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α_1-Acid glycoprotein (orosomucoid): pathophysiological changes in glycosylation in relation to its function

W. VAN DIJK*, E. C. HAVENAAR and E. C. M. BRINKMAN-VAN DER LINDEN

Department of Medical Chemistry, Faculty of Medicine, Vrije Universiteit Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

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The aim of this review is to summarize the research efforts of the last two decades with respect to (i) the determination and characterization of the changes in glycosylation of AGP under various physiological and pathological states; and (ii) the effects of such changes on its possible anti-inflammatory functions. It will become clear that the heterogeneity observed in the glycosylation of AGP in serum, represents various so-called glycoforms of AGP, of which the relative amounts are strictly determined by the (patho) physiological conditions.

Keywords: α_1-acid glycoprotein, orosomucoid, glycosylation, acute inflammation, rheumatoid arthritis, fucosylation

General properties of AGP

Human AGP, also called orosomucoid, is a plasma glycoprotein of 41–43 kDa molecular weight with a high carbohydrate content (45%). The carbohydrate part consists of five complex-type N-linked glycans (Fig. 1) [1, 2]. AGP has been thoroughly investigated because this is one of the few serum glycoproteins that contains tetraantennary as well as di- and triantennary, N-linked glycans. This high degree of branching, in general, also results in a high degree of sialylation, giving rise to a very low pI of 2.8–3.8 [3]. Rat and mouse AGP contain 4–6 N-linked complex-type glycans which are also heavily glycosylated [2, 4]. At least 12 glycoforms of AGP can be detected in normal human serum [5]. They differ in the degree of branching (di-antennary *versus* tri- or tetraantennary glycans) as well as in the degree of fucosylation and sialylation. Studies from various laboratories (reviewed below) have shown that this microheterogeneity of AGP does not represent a non-specific process but is strongly dependent on the (patho) physiological conditions.

Various genetic variants of human AGP can be detected in serum by isoelectric focusing (IEF) after desialylation of AGP [6]. These variants are determined by a cluster of three genes of which the *AGP-A* gene encodes the variant ORM1 and the *AGP-B/B′* genes encode the variant ORM2 [7]. The *AGP-B* and *AGP-B′* genes are identical; the *AGP-A* gene is structurally similar but contains 22 base substitutions.

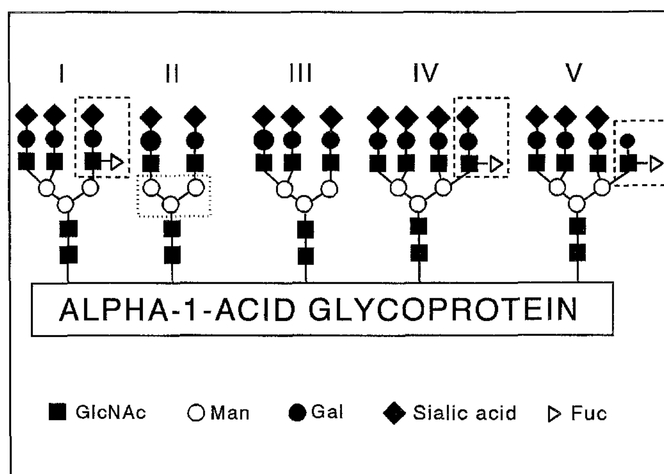

Figure 1. Schematic representation of the carbohydrate structures as they may occur on a molecule of human AGP containing one diantennary glycan. Five glycosylation sites on human AGP contain N-linked structures. The box in II surrounds the Con A binding site of the diantennary glycan. The boxes in I and IV surround the structure SLEX and the box in V the structure LEX which both are ligands for AAL. (Slightly modified from de Graaf TW *et al.* (1994) *J Rheumatol* **21** (12): 2209–16. With permission.)

The serum concentration of AGP increases several-fold during acute-phase reactions e.g. severe burning or trauma, but

*To whom correspondence should be addressed.

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also under chronic pathological conditions, like rheumatoid arthritis and tumour growth [8]. This occurs in man as well in a variety of other species [4, 9–13]. Corticosteroid hormones and inflammatory cytokines are involved in the regulation of these changes, by affecting the rate of synthesis of AGP by the parenchymal cells of the liver, the so-called hepatic acute-phase response [14]. Therefore, AGP is included among the positive acute-phase proteins. Two distinct phases have been found for the turnover of ^{125}I-labelled human AGP injected in man, with $t_{1/2}$ of 6 h and 65 h [15]. For human AGP injected in rats, a turnover rate of about 15 h has been reported [16, 17]. No data are available regarding the effect of inflammation-induced changes on the turnover rate, or the mechanisms involved in AGP turnover.

