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Glycosylation of α_1-acid glycoprotein in septic shock: changes in degree of branching and in expression of sialyl Lewis^x groups

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The occurrence of differences in acute-phase response, with respect to concentration and glycosylation of α_1-acid glycoprotein (AGP) was studied in the sera of patients surviving or not from septic shock. Crossed affino-immunoelectrophoresis was used with concanavalin A and *Aleuria aurantia* lectin for the detection of the degree of branching and fucosylation, respectively, and the monoclonal CSLEX-1 for the detection of sialyl Lewis^x (SLeX) groups on AGP. Septic shock apparently induced an acute-phase response as indicated by the increased serum levels and changed glycosylation of AGP. In the survivor group a transient increase in diantennary glycan content was accompanied by a gradually increasing fucosylation and SLeX expression, comparable to those observed in the early phase of an acute-inflammatory response. Remarkably, in the non-survivor group a modest increase in diantennary glycan content was accompanied by a strong elevation of the fucosylation of AGP and the expression of SLeX groups on AGP, typical for the late phase of an acute-phase response. Our results suggest that these changes in glycosylation of AGP can have a prognostic value for the outcome of septic shock.

Keywords: α_1-acid glycoprotein, orosomucoid, septic shock, sepsis, sialyl Lewis^x, fucosylation, concanavalin A, *Aleuria aurantia* lectin

Abbreviations: AAL, *Aleuria aurantia* lectin; AGP, α_1-acid glycoprotein; CAIE, crossed affino-immunoelectrophoresis; Con A, Concanavalin A; HSPC, human serum protein calibrator; IL-1, interleukin 1; IL-6, interleukin 6; LIF, leukaemia inhibitory factor; LPS, lipopolysaccharide; SLeX, sialyl Lewis^x; TNF, tumour necrosis factor.

Introduction

Septic syndrome is the systemic response to bacterial infection and is characterized by fever, tachycardia, tachypnea and multiple organ failure [1]. In about 40% of the cases this syndrome results in septic shock leading to an increased lethality. The disease is caused by the presence of bacteria or products of these bacteria, like lipopolysaccaride (LPS) or endotoxin, in the circulation. LPS induces several cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), which play a crucial role in the disease [2]. Interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF) are other cytokines induced during septic shock; they are considered as markers of severity and can be used as prognostic indicators [3–5].

It has been reported that α_1-acid glycoprotein (AGP, orosomucoid) is able to protect mice from lethal shock induced by TNF or LPS [6]. This positive acute-phase protein, which is a glycoprotein of 43 kDa containing five N-linked glycans of the di,-tri- and/or tetraantennary type [7], is subject to a cytokine-induced increase in diantennary glycan content during acute inflammation (see [8] for review) and in intercurrent infections, e.g. in systemic lupus erythematosus [9] and rheumatoid arthritis [10]. A decrease in diantennary glycan content has been described during chronic inflammation such as rheuma-

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toid arthritis [11] and also during estrogen treatment [12, 13]. Changes in the diantennary glycan content of AGP have been shown to correlate with the severity and duration of disease [11, 14]. Another change in glycosylation of AGP was described by us and concerns the degree of $\alpha 1 \rightarrow 3$ fucosylation during acute inflammation [14] and rheumatoid arthritis [15, 16]. The increased $\alpha 1 \rightarrow 3$ fucosylation was accompanied by an increased expression of blood group determinant sialyl Lewis^x (SLeX; NeuAc-$\alpha 2 \rightarrow 3$Gal$\beta 1 \rightarrow 4$(Fuc$\alpha 1 \rightarrow 3$)-GlcNAc-R) [14].

In this study we have analysed to what extent these changes in glycosylation of AGP occur in septic shock and also whether differences exist in this respect between surviving and non-surviving patients, to evaluate a potential prognostic value of these changes for the outcome of septic shock.

Materials and methods

Materials

Aleuria aurantia mushrooms were collected locally and AAL was isolated as detailed earlier [14]. Con A (Type V), Coomassie Brilliant Blue R250, methyl-α-D-glucopyranoside and methyl-α-D-mannopyranoside were purchased from Sigma (St Louis, MO, USA). Human serum protein calibrator (HSPC) and rabbit anti-human AGP IgG (RAH-AGP-IgG) were obtained from Dakopatts (Glostrup, Denmark), polyacrylamide and agarose M from BioRad (Richmond, CA, USA), mouse anti SLeX IgM CSLEX-1 from ATCC (HB 8580), alkaline phosphatase-conjugated goat anti-mouse IgM from Zymed (San Francisco, CA, USA), and *Vibrio cholerae* neuraminidase from Boehringer (Mannheim, Germany). All other materials used were of analytical grade and obtained from commercial sources.

