Specific glycosylation of α_1 -acid glycoprotein characterises patients with familial Mediterranean fever and obligatory carriers of MEFV

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Abstract

Background—Familial Mediterranean fever (FMF) is a periodic febrile disorder, characterised by fever and serositis. The acute phase response during attacks of FMF results from the release of cytokines, which in turn induce increased expression and changed glycosylation of acute phase proteins. A recent study indicated that attacks in FMF are accompanied by a rise of plasma concentrations of serum amyloid A (SAA) and C reactive protein (CRP), which remain significantly raised during remission relative to healthy controls. Another study suggested that obligatory heterozygotes also display an inflammatory acute phase response.

Objective—To determine the state of inflammation in homozygotic and heterozygotic MEFV genotypes.

Methods—CRP and SAA were studied by enzyme linked immunosorbent assay (ELISA). The glycosylation of the acute phase protein, α_1 -acid glycoprotein (AGP), was visualised with crossed affinoimmuno-electrophoresis with concanavalin A as diantennary glycan-specific component and Aleuria aurantia lectin as fucose-specific affinity component.

Results—FMF attacks were associated with an increase ($p < 0.05$) in the serum inflammation parameters CRP, SAA, and AGP. The glycosylation of AGP showed an increase ($p < 0.05$) in fucosylated AGP glycoforms, whereas the branching of the glycans remained unaffected. The glycosylation of AGP in the MEFV carrier group, compared with that in a healthy control group, was characterised by a significant increase ($p < 0.05$) in branching of the glycans, whereas the fucosylation remained unaffected.

Conclusion—The findings suggest an FMF-specific release of cytokines, resulting in a different glycosylation of AGP between a homozygotic and heterozygotic MEFV genotype.

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Familial Mediterranean fever (FMF) is an inherited autosomal recessive inflammatory disease affecting mainly north African Jewish, Armenian, Turkish, and Arab populations.¹ In more than 80% of the cases, one or two conservative missense mutations are identified. Most of the mutations are in exon 10 at the carboxy terminal portion of the putative

protein encoded by the MEFV gene on chromosome 16p.^{2,3} The MEFV gene encodes a protein of 781 amino acids termed pyrin or marenostin, which is mainly expressed in mature granulocytes and is supposed to play a part in the down regulation of inflammation mediators.²⁻⁴ Mutations in the MEFV gene are thought to lead to uncontrolled neutrophil activation and migration to serosal tissues. Clinically, FMF is characterised by recurrent attacks of fever, with one or more of the following manifestations: peritonitis, pleuritis, arthritis, myalgia, and erysipelas-like skin lesions.⁵⁻⁷ Colchicine is the preferred treatment in FMF and 65% of patients obtain a complete remission, some 20-30% show significant improvement with a reduction in the number and severity of attacks, but between 5 and 10% do not respond to the drug.^{8,9} Attacks of FMF are associated with an intense inflammatory response and as a result the acute phase reactants C reactive protein (CRP), fibrinogen, and serum amyloid A (SAA), the erythrocyte sedimentation rate, and white blood cell count rise sharply.^{10,11} α_1 -Acid glycoprotein (AGP), another positive acute phase protein, has not been studied in detail before in FMF.

AGP not only rises during inflammation but also undergoes structural modifications, resulting in a change of both the degree of branching and the extent of α_3 -fucosylation of its glycans. This results in the appearance of a variety of AGP glycoforms in plasma.¹²⁻¹⁵ The change in extent of α_3 -fucosylation is not unique for AGP but has also been described for haptoglobin and α_1 -protease inhibitor.¹⁶ An increase in α_3 -fucosylation is accompanied by an increased expression of serum blood group determinant sialyl Lewis X.¹⁷ The state of inflammation—that is, acute or chronic, can be determined by analysing the glycosylation of AGP. During an acute inflammation, as found after an acute insult such as extensive burns or bacterial sepsis, a decrease in branching of the glycans in combination with increased α_3 -fucosylation has been found.^{12,13,17-20} In contrast, in patients with chronic inflammatory disorders like rheumatoid arthritis or insulin dependent diabetes mellitus, an increased branching with a further increase of α_3 -fucosylation can be found.^{14,27,28} Therefore, the changes in relative occurrence of AGP glycoforms, differing in degree of branching and extent of fucosylation, can shed more light on the state of inflammation than the CRP and SAA concentrations alone.