The biological function of AGP is still obscure. However, a number of activities of possible physiological significance have been described, such as various immunomodulating effects [19–28], the ability to bind basic drugs like warfarin [29] and tamoxifen [30], and the ability to bind vanilloids [31], phorbol esters [31], plasticizers [32], IgG3 [27, 28], heparin [33], serotonin [34] and certain steroid hormones [32, 35, 36]. Because of the latter ability, AGP has been suggested to be a functional member of the lipocalin family [37]. This family comprises a group of proteins that are structurally related to serum retinol-binding protein, with regard to amino acid sequence, disulphide bond arrangement and gene structure. Other physiological functions for AGP are also possible, because part of its nucleotide sequence is significantly similar to regions of the extracellular domain of the EGF receptor [38]. A number of the activities of AGP have been shown to be dependent on the glycoform of AGP, i.e. the composition of the five asparagine-linked glycans of the molecule [18, 19, 39, 40]. This is very interesting, because, as outlined below, the serum concentration of specific glycoforms of AGP change markedly under acute or chronic inflammatory conditions, as well as in pregnancy and tumour growth. This may result in a (patho)physiological dependent change in the carbohydrate-dependent activities of AGP.

In this review, results of studies on the occurrence, the regulation and the possible anti-inflammatory aspects of changes in glycosylation of AGP will be discussed. The studies have revealed that the (patho) physiological variations in the glycosylation of AGP are under strict physiological control, and independent of the changes in its serum concentration, underscoring the importance of this phenomenon.

Detection and quantification of AGP glycoforms

In most studies, the detection and quantification of different glycoforms of AGP in serum or cell culture media have been performed using the elegant Bøg-Hansen technique of crossed affino-immunoelectrophoresis (CAIE) [41], with lectins used as the carbohydrate-binding component in the first dimension gel and a polyclonal anti-AGP IgG used for immunoprecipitation in the second dimension gel. In this technique glycoforms

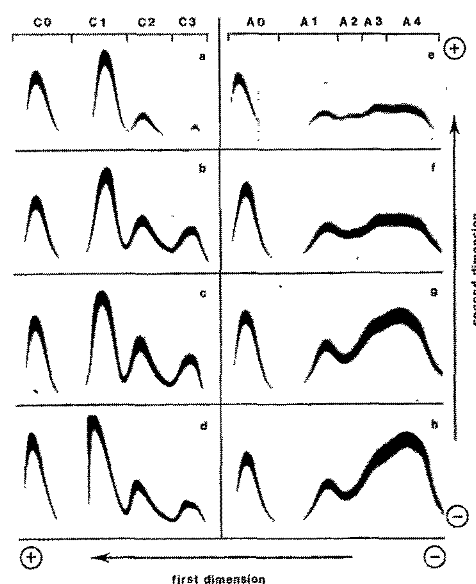

Figure 2. Reactivity of AGP with Con A and AAL before, and at various days after laparotomy. Sera were obtained 1 day before (a, e) and 1 (b, f), 2 (c, g) and 4 days (d, h) after the removal of a benign tumour of the uterus from an otherwise healthy woman. One µl of serum was analysed by CAIE with Con A or AAL. Only the second dimension gels are shown; the application sites in the first dimension gels coincide with the right hand side of each Figure. A0 and C0 are the AGP fractions non-reactive with AAL and Con A respectively; A1–A4 and C1–C3, are the AGP fractions reactive with AAL and Con A respectively, in increasing order of reactivity. (From de Graaf TW *et al.* (1993) *J Exp Med* **177**:657–66. With permission.)

expressing lectin-reactive properties form complexes with the lectin. These complexes are retarded relative to the non-reactive glycoform, because the electrophoresis is performed at a pH close to the isoelectric point of the lectin. The extent of retardation is a measure for the number of lectin-binding sites on the glycoform [41, 42]. Most CAIE studies have been performed using the lectin, Concanavalin A (Con A), as the affino-component. In this way AGP is fractionated according to its diantennary glycan content, as was established by us by detailed analyses of the carbohydrate structures on the various fractions [43]. Fractions lacking (C0) or containing one or more diantennary glycans (C1–C3; see below) are well separated and can be quantified by measuring the areas under the precipitin lines in the second dimension gels (Fig. 2A). Recently, we showed that AGP can also be fractionated according to its fucose content, using the fucose-specific *Aleuria aurantia* lectin (AAL) as an affino-component in CAIE (Fig. 2E) [44]. This is the only lectin to date that will react with the fucosylated lactosamine units present on AGP (see Fig. 1). Both methods can be applied directly to the analysis of serum (Fig. 2), and also for preparative affinity elec-

trophoresis (Table 1) [5, 44]. From our studies it appeared that: (i) in control male serum 33 ± 6% of human AGP did not contain fucose at all (A0); and (ii) that a high degree of fucosylation (A3 and A4) was associated with a low content or a total absence of diantennary glycans, and with a high content of tri- and/or tetraantennary glycans [5].

