Inflammation-induced changes in expression and glycosylation of genetic variants of α_1 -acid glycoprotein

Studies with human sera, primary cultures of human hepatocytes and transgenic mice

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The relative occurrence of genetic variants of human α_1 -acid glycoprotein (AGP) in relation to changes in glycosylation was studied in sera of patients with burn injury, media of cytokine-treated primary cultures of human hepatocytes and Hep 3B cells, and sera of transgenic mice expressing the human AGP-A gene. It is concluded (i) that the glycosylation of AGP was not dependent on its genetic expression and (ii) that both the variants determined by the AGP-A gene as well as by the AGP-B/B' genes are increased after inflammation or treatment with interleukins 1 and 6.

INTRODUCTION

Human α_1 -acid glycoprotein (AGP) is a polymorphic glycoprotein containing five complex-type glycans [1]. At least three molecular forms can be distinguished by their interaction with concanavalin A (Con A) [2,3], revealing differences in biantennary glycan content (no, one or two biantennae) [4]. Furthermore, various genetic variants can be detected in serum by isoelectric focusing (i.e.f.) of desialylated AGP [5,6]. These variants are determined by a cluster of three genes [7] of which the AGP-A gene encodes the variants ORM1 and the AGP-B/B' genes the variants ORM2 [8]. The AGP-B and AGP-B' genes are identical; the AGP-A gene is structurally similar, but contains 22 base substitutions. Some of the amino acid substitutions (at positions 20, 73, 76, 77 and 88) corresponding to these are located in the vicinity of glycosylation sites (positions 15, 75 and 85). It is not known, however, whether these amino acid substitutions cause differences in glycosylation of the various gene products.

During the acute-phase response the serum concentration of AGP is increased as a response of the liver hepatocytes to cytokines [e.g. interleukin 1 α (IL-1 α) and interleukin 6 (IL-6)] that are released by monocytes and other cells in the early phase of inflammation or infection [e.g. 9,10]. Concomitantly, the fraction of AGP containing one, but especially two, biantennary glycans is increased [2–4,11]. We and others have shown that this change in glycosylation results from alterations in the biosynthesis within hepatocytes, and that the cytokines IL-1 α and IL-6 are involved in the regulation of these post-translational changes [11–14]. Dente *et al.* [7] have demonstrated that, in human liver, the tautology majority of the AGP mRNA is transcribed from the AGP-A gene and that only this gene is strongly expressed in the human hepatocarcinoma cell line Hep 3B after treatment with conditioned medium of lipopolysaccharides (LPS)-stimulated monocytes. Taken together, these

studies suggest that, during the acute-phase response, an increase of the ORM1 variants is responsible for the elevated serum level of AGP, and moreover, that the ORM1 and ORM2 variants might differ in their biantennary glycan content.

In the present paper we describe further investigations into the occurrence and glycosylation of the various genetic variants of human AGP in studies with sera of burnt patients, with media of primary cultures of human hepatocytes treated with cytokines, and with sera of transgenic mice in which only the human AGP-A gene was expressed.

MATERIALS AND METHODS

Materials

Concanavalin A (Con A) was purchased from EY Labs, San Mateo, CA, U.S.A. Different glycosylated fractions (non-reactive, weakly reactive and reactive with Con A) of human AGP isolated from pooled human sera were prepared by Con A-Sepharose chromatography as described previously [4]. Recombinant murine IL-1 α (sp. activity 3×10^4 units/mg) was obtained from Dr. I. Otterness, Pfizer, Croton, NY, U.S.A.; recombinant human IL-6 (sp. activity 10^6 units/mg) was a gift of Dr. L. Aarden, CLB, Amsterdam, The Netherlands. A crude extract of monocytic products (CM) was prepared by incubation of activated macrophages, isolated from peritoneal exudates induced by glycogen in New Zealand White rabbits, as described by Ritchie & Fuller [15]. It was proven to be endotoxin-free and was shown to have IL-1 activity as determined by the thymocyte-proliferation assay [16]. The other materials were obtained as detailed previously [11,16,17].

Source of sera and secretion media

Serum samples of previously healthy burnt patients were taken within hours of the injury and at regular intervals within the succeeding 2 months; the samples were stored at -70°C . The

Abbreviations used: AGP, α_1 -acid glycoprotein; CM, crude extract of monocytic products; Con A, concanavalin A; CAIE, crossed affino-(ConA)-immunoelectrophoresis; i.e.f., isoelectric focusing; IL-1 α , recombinant interleukin 1 α ; IL-6, recombinant interleukin 6; LPS, lipopolysaccharides; RC, reactivity coefficient.

