabetes duration (years)	11.0 ± 7.8	17.9 ± 8.6	18.9 ± 10.9	0.028
Albuminuria (μg/min)	7 ± 5	77 ± 45	1212.0 ± 819	< 0.0005
A1-5 (%)	72.9 ± 9.5	76.4 ± 12.4	83.7 ± 16.6	0.034
A4/A1 ratio	1.2 ± 0.3	1.5 ± 0.7	2.7 ± 2.6	0.005

Data are expressed as mean ± S.D.

result, an increased fucosylation of AGP may provide a humoral mechanism for feedback inhibition of granulocyte extravasation and increased endothelial permeability.

In conclusion, the type of changes in AAL reactivity of AGP in Type I diabetic patients, resulted from an increase in α3-fucosylation which gave rise to the formation of sLe^x-groups. Since the increase in fucosylation of AGP correlated with increasing urinary albumin excretion, these results suggest a relation between the state of inflammation and endothelial dysfunction in Type I diabetic patients. Therefore, the increased fucosylation of AGP might be used as an additional marker for the development of vascular complications in Type I diabetic patients.

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