Table 1. Sequential fractionation of AGP glycoforms by AAL and Con A.

	Percentage of total AGP					
	Total AGP	*A0*	*A1*	*A2*	*A3*	*A4*
Total AGP	100	28	15	11	22	24
C0	42	11	6	5	9	11
tC1	43	12	7	5	9	10
C2 + C3	15	5	2	2	4	2

Preparative affinity electrophoresis with AAL as a lectin was used to fractionate human serum AGP into non-fucosylated (A0) and various fucosylated fractions (A1–A4). The five fractions obtained were then subjected to CAIE with Con A [5]. The percentages of the resulting glycoforms were calculated using the AAL reactivity of total AGP (*cf.* line 1). Results of a typical experiment are given as obtained for AGP purified from pooled sera (De Graaf TW, Van Dijk W, unpublished results).

Occurrence of variations in AGP glycoforms

Acute inflammation

Substantial increases in glycoforms expressing diantennary glycans are apparent in the early phase of an acute-phase reaction [3, 5, 8, 10, 11, 44–48]. This is illustrated by the large increase of AGP-C2 and AGP-C3 in the sera of a patient suffering from surgical trauma following laparotomy (Fig. 2 A–D). This increase is transient reaching a maximum value at the 2nd day, after which it gradually decreases to control levels between the 15 and 30 days, whereas the plasma level of total AGP is still increased [44, 46].

Acute inflammation also induces large changes in the fucosylation of AGP as is illustrated for the same patient by the strong increase of highly fucosylated AGP-A3 and AGP-A4 and the decrease in the proportion of non-fucosylated AGP-A0 (Figs 2E-H) [44]. It can be seen that the kinetics of these changes in fucose glycoforms differ from the variation in diantennary glycan content. Maximum values were reached at the fourth day and persisted to the end of the period studied. Corresponding time-dependent changes in fucosylation and the degree of branching of the glycans were detected by us under other sterile acute inflammatory conditions, like severe trauma following burning or traffic accidents [5, 44, 47]. In all patients studied the degree of fucosylation was still strongly increased when they were released from the hospital after 10–30 days, whereas the diantennary glycan content had returned to normal values within 9–14 days after hospitalization. The serum level of total AGP was also still increased at the time of release from the hospital. This long-term acute-phase effect on the fucosylation of AGP, as well as on the serum level of total AGP, was also found by us during the 4–6 weeks remissions between recurrent febrile attacks in patients suffering from hyperimmunoglobulinaemia D and periodic fever syndrome [49]. Again, the febrile attack induced an increase in diantennary glycan content of AGP which returned to near control values during remissions. Taken together, these studies show that the paired measurement of Con A and AAL reactivity with AGP are useful clinical parameters in the determination of the recovery of a patient from severe trauma.

Using the monoclonal IgM, CSLEXI, we have demonstrated that the acute-phase-induced increase in fucosylation of AGP is at least partly due to sialyl Lewis^x (SLEX; NeuAcα2 $\rightarrow$ 3Galα1 $\rightarrow$ 4(Fucα1 $\rightarrow$ 3)GlcNAc-R) [5, 44]. The SLEX content of AGP is very low in control serum, and as expected, no SLEX could be detected on non-fucosylated AGP-A0 [5]. The highest SLEX content was found on the strongly fucosylated AGP-A3 and AGP-A4. The Con A reactivity, and thus, the diantennary glycan content of the latter two fractions is also lower than for AGP-A0, plus AGP-A1, plus AGP-A2 (Table 1) [5]. An inverse biosynthetic relationship exists between the diantennary glycan content of AGP and SLEX expression, i.e. the increase in SLEX content is especially expressed on tri- or tetraantennary glycans. This is in line with existing knowledge about the branch specificity of the liver enzymes, α2-3 sialyltransferase, α2-6 sialyltransferase and α1-3 fucosyltransferase [50–53]. The α2-6 sialyltransferase has a decreased ability to sialylate N-linked glycans with more tri and tetra-antennary than diantennary structures. In contrast, α2-3 sialyltransferase and α1-3 fucosyltransferase, responsible for the expression of SLEX, show a slightly elevated activity for the higher branched glycans. Furthermore, the action of these two enzymes are mutually exclusive with the action of α2-6 sialyltransferase. So, inflammation-induced changes in the relative activities of these enzymes, as well as the transient change in degree of branching of the glycans, could determine the SLEX expression on AGP. Further studies are needed to delineate these processes.