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patients had burns covering 32–50% of the total body surface area. Initial therapy was directed towards repletion of fluid losses, mainly with saline infusions. The patients displayed acute-phase responses, reflected by prolonged temperature elevation, tachycardia and sharp increases in C-reactive-protein levels.

Human hepatocytes were isolated from post-mortem human liver tissue that was obtained from a kidney donor aged 16 years and were cultured as described previously [11,16]. After isolation and attachment (day 1), cells were cultured in Williams medium E containing 10% (v/v) fetal-calf serum in the presence of 50 nM-dexamethasone (days 1 and 2). On day 3, test substances [IL-1 α (final concn. 100 units/ml), IL-6 (final concn. 2000 units/ml) or CM (final concn. 250 μ g/ml)] were added in refreshed medium containing 1 μ M-dexamethasone. This medium was refreshed every 24 h. Secretion media from day 5 of culture were collected and dialysed for 24 h against 50 mM-NH₄HCO₃/0.1 mM-phenylmethanesulphonyl fluoride/0.02% NaN₃. The resultant solution was freeze-dried and stored frozen at -20 °C.

Transgenic mice in which intact copies of the human AGP-A gene were expressed were generated as described previously [18]. The transgenic mice carried the human gene stably integrated into their genome and could transmit it to their progeny. Although human AGP was secreted into the serum, full expression was reached by injection of LPS (100 μ g) 24 h before serum samples were collected.

Hepatocarcinoma Hep 3B cells were cultured as described previously [7]. Induction was obtained by incubating the cells in the presence of conditioned medium of LPS-stimulated monocytes [7] or with IL-6 for 24 h.

Crossed affino (Con A)-immunoelectrophoresis (CAIE)

CAIE was performed as in [19] as described previously [11], using 1 mg of Con A/ml of agarose in the first-dimension gel and monospecific rabbit anti-(human AGP) antiserum (Dakopatts, Copenhagen, Denmark) in the second-dimension gel. The antiserum did not cross-react with mouse AGP (results not shown). In the second-dimension gel, α -methyl D-glucopyranoside (40 mg/ml) was included to decompose the complexes with Con A. A 0.3–1 μ l portion of serum from burnt patients, 20 μ l of 10-times-concentrated secretion media of primary cultures of human hepatocytes or 8 μ l of serum of transgenic mice was applied. Gels were stained with Coomassie Brilliant Blue R 250; thereafter the area of each AGP form under the precipitation line was measured by planimetry. The total amounts of AGP as present in the various samples were determined by circular immunodiffusion.

Detection of genetic variants of AGP

Variants of human AGP were determined as described by Eap & Baumann [17]. After desialylation with neuraminidase (type V; Sigma, St. Louis, MO, U.S.A.), the samples were subjected to isoelectric focusing (i.e.f.) followed by immunoblotting using rabbit anti-(human AGP) antiserum and goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) for detection. Each sample was analysed in duplicate after serial dilutions to avoid an excess of antigen. The type of variant of the bands obtained was identified by running standard sera [17]. Relative intensities of the three main variants were determined by laser densitometry; each track was determined in two positions.

Table 1. Con A-reactivity of human AGP and relative occurrence of the genetic variants ORM1 S, ORM1 F1 and ORM2 A in sera and secretion media of primary cultures of human hepatocytes and Hep 3B cells under various conditions

I.e.f. patterns (cf. Fig. 2) of human AGP in sera of burnt patients at various days after injury and in secretion media of control and cytokine-treated primary cultures of human hepatocytes were analysed by laser densitometry. Each value is the mean of four determinations. The concentrations of AGP are given relative to the values at day 1 (sera of burnt patients) or to control media of isolated human hepatocytes (HH) and Hep 3B cells (H3B). The reactivity of AGP with Con A (RC) is expressed as the ratio of the sum of the areas of Con A-reactive forms to the area of the non-reactive form. See the Materials and methods section for further details.