Source of sera

Ten patients suffering from septic shock (according to international definitions [1]) were studied. Five patients survived (three women and two men) and five patients did not (one woman and four men). Sources of sepsis in the survivors: two pulmonary, two skin and one urinary tract and in the non-survivors: two pulmonary, two abdominal and one mediastinal. The mean age in the survivor group was 51 ± 20 and in the non-survivor group: 62 ± 10 years. The mean APACHE score in the survivor group was 29 ± 6 and in the non-survivor group 30 ± 6. The day of death of the five non-survivors varied between 3 and 14 days after admission into the intensive care unit. Four of the five survivors were discharged from the intensive care unit within the first week of admission. Sera were stored at -20 °C until analysis. HSPC consisting of pooled sera from healthy blood donors was used as a standard for determination of control values.

AGP-concentration

Concentrations of AGP were determined by single radial immunodiffusion, according to Mancini [17], using monospecific RAH-AGP-IgG for precipitation; HSPC was used as a standard.

Crossed affino-immunoelectrophoresis (CAIE)

CAIE was performed according to a modification of the Bøg-Hansen method [18], using, instead of 1% agarose in the first dimension gel, 8% polyacrylamide in a 24.3 mM diethylphenobarbituric acid/Tris buffer (pH 8.6) containing 0.4 mM calcium lactate and 0.02% NaN_3. Two mg ml^{-1} Con A was included in the first dimension gel as the diantennary-specific affinocomponent. Con A binds the unsubstituted groups of α-linked, 2-O-substituted mannose residues at carbons 3, 4, and 6 with at least two interacting mannose molecules being required for the binding. As a result, Con A binds with di- but not with tri- or tetraantennary glycans. 2.5 mg ml^{-1} of an AAL preparation (with a haemagglutination titre of 1024) was included as the fucose-specific affinocomponent [14]. Although the binding specificity of AAL is not restricted to the $\alpha 1 \rightarrow 3$ linked fucose residues, in the case of reactivity of AAL with AGP only this type of linkage is detected, because this is the only one present on AGP, as discussed earlier [14]. Separation of the different glycoforms of AGP was obtained by electrophoresis of sera (0.3–1.3 μl) through a lectin containing polyacrylamide slab gel using a Mini-Protean II dual slab gel apparatus (BioRad). Detection of the separated glycoforms was achieved by immunoelectrophoresis in the second perpendicular dimension using the precipitating monospecific antiserum (RAH-AGP-IgG) in a 1% agarose gel [14]. The resulting precipitation curves were visualized by staining with Coomassie Brilliant Blue R250. The areas under the curves indicate the relative amounts of glycoprotein, which were determined in triplicate using a Summagraph (ACECAD D-9000) coupled to a 486 DX PC equipped with an area measurement programme [16].

Partial purification of AGP and detection of expression of SLeX on AGP

AGP was isolated from 20–100 μl of the indicated sera by immunoprecipitation in a micromethod [19] using 300 μl anti-AGP:Sepharose beads (1:1, v/v) in phosphate buffered saline. The eluting buffer was 0.05 M sodium citrate (pH 3.0). SDS-PAGE was performed and the AGP preparations were subsequently blotted onto nitrocellulose by electrophoretic transfer [20]. SLeX determinants were detected by incubating nitrocellulose blots of AGP preparations with the mouse monoclonal anti SLeX IgM CSLEX-1, as previously described [20]. AGP isolated from Cohn fraction V according to Hao and Wickerhauser [21] from pooled normal human serum (a kind gift from Dr D.H.

van den Eijnden) was used as a standard. AGP desialylated with neuraminidase (*Vibrio cholerae*) was used as a negative control [14].

Statistics

All values were tested for significance using the two-sided Student's *t*-test.

Results

Concentration of AGP in septic shock sera

The AGP concentrations in the sera of patients suffering from septic shock were increased compared to control sera, with large interindividual differences. No difference in AGP concentration was found between the surviving and non-surviving patients (Table 1).