So far, studies of AGP in species other than man have been restricted to changes in Con A reactivity in lipopolysaccharide-, turpentine- or laparotomy-treated animals. An experimentally-induced acute-phase response resulted in an increase in the Con A reactivity of AGP in the sera of rats [10, 12, 45, 54] and cows [13] that was comparable to those found for human sera AGP. The glycosylation of mouse AGP can also be influenced by experimental acute inflammation, but both increased and decreased Con A reactivity occur [12]. A prominent increase in Con A reactivity, but on a different time-scale, was also found when phenobarbital was used to induce increased serum levels of AGP in rats [55]. However, this involved a different mechanism of induction, because pheno-

barbital can induce cytochrome P 450 related pathways, which decrease during acute inflammation [56].

Other conditions

Changes in glycosylation of AGP are not restricted to acute inflammatory conditions but also occur in a wide variety of other (patho)physiological conditions [8, 48]. However, the quality as well as the quantity of the changes are different from the acute condition, in that frequently, a moderate to strong decrease in Con A reactivity, i.e. a decreased diantennary glycan content, is observed for AGP. Examples are late pregnancy [57], severe (Grade III and IV) rheumatoid arthritis [58], alcoholic liver cirrhosis [59] and hepatitis [60, 61]. Occurrence of acute inflammation, like intercurrent infections in rheumatoid arthritis and systemic lupus erythematosus, however, induce a strong increase in Con A reactivity of AGP [62, 63]. This indicates that different inflammatory mediators are involved in the regulation of the degree of branching of AGP in acute and chronic inflammation.

Strongly elevated levels of highly fucosylated AGP-A3 and AGP-A4 were also found by us in patients suffering from rheumatoid arthritis. The degree of fucosylation decreased in patients responding to treatment with low-dose methotrexate [64]. As in acute inflammation, the increase in fucosylation appeared to reflect an increase in SLEX content of AGP fractions (Havenaar EC and Van Dijk W, in preparation). Increased fucosylation and SLEX expression on AGP have been described for another chronic condition, alcoholic liver cirrhosis, by Durand and co-workers [65]. So, it appears that high levels of SLEX-expressing glycoforms of AGP can occur in the late phase of acute inflammatory reactions as well as in a number of chronic inflammatory conditions.

Regulation of the changes in glycosylation

The changes in glycosylation described above clearly exceed basal levels, because the variation observed in the occurrence of the various Con A- and AAL-reactive glycoforms of AGP in the sera of apparently healthy individuals was about 5%, when studied over a period of 30 days [66]. Inter-individual differences in Con A reactivity were found, but these were largely due to the use of oral contraceptives [66, 67]. The latter resulted in a decreased Con A reactivity for AGP and a somewhat lower concentration of total AGP relative to non-users and men. These changes could be induced directly or indirectly by the estrogen, because the same type of change was found during late pregnancy, and also in the treatment of *prostate* tumours with estrogen in males [57, 66–69]. These findings suggest that estrogen may particularly affect the biosynthesis or increase the turnover rate of diantennary containing AGP glycoforms. The mechanism for its action is unknown, because these effects have not been studied *in vitro*.

Most studies on the regulation of (patho)physiological changes in glycosylation of AGP have focused on changes in branching of AGP, because changes in fucosylation and SLEX expression have only been described recently [5, 44, 47, 49]. Besides estrogen, glucocorticoids and the cytokines IL-6, IL-1β and TNF-α [10, 70–74] have been shown to be involved in the induction of diantennary containing glycoforms of AGP. This has been shown *in vivo* by injection of the various compounds in rats [71, 72], but also in studies using isolated rat [10, 73, 74] and human hepatocytes [71]. The *in vitro* studies clearly showed that the hepatocytes can secrete all the glycoforms of AGP detectable in human serum by CAIE with Con A [71] or AAL [unpublished results]. Furthermore, incubation of rat and human hepatocytes in the presence of IL-6, IL-1 and dexamethasone could mimic the acute-phase induced changes in diantennary glycan content in rat and human sera. This is an important finding, because it indicates that the changes in glycosylation most probably are biosynthetic in nature. However, changes in the turnover rate of specific glycoforms [17], as well as contributions from extrahepatic cells, cannot be totally excluded. Several reports have indicated that AGP can be secreted by human lymphocytes, granulocytes and monocytes [75, 76]. Since inflammation is associated with proliferation of leukocytes, part of the observed changes in glycosylation and synthesis of AGP in inflammation could originate from these cells [76].