Source of AGP	Condition	[AGP] (%)	RC	Occurrence of genetic variants (%)		
				AGP-A gene product		AGP-B/B' gene product ORM2 A
				ORM1 F1	ORM1 S	
Patient 1	Day 1	100	0.7	31.2 $\pm$ 0.3	40.3 $\pm$ 0.4	28.5 $\pm$ 0.6
	Day 3	125	2.2	30.1 $\pm$ 1.9	47.6 $\pm$ 1.3	22.3 $\pm$ 1.0
	Day 12	320	1.8	33.1 $\pm$ 0.1	42.8 $\pm$ 0.6	24.1 $\pm$ 0.6
	Day 60	154	0.9	35.6 $\pm$ 1.6	40.4 $\pm$ 0.0	24.0 $\pm$ 1.6
Patient 2	Day 1	100	2.1	40.7 $\pm$ 1.2	35.9 $\pm$ 0.9	23.4 $\pm$ 0.7
	Day 5	311	2.7	42.4 $\pm$ 1.1	37.7 $\pm$ 0.6	19.9 $\pm$ 0.6
	Day 22	307	1.0	42.0 $\pm$ 2.3	33.3 $\pm$ 2.3	24.7 $\pm$ 0.7
	Day 50	215	0.9	41.1 $\pm$ 2.4	32.7 $\pm$ 0.9	26.2 $\pm$ 1.7
Patient 3	Day 1	100	0.4	36.0 $\pm$ 0.4	30.5 $\pm$ 0.9	33.5 $\pm$ 0.6
	Day 4	208	1.7	44.0 $\pm$ 1.4	35.4 $\pm$ 1.0	20.6 $\pm$ 0.8
	Day 14	526	1.1	39.1 $\pm$ 0.9	36.4 $\pm$ 0.9	24.5 $\pm$ 0.7
Medium HH	Control	100*	1.0*	46.0 $\pm$ 1.7	33.9 $\pm$ 2.7	20.1 $\pm$ 1.3
	IL-1 α	145*	2.8*	41.6 $\pm$ 1.8	37.3 $\pm$ 0.7	21.1 $\pm$ 1.7
	IL-6	182*	3.8*	45.0 $\pm$ 3.0	35.9 $\pm$ 2.6	19.1 $\pm$ 0.5
	CM	191*	2.7*	43.1 $\pm$ 2.1	37.8 $\pm$ 2.1	19.1 $\pm$ 1.3
Medium H3B	Control	100	0	88†	n.d.‡	12†
	CM	450	0	87†	n.d.‡	13†

* Taken from Pos *et al.* [11].

† Means of duplicate measurements.

‡ n.d., not detectable.

Fig. 1. Con A-reactivity of human AGP in serum of a burnt patient (*a*, *b*) and in the serum of a transgenic mouse in which only the A-gene of human AGP is expressed (*c*)

The Con A-reactivity was analysed by CAIE using Con A as an affinity component in the first-dimension gel. The sera were obtained from burnt patient 1 at day 1, immediately after injury, (*a*) and 3 days later (*b*); 1 and 0.3 μ l respectively was applied to the first-dimension gel. In (*c*) 8 μ l of serum of a LPS-treated transgenic mouse was applied. (A) AGP non-reactive with Con A; (B,D) (weakly) reactive forms of AGP.

RESULTS

Effect of inflammation on genetic variants and Con A reactivity of AGP

The reactivity with Con A, as deduced from CAIE patterns, as well as the total amount of AGP showed considerable changes in sera of severely burnt persons during the period after injury (Table 1). At 1 month after the injury, the CAIE patterns of AGP as present in serum were comparable with those of healthy persons, but the serum level of total AGP was still increased in most patients. The shift in Con A-reactivity as determined by CAIE is illustrated for patient no. 1 in Figs. 1(*a*) and 1(*b*).

Three genetic variants of AGP, namely ORM1 F1 and S (AGP-A gene products [8]) and ORM2 (AGP-B/B' gene product [8]), were detected by i.e.f. in the sera of burnt patients. The sera had been treated with neuraminidase to eliminate the charge variability caused in AGP by the carbohydrate structures [17] (cf.

Fig. 2. I.e.f. analysis after desialylation of AGP in sera from a burnt patient (*a*), of isolated AGP forms differing in reactivity towards Con A (*b*), of AGP secreted by primary cultures of human hepatocytes (*c*) and of AGP secreted by Hep 3B cells (*d*)