In this study we used the serum concentrations of CRP, SAA, and AGP and the

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Table 1 Concentrations of α_1 -acid glycoprotein (AGP), C reactive protein (CRP), and serum amyloid A (SAA), and reactivities with concanavalin A (Con A) and Aleuria aurantia lectin (AAL) of AGP in controls, MEFV carriers, attack and remission. Values are given as means (SD)

	Controls (n=38)	MEFV carriers (n=11)	Attack (n=8)	Remission (n=11)
AGP (g/l)	0.9 (0.2)	0.9 (0.2)	1.3 (0.3)	0.9 (0.3) [†] ^w
CRP (mg/l)	3.1 (3.2)	5.0 (9.4)	101.7 (110.1)	23.7 (51.6) [†] ^w
SAA (mg/l)	1.1 (0.8)	6.4 (13.7)	214.3 (200.8)	54.1 (138.5) [†] ^w
C0 (%)	49.9 (11.0)	53.2 (11.0)	42.7 (9.4)	50.4 (17.2)
Cw (%)	39.7 (6.5)	40.7 (7.8)	43.8 (4.8)	39.5 (9.9)
Cs (%)	10.4 (5.2)	6.2 (3.9) [†] ^t	13.5 (6.1)	10.1 (9.1)
A0 (%)	37.2 (15.4)	43.0 (24.6)	14.4 (6.4)	28.5 (17.3) [†] ^t
Aw+As (%)	62.8 (15.4)	57.0 (24.6)	85.6 (6.4)	71.5 (17.3) [†] ^t

*Significantly different from control value $p \leq 0.05$ (two tailed); †significantly different from attack value $p \leq 0.05$ (2-tailed); ^w Wilcoxon test; ^t Student's *t* test.

C0 = Con A non-reactive fraction; Cw = Con A weakly reactive fraction; Cs = Con A strongly reactive fraction; A0 = AAL non-reactive fraction and Aw+As = total AAL reactive fractions.

glycosylation pattern of AGP to investigate the state of inflammation in patients with FMF. Further, we also tested these variables in unaffected MEFV carriers as it was recently suggested that this group had significantly higher CRP and SAA concentrations.¹⁰

Material and methods

SOURCE OF SERUM SAMPLES

Serum samples of eight patients with FMF (six male, two female) during attacks, 11 patients with FMF (six male, five female) during remission, 11 MEFV carriers (three male, eight female) were collected for this study. A well defined group, consisting of 38 healthy people with the same ethnic background (non-Ashkenazi Jews) and found negative for the three most commonly encountered mutations (M694V, E148Q, V726A) in this group, were used as controls. The mean age of the patients with attacks was 25 years (range 19–38), the mean age of the patients in remission was 30 years (range 20–46), and the mean age of the control group was 38 years (range 18–56). Serum samples were collected from patients seen in the FMF clinic in the Sheba Medical Centre, Tel Hashomer. FMF was diagnosed according to an established set of criteria.²⁹

DETERMINATION OF ACUTE PHASE REACTANTS

AGP concentration

Concentrations of AGP were determined by single radial immunodiffusion, according to Mancini *et al*, using monospecific precipitating anti-AGP antiserum.³⁰ Human serum protein calibrator with known AGP concentration, consisting of pooled serum samples from healthy blood donors, was used as a standard for the determination of the AGP concentration.

CRP and SAA concentration

CRP and SAA concentrations were determined by a modification of a previously described enzyme linked immunosorbent assay (ELISA).^{31–32} For CRP measurement, goat antihuman CRP, IgG fraction diluted 1:1000 (vol/vol), and biotinylated sheep antihuman CRP (1 ng/ml) were used as the capture and detection antibody, respectively.

For SAA measurement, the IgG fraction of Reu 86.5 diluted 1:1500 (vol/vol) was used as a capture antibody, and for detection a peroxidase labelled IgG fraction of the monoclonal

anti-SAA Reu 86.1 diluted 1:2000 (vol/vol) was used.

CROSSED AFFINOIMMUNOELECTROPHORESIS (CAIE)

This two dimensional electrophoretic technique was performed according to the modified method of Bøg-Hansen.³³ Two different lectins, concanavalin A (Con A) (1 mg/ml) (Sigma, St Louis, MO, USA) and Aleuria aurantia lectin (AAL) (2.5 mg/ml, with a haemagglutination titre of 1/512) (Biomed Labs, New Castle, UK), were used for the binding of diantennary or fucose containing AGP glycoforms. AGP 0.8 μ g (1–4 μ l serum) was electrophoresed through the lectin containing the first dimension gel, and the separated AGP glycoforms underwent immunoelectrophoresis in the second dimension against a precipitating monospecific antiserum. The precipitation curves were visualised by Coomassie brilliant blue R250 staining (Sigma, St Louis, MO, USA). From the areas under the curves the relative amounts of AGP glycoforms were determined by Summagraph (ACECAD D-9000) analysis coupled with an area measurement programme.

STATISTICS

Results are expressed as mean (SD). Statistical differences between groups were tested by Wilcoxon's test for non-parametrically distributed data and Student's *t* test for normally distributed data. A two tailed *p* value of less than 0.05 was considered significant. All calculations were done using SPSS version 9.0.

Results

CONCENTRATIONS OF AGP, CRP, AND SAA

Table 1 shows that the AGP concentration in the attack group was significantly increased compared with values in the remission group ($p < 0.05$). The CRP concentration was significantly higher during attacks than during remission ($p = 0.05$). The same significant difference was found in SAA concentration between the attack group and the remission group. No significant difference could be detected in AGP, CRP, and SAA concentrations between the control group and the MEFV carrier group.