Another conclusion emerging from previous experiments is that the induction of the changes in glycosylation is uncoupled in time from the changes in secretion [44, 46, 71, 73, 74], i.e. different signalling pathways must exist for the induction of these changes, and the induction of AGP mRNA by the same inflammatory mediators. Our studies with human AGP support the latter conclusion, because changes in the relative proportions of the products of the three *AGP* genes induced by acute inflammation could not account for the alterations in the diantennary glycan content of AGP [77–79]. However, the effect of increased protein production on glycosylation cannot be totally excluded, because this was suggested by studies with isolated hepatocytes from transgenic mice, in which the rat *AGP* gene was overexpressed [11]. Furthermore, an altered rate of intracellular transport, or an altered route of transit of AGP, has been suggested to exist in the Golgi system during inflammation [80–82].

Overall, the studies support the idea that pathophysiological variations in different glycoforms of AGP in serum result from changes in the glycosylation process during their biosynthesis in the parenchymal cells of the liver. Studies with the human hepatoma cell lines Hep G2 and Hep 3B have confirmed that hepatic glycosylation to acute-phase proteins is strongly dependent on the interplay of cytokines, growth factors, and hormones [83, 84]. The mechanism is still unknown and is subject to further studies (see [48] for recent review of possible mechanisms).

Glycosylation dependent properties of AGP

A great number of properties have been described for AGP, but the exact physiological function of the molecule is not

known yet. The best described properties are related to its ability to bind basic lipophilic compounds. This includes a large group of molecules ranging from steroid hormones to plasticizers (see General Properties of AGP). It has been shown that changes in the plasma level of AGP during inflammation can affect the distribution of free and protein-bound drugs, and therefore the effective concentration of the drug in blood [34, 35]. Studies performed so far do not show that the drug-binding ability of AGP is affected by changes in glycosylation. However, Parivar *et al.* [17] have reported that acute inflammation can induce differences in the distribution volume of various glycoforms of AGP. This was observed in studies in which different glycoforms of human AGP were injected intravenously into control rats and ones in which the acute-phase reaction was activated. In control rats, no differences existed between the steady-state distribution volume (about 90 ml kg^{-1}) of glycoforms containing only tri- and tetraantennary glycans (C0) and glycoforms containing also diantennary glycans (C1 and C2). During acute inflammation the distribution volumes of C0 and C1/C2 increased to 152 ± 69, and 262 ± 81 ml kg^{-1} respectively. Inflammation induced increased capillary leakage [85] but did not affect the different glycoforms to the same extent. This indicates that additional factors are involved, e.g. lectins specifically recognizing diantennary glycans [86] or differences in glycan-dependent rheological properties of the glycoforms [87]. The inflammation-induced differences in distribution have implications for the distribution of drugs, especially because the plasma levels of the diantennary containing glycoforms of AGP are elevated during inflammation.

Other activities of AGP are related to its ability to modulate a variety of immunological reactions [18–28, 39, 40]. These activities are dose-dependent, in the physiological concentration range, and frequently glycosylation-dependent. Thus, AGP can inhibit the anti-CD3-induced proliferation of human peripheral blood mononuclear lymphocytes [18, 19, 23, 39], and can also induce the secretion of an IL–1 antagonist in murine peritoneal macrophages [26, 40]. The Con A unreactive AGP-C0 glycoform, containing only highly branched glycans, proved to be more effective than the diantennary containing glycoforms AGP-C1 and AGP-C2 [39, 40]. In fact, no inhibitory effect at all of AGP-C2 could be detected on the anti-CD3-induced proliferation of lymphocytes [39]. These and other immunomodulatory activities have been determined *in vitro*, but it is unknown whether different glycoforms of AGP can exert the same effects *in vivo*. If this is possible then the changes in glycosylation described above could be part of a humoral feed-back response to dampen down cellular inflammatory reactions. Our recent findings on inflammation-induced expression of SLEX on AGP [5, 44, 47, 49] support such a conclusion, because these molecules may influence the E- or P-selectin-mediated influx of SLEX-expressing leukocytes into inflamed areas. The selectin-mediated primary adhesion of leukocytes occurs especially at the start of the acute-phase reaction, but also in chronic inflammation like RA [88, 89]. The hyperfucosylation of AGP *in vitro*, has been reported to turn AGP into a ligand for E-selectin [90]. Studies are in progress in our laboratory to investigate such a role for naturally-occurring SLEX containing glycoforms of AGP which are present in serum during acute and chronic inflammation.

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