(*a*) Patterns obtained for burnt patient 1 at various days after injury. For each serum about the same amount of AGP was applied. The dilutions used corresponded to 0.3 μ l serum for days 1 and 60, and to 0.12 μ l serum for days 3 and 12. (*b*) Patterns of different glycosylated forms of desialylated AGP isolated from pooled human sera, non-reactive (A), weakly reactive (B) and reactive with Con A (C). (*c*) Patterns of AGP secreted by primary monolayer cultures of human hepatocytes in the absence (CTL) and the presence of cytokines IL-1 α and IL-6 and conditioned medium (CM) of glycogen-stimulated rabbit macrophages. (*d*) Patterns of AGP secreted by primary monolayer cultures of human hepatocytes in the absence (CTL) and the presence of IL-6 and conditioned medium (CM) of LPS-stimulated monocytes. The last three lanes represent the i.e.f. patterns of mixtures of the genetic variants ORM1 F1, ORM1 S and ORM2 A. In (*a*), (*b*) and (*c*) the position of the standards is indicated by the arrows. See the Materials and methods section for details of the i.e.f. and immunoblotting procedure. New bands appearing on the blot of the media of primary cultures of human hepatocytes are probably due to incomplete desialylation [8].

Fig. 2a). The results presented in Table 1 indicate that the gene products of both the AGP-A gene and the AGP-B or -B' gene were increased by the inflammatory process, since after injury minor changes in the relative occurrence of the genetic variants were accompanied by substantial rises and falls of the total AGP level. The contribution of the products of the AGP-A gene, however, was somewhat higher than those of the other gene(s).

The results further suggested that the small changes in relative occurrence of the genetic variants could not account for the large changes in the Con A-reactivity of AGP. Indeed, all three variants appeared to be present in preparations of AGP isolated from pooled human serum that were fractionated by Con A-Sepharose chromatography in AGP non-reactive (Fig. 2b, A), weakly reactive (Fig. 2b, B) and reactive with Con A (Fig. 2b, lane C). This was further substantiated in studies with sera of transgenic mice in which the AGP-A gene was expressed. Although only one human variant of AGP (ORM1 F1) was present in these mouse sera [8], up to four different molecular forms could be distinguished in CAIE with Con A as the affinity component (Fig. 1c).

Effect of cytokines on genetic variants and Con A-reactivity of AGP secreted by human hepatocytes or Hep 3B cells *in vitro*

In order to investigate whether the concentration of the three genetic variants in serum was influenced by, for instance, differences in turnover, the ability of primary monolayer cultures of human hepatocytes to secrete the different variants was studied. Inflammatory conditions were simulated *in vitro* using IL-1 α , IL-6 or a mixture of crude cytokines (CM) [11,16,20], as illustrated (Table 1) by the increase in Con A-reactivity of AGP [11]. The hepatocyte cultures were able to secrete the ORM1 and ORM2 variants, the latter representing about 20% of total AGP. This value is well comparable with the relative occurrence of the variants observed in the human sera. The proportion of ORM2 A was not influenced by cytokines, despite the fact that the total amount of secreted AGP was increased under the same conditions. Comparable results were obtained for AGP secreted by Hep 3B cells. The amount of AGP secreted was strongly increased by incubating the Hep 3B cells in the presence of conditioned medium, but the relative occurrence of the ORM1 and ORM2 variants was not affected (Table 1 and Fig. 2d). No reactivity of AGP with Con A could be detected under the various conditions, which might be a reflection of the abnormal glycosylation process in the hepatocarcinoma cells [21].

DISCUSSION

Our studies with sera of burnt patients, with primary cultures of human hepatocytes and with Hep 3B cells clearly demonstrate that the gene products of both the human AGP-A gene and the AGP-B/B' genes (the variants ORM1 and ORM2 respectively [8]) contribute to the increased serum level of human AGP during the acute phase of inflammation. This result was unexpected, since in previous studies it had been shown that, in human liver, the level of mRNA transcribed from the AGP-A gene is higher than that transcribed from the AGP-B or -B' gene, and, furthermore, only AGP-A mRNA could be induced by conditioned medium in Hep 3B cells [7]. Our new results, therefore, suggest that altered synthesis of human AGP is also regulated at a post-transcriptional level. Regulation at that level has been reported by Carter *et al.* to occur in rat liver, showing that the turnover [22], as well as microtubule-mediated transport

of rat AGP mRNA [23], is influenced by the inflammatory factors dexamethasone and/or cytokines.