REACTIVITY OF AGP WITH CON A AND AAL

To investigate the glycosylation of AGP with respect to its branching and its α_3 -fucosylation, CAIE was performed with Con A and AAL as

Figure 1 Crossed affinity immunoelectrophoresis patterns of α_1 -acid glycoprotein (AGP) with (A-D) concanavalin A (Con A) and (E-H) *Aleuria aurantia* lectin (AAL) in serum of a representative control (A, E), MEFV carrier (B, F), patient with familial Mediterranean fever (FMF) during attack (C, G), and a patient with FMF during remission (D, H). The first dimension gel, containing the lectin, was electrophoresed from right to left, the second dimension gel, containing the anti-AGP antiserum, from bottom to top; see "Material and methods" for experimental details. C0 and A0 = AGP glycoforms that are non-reactive with Con A and AAL, respectively; Cw and Aw = AGP glycoforms that are weakly reactive with Con A and AAL, respectively; Cs and As = AGP glycoforms that are strongly reactive with Con A and AAL, respectively.

affinity components, respectively. Figure 1 shows CAIE patterns of AGP with Con A and AAL of a representative example of each group. The reactivity of AGP with AAL (Aw+As) was significantly higher in the attack group than in the remission group ($p < 0.05$), whereas no significant differences in Con A reactivity could be detected (fig 1 and table 1). This suggests that with attacks, the fucosylation of AGP is increased but that the degree of branching remains unchanged. There was a significantly lower Con A reactive fraction (Cs) in the MEFV carrier group than in the control group, indicating a lower degree of diantennary branching of AGP in the MEFV carriers ($p < 0.05$). No significant differences could be shown in AAL reactivity between the two groups (fig 1 and table 1).

Discussion

FMF is characterised by short attacks of fever and serositis associated with an increase of inflammatory mediators such as CRP, SAA.^{5-7 34 35} In this paper we describe the changes in concentration and glycosylation of another acute phase protein, AGP, in patients with FMF during attack and remission, and in carriers of MEFV. Our patients with FMF were clinically diagnosed using rigorous criteria, and in the population studied these criteria have a sensitivity and specificity of 99%.²⁹ In general, genetic analysis

of patients with a clinical diagnosis of FMF will yield two mutated alleles in 70%, while in the remaining 30% only one or even no mutations may be found (data not shown). In the group studied we detected two mutations in 12 patients, one mutated allele in three patients, and none in the remaining four patients (data not shown). Accordingly, we could detect MEFV mutations in seven of the parents of MEFV carriers (data not shown). Our genetic results might be biased because we only searched for three (M694V, V726A, E148Q) mutations. However, these mutations represent the most prevalent MEFV alterations in the studied population.³⁶

As indicated by the AAL profiles, an increase in highly fucosylated AGP glycoforms (As), was detected during febrile attacks, whereas in the remission period of FMF the relative occurrence of the highly fucosylated AGP glycoform appears normal. FMF attacks did not influence the degree of branching of the glycans of AGP. The changes in glycosylation of AGP during an attack of FMF point to a hepatic response to chronic inflammation reminiscent of disorders such as rheumatoid arthritis and insulin dependent diabetes mellitus.^{14 27 28} The glycosylation pattern of AGP in patients with FMF differs from that of patients with another autosomal recessive inherited periodic febrile disorder: hyper-IgD syndrome (HIDS).³⁷ In HIDS we detected an increased α_3 -fucosylation of AGP not only during attacks but also in remission, suggesting a constant chronic hepatic inflammatory response.^{18 38 39} Furthermore, attacks in HIDS are associated with an increase in diantennary branching of AGP, which returns to normal values in between attacks. Therefore in HIDS, increased α_3 -fucosylation in combination with an increased diantennary glycan content of AGP is evidence for a period of acute inflammation.

The difference in response between both periodic febrile disorders may originate in the completely different profile of cytokine activation during attacks. Cytokines such as interleukin 1 (IL1), IL6, and tumour necrosis factor α (TNF α) are held responsible for the changed glycosylation of acute phase glycoproteins such as AGP.^{35 40} In patients with HIDS, a release of IL6, IL1ra, sTNF α p55 and p75, TNF α , and interferon γ can be found, whereas during an attack of FMF IL1ra does not rise and appreciable amounts of IL6, sTNF α p55 and p75 only have been detected.³⁵

We detected no significant difference in CRP and SAA concentrations in plasma in the MEFV carrier group relative to the healthy control group, which is in contrast with earlier results.¹⁰ This may be the result of a type 2 error because the concentration of CRP and SAA in plasma in a recently described Dutch control group was shown to be significantly lower than in an MEFV carrier group (data not shown).⁴¹ Despite similar AGP concentrations in healthy controls and MEFV carriers we found significantly lower amounts of diantennary glycan containing AGP glycoforms in the MEFV carrier group. In contrast, the amount of AGP fucosylation was comparable in both

groups. This study may indicate that in an MEFV carrier a specific release of cytokines occurs which can modify the branching of AGP but cannot change the fucosylation of AGP.

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