Reactivity of AGP with Con A and AAL

To establish whether changes occurred in the diantennary glycan content and/or degree of $\alpha 1 \rightarrow 3$ fucosylation in the sera of patients suffering from septic shock, different AGP glycoforms were fractionated by CAIE with Con A and AAL, respectively, as affinocomponents. Con A fractionates AGP in a non-reactive form C0, containing only tri- and/or tetraantennary glycans, in a weakly reactive form C1, containing one diantennary glycan and in the strongly reactive forms C2 + C3, containing two or more diantennary glycans (see Materials and methods). AAL fractionates AGP in a non-reactive form A0, containing no fucose and in the reactive forms A1–A4 containing fucose in increasing amounts. In Fig. 1 representative precipitation curves are shown for a surviving septic shock patient. Tables 1 and 2 give the distribution of the various glycoforms of AGP in the sera of patients suffering from septic shock, as well as their changes in absolute amounts.

On the first day of septic shock the diantennary glycan content was significantly increased compared to control values (Table 1). However, the absolute amounts of all Con A-fractionated AGP glycoforms were increased compared to control values, except for AGP-C0 of the survivors at day 1, as can be calculated from the data in Table 1. Remarkably, the increase in diantennary glycan content was higher in the survivor group than in the non-survivor group. This can be seen from a significant higher percentage of the strongly reactive fractions with

Table 1. Effect of septic shock on concentration and degree of branching of AGP. Fractionation of AGP in glycoforms differing in degree of branching was performed by CAIE with Con A. Values are given as the mean ± SD. C0, glycoform of AGP non-reactive with Con A, C1–C3 glycoforms containing diantennary glycans in increasing amounts (cf. Fig. 1). The ratios S/W were determined by dividing the sum of the percentages of the strongly reactive glycoforms (C2 + C3) by the percentages of the weakly reactive glycoform (C1). Values of HSPC were determined by five replicate determinations, performed on different days.

	AGP ($mg\,ml^{-1}$)	*Relative occurrence of AGP glycoforms (%)*			*Ratio S/W*
		C0	*C1 (W)*	*C2 + C3 (S)*	
Control (HSPC, $n = 5$)	0.81	41 ± 2	42 ± 2	17 ± 1	0.40 ± 0.03
Survivors Day 1 ($n = 5$)	1.58 ± 0.83	25 ± 4****	39 ± 4	36 ± 5****	0.92 ± 0.19****
Survivors Day 7 ($n = 5$)	1.66 ± 0.59*	34 ± 8	43 ± 3	23 ± 6‡	0.53 ± 0.11*‡‡
Non-survivors Day 1 ($n = 5$)	1.71 ± 0.93	33 ± 5**§	42 ± 4	25 ± 5***§§§	0.60 ± 0.14**§§
Non-survivors Day 7/day of death ($n = 5$)	1.69 ± 1.14	34 ± 4***	41 ± 1	25 ± 5****	0.61 ± 0.08****

*Significantly different from control value $p < 0.05$, **$p < 0.02$, ***$p < 0.01$, ****$p < 0.001$; ‡Significantly different from day 1 $p < 0.01$, ‡‡$p < 0.005$; §Significantly different from day 1 survivors $p < 0.05$, §§$p < 0.02$, §§§$p < 0.01$.

Table 2. Effect of septic shock on concentration and degree of fucosylation of AGP. Fractionation of AGP in glycoforms differing in degree of fucosylation was performed by CAIE with AAL. Values are given as the mean ± SD. A0, glycoform of AGP non-reactive with AAL, A1–A4 glycoforms of AGP containing fucose in increasing amounts (cf. Fig. 1). The ratios S/W were determined by dividing the sum of the percentages of the strongly reactive glycoforms (A3 + A4) by the sum of the percentages of the weakly reactive glycoforms (A1 + A2). Values of HSPC were determined by five replicate determinations, performed on different days.