Our study, furthermore, clearly shows that the occurrence of various glycosylated forms of human AGP is independent of the genetic expression of AGP. The sole AGP variant in transgenic mouse serum (ORM1 F1) can be distinguished as four differently glycosylated molecular forms (Fig. 1c). Furthermore, in normal pooled human serum, the individual glycosylated forms each expressed the variants ORM1 F1, ORM1 S and ORM2 A (Fig. 2b). Moreover, the substantial changes in Con A-reactivity during inflammation or after induction *in vitro* by cytokines were accompanied by only minor changes in variant distribution. This implies that the amino acid substitution between the various gene products in the vicinity of the glycosylation sites (see the Introduction) do not influence the final product. This conclusion further substantiates the idea that the post-translational regulation of changes in glycosylation of AGP is dissociated from the regulatory mechanisms of the AGP-gene expression during inflammation. In both processes cytokines appeared to be involved, as revealed in the present, and previous [11,13,14], studies. Further research towards the understanding of the inflammation-induced alterations in glycosylation of AGP should therefore be focused on the way cytokines influence the post-translational processes occurring in the endoplasmic reticulum and in the Golgi system.

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REFERENCES

- Schmid, K., Nimberg, R. B., Kimura, A., Yamaguchi, H. & Binette, P. (1977) *Biochim. Biophys. Acta* **492**, 291-302
- Nicollet, I., Lebreton, J.-P., Fontaine, M. & Hiron, M. (1981) *Biochim. Biophys. Acta* **668**, 235-245
- Mackiewicz, A., Marcinkowska-Pieta, R., Ballou, S., Mackiewicz, S. & Kushner, I. (1987) *Arthritis Rheum.* **30**, 513-517
- Bierhuizen, M. F. A., De Wit, M., Govers, C. A. R. L., Ferwerda, W., Koeleman, C., Pos, O. & Van Dijk, W. (1988) *Eur. J. Biochem.* **175**, 387-394
- Berger, E. C., Wyss, S. R., Nimberg, R. B. & Schmid, K. (1980) *Hoppe Seyler's Z. Physiol. Chem.* **361**, 1567-1572
- Eap, C. B., Cuendet, C. & Baumann, P. (1988) *Hum. Genet.* **80**, 183-185
- Dente, L., Pizza, M. G., Metspalu, A. & Cortese, R. (1987) *EMBO J.* **6**, 2289-2296
- Tomei, L., Eap, C. B., Baumann, P. & Dente, L. (1989) *Hum. Genet.* **84**, 89-91
- Baumann, H., Jahreis, G. P., Saunders, D. N. & Koj, A. (1984) *J. Biol. Chem.* **259**, 7331-7342
- Gauldie, J., Richards, C., Harnish, D., Lansdorp, P. & Baumann, H. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7251-7255
- Pos, O., Moshage, H. J., Yap, S. H., Snieders, J. P. M., Aarden, L. A., Van Gool, J., Boers, W., Brugman, A. M. & Van Dijk, W. (1989) *Inflammation* **13**, 415-427
- Pos, O., Van Dijk, W., Ladiges, N., Linthorst, C., Sala, M., Van Tiel, D. & Boers, W. (1988) *Eur. J. Cell Biol.* **46**, 121-128
- Mackiewicz, A., Ganapathi, M. K., Schultz, D. & Kushner, I. (1987) *J. Exp. Med.* **166**, 253-258
- Mackiewicz, A. & Kushner, I. (1989) *Ann. N.Y. Acad. Sci.* **557**, 515-517
- Ritchie, D. G. & Fuller, G. M. (1981) *Inflammation* **5**, 275-267

16. Moshage, H. J., Rijntjes, P. J. M., Hafkenscheid, J. C. M., Roelofs, H. M. J. & Yap, S. H. (1988) *J. Hepatol.* **7**, 34–44
17. Eap, C. B. & Baumann, P. (1988) *Electrophoresis* **9**, 650–654
18. Dente, L., Ulrich, R., Tripodi, M., Wagner, E. F. & Cortese, R. (1988) *Genes Dev.* **2**, 259–266
19. Bøg-Hansen, T. C., Prahl, P. & Løvenstein, M. (1978) *J. Immunol. Methods* **22**, 293–307
20. Moshage, H. J., Roelofs, H. M. J., Van Pelt, J. F., Hazenberg, B. P. C., Van Leeuwen, M. A., Limburg, P. C., Aarden, L. A. & Yap, S. H. (1988) *Biochem. Biophys. Res. Commun.* **155**, 112–117
21. Goldberger, G., Arnaout, M. A., Aden, D., Kay, R., Rits, M. & Colton, H. R. (1984) *J. Biol. Chem.* **259**, 6492–6497
22. Carter, K. C., Bryan, S., Cadson, P. & Papaconstantino, J. (1989) *J. Biol. Chem.* **264**, 4112–4119
23. Carter, K. C., Cooper, R., Papaconstantino, J. & Ritchie, D. G. (1989) *J. Biol. Chem.* **264**, 515–519

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