	AGP ($mg\,ml^{-1}$)	*Relative occurrence of AGP glycoforms (%)*			*Ratio S/W*
		A0	*A1 + A2 (W)*	*A3 + A4 (S)*	
Control (HSPC, $n = 5$)	0.81	34 ± 2	31 ± 2	35 ± 3	1.10 ± 0.13
Survivors Day 1 ($n = 5$)	1.58 ± 0.83	29 ± 11	32 ± 4	39 ± 13	1.23 ± 0.55
Survivors Day 7 ($n = 5$)	1.66 ± 0.59*	20 ± 8**	34 ± 5	46 ± 13	1.42 ± 0.62
Non-survivors Day 1 ($n = 5$)	1.71 ± 0.93	17 ± 7****	26 ± 5	57 ± 13**	2.28 ± 0.92*
Non-survivors Day 7/day of death ($n = 5$)	1.69 ± 1.14	14 ± 4****	26 ± 4*	60 ± 7****	2.42 ± 0.58***

*Significantly different from control value $p < 0.05$, **$p < 0.01$, ***$p < 0.002$, ****$p < 0.001$.

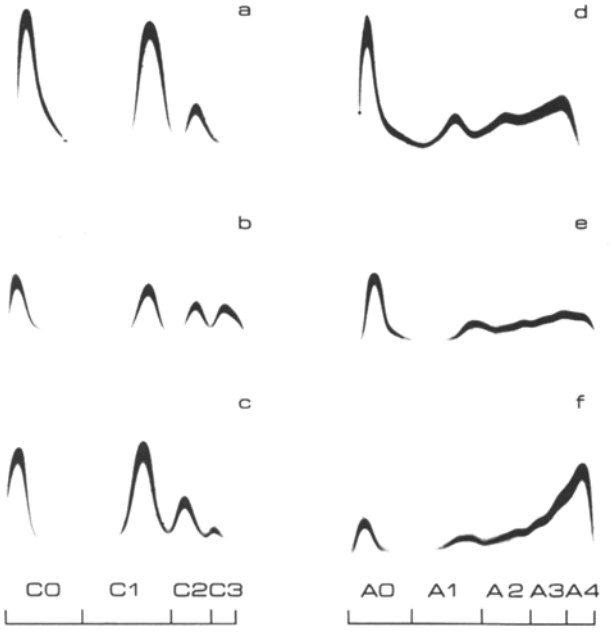

Figure 1. Reactivity of AGP with Con A (a–c) and AAL (d–f) in a patient surviving septic shock. Sera were subjected to CAIE as described in Materials and methods. Only the second dimension gels are shown. The lower right corner of each gel corresponds to the site of application in the first dimension gel. Electrophoresis was performed from right to left for the first dimension, and from bottom to top for the second dimension. C0 and A0: AGP-fractions that are non-reactive with Con A and AAL respectively. a, d: HSPC; b, e: a survivor at day 1; c, f: the same survivor as in b, e at day 7. In the absence of Con A or AAL all AGP was recovered at the site of C0 or A0, respectively.

Con A (C2 + C3), 36 ± 5% vs 25 ± 5% ($p < 0.01$), as well as a higher S/W ratio, 0.92 ± 0.19 vs 0.60 ± 0.14 ($p < 0.02$) (Table 1). The higher increase in diantennary glycan content in the survivor group is confirmed by a significant lower percentage of C0 in this group: 25 ± 4% vs 33 ± 5% ($p < 0.05$) (Table 1). Another difference between the two groups of patients was that only in the survivor group a change towards less reactivity with Con A in comparison to the first day of sepsis became apparent during the period studied (Table 1).

In the course of determining the degree of fucosylation of AGP by CAIE with AAL it became apparent that in this case it was the non-survivor group that expressed an aberrant glycosylation of AGP on day 1. This is clearly illustrated by the significant increase in the strongly fucosylated glycoforms of AGP (A3 + A4) compared to control values, 57 ± 13% vs 35 ± 3% ($p < 0.01$), as well as by the significant increase in S/W ratio and the significant decrease in percentage A0 (Table 2). This increase is not only qualitative (as can be calculated from the data in Table 2) as a two- to three-fold elevation in absolute amounts of the fucosylated glycoforms compared to control values occurred – whereas no increase was found in the nonfucosylated glycoform A0 of AGP, except for the survivors on day 1. During the period studied the degree of fucosylation of AGP remained at this high level in the non-survivor group, whereas in the survivor group an increase in the degree of fucosylation only became apparent during the first week of hospitalization (Table 2, Fig. 1d–f).

Expression of sialyl Lewis x (SLeX) on AGP in septic shock sera

In order to determine if the increases in $\alpha 1 \rightarrow 3$ fucosylation coincided with an increased expression of SLeX, partially purified AGP from the indicated sera was subjected to SDS-PAGE, blotting and subsequent staining with a monoclonal antibody directed against SLeX (CSLEX-1). No enhanced staining compared to control values was found on AGP isolated from sera of the survivors on day 1 of septic shock, but an increase in staining was found on day 7 (Fig. 2, lanes 3–6). A strongly enhanced staining was observed for AGP isolated from sera of a non-survivor on the first day of septic shock, which remained elevated until the day of death (Fig. 2, lanes 7 and 8).

Discussion

A variety of tissue injuries as well as bacterial infections result in a cytokine-induced acute-phase response in mammalian organisms [22]. Characteristic for this response is the increased hepatic synthesis and changed glycosylation of various plasma glycoproteins, one of which is AGP [8, 22]. It is generally assumed that acute-

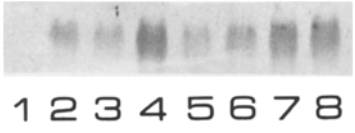

Figure 2. Expression of SLeX on partially purified AGP from septic shock sera. Equal amounts of affinity purified AGP (2 μg) were subjected to SDS-PAGE, subsequent blotting and detection of SLeX with a specific monoclonal antibody (CSLEX-1) as described in Materials and methods. Only the part of the blot containing AGP-bands is reproduced. (1) desialylated-AGP (from pooled normal human sera); (2) AGP from pooled normal human sera (A3 + A4: 35%); (3) AGP from serum of a survivor at day 1 (A3 + A4: 33%); (4) AGP from serum of the same survivor as in lane 3 at day 7 (A3 + A4: 55%); (5) AGP from serum of another survivor at day 1 (A3 + A4: 33%); (6) AGP from serum of the same survivor as in lane 5 at day 7 (A3 + A4: 40%); (7) AGP from serum of a non-survivor at day 1 (A3 + A4: 72%); (8) AGP from serum of the same non-survivor as in lane 7 at the day of death (day 4) (A3 + A4: 71%).

phase proteins neutralize the harmful consequences of the inflammatory reaction. In septic shock, this neutralizing effect has most probably been insufficient, especially in those patients that do not survive, because of the extreme systemic inflammatory reaction. For this reason we have studied the occurrence of differences in acute-phase response, with respect to concentration and glycosylation of AGP, in surviving and non-surviving patients with septic shock.

In both groups of patients the occurrence of an acute-phase response was indicated by the increased serum level and changes in glycosylation of AGP. Remarkable differences were found in the glycosylation of AGP between the two groups, with respect to diantennary glycan content, degree of fucosylation and expression of SLeX. The changes in glycosylation of AGP detected in the survivor group are comparable to those observed in the early phase of (e.g. laparatomy-induced) acute-inflammatory response, i.e. a transient increase in diantennary glycan content for the first few days, accompanied by a gradually increasing fucosylation and SLeX expression, remaining elevated during the study period [8, 14, 20]. Contrary to this, in the non-survivor group the changes are typical for the late phase of such an acute-phase response, i.e. the diantennary glycan content is only elevated to suboptimal levels, and at the same time the fucosylation is strongly increased, as is the expression of SLeX groups on AGP.

We also found a correlation between the severity of disease and the extent of fucosylation and SLeX expression on AGP in rheumatoid arthritis [15, 16], in which the systemic inflammatory reaction also has harmful consequences. These are remarkable findings, for which the implications are not known. However, differences in glycosylation of AGP can influence a number of its immunomodulatory activities and may thereby affect its anti-inflammatory action [23–26]. For example, the diantennary glycan content of AGP affects its inhibitory action on the proliferation of lymphocytes [23, 24] and the AGP-dependent induction of IL-1 inhibiting activity in macrophages [25]. The aggregation of platelets is affected by the degree of sialylation of AGP [26]. More recently, SLeX-containing AGP has been suggested to interfere with leukocyte extravasation by binding to adhesion molecules like the selectins [14]. The functional implications of these changes in glycosylation of AGP in inflammation are as yet unknown and are the subject of further studies in our laboratory. The changes in degree of diantennary glycan content, the fucosylation of AGP and the expression of SLeX groups on AGP can have a prognostic value for the outcome of septic shock.

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