Carbohydrate composition and immunomodulatory activity of different glycoforms of $\alpha_1$-acid glycoprotein

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The acute phase protein, $\alpha_1$-acid glycoprotein (AGP), is a normal constituent of human blood (0.2–1 mg ml$^{-1}$) and its glycosylation and concentration in the blood change during inflammation. In this review of our recent work, we discuss the immunomodulatory properties of AGP in connection with the structure of its carbohydrate chains.

AGP samples prepared from normal donor serum (nAGP), serum obtained during abortion (fAGP), serum of cancer patients (cAGP), and ascitic fluid of patients with stomach cancer (sAGP) were subjected to analysis. All the samples except for fAGP had five N-linked chains of the 'complex' type, however, the numbers of bi-, tri-, and tetra-antennary chains, as well as glycan structures terminating these chains, were different. fAGP had three N-linked chains of the lactosamine and polylactosamine type and three O-chains which were not present in AGP isolated from the other sources. The glycoforms of nAGP and sAGP that were isolated using a ConA affinity column were similar in respect to their branching, but differed in their terminal oligosaccharides. sAGP was enriched in units ending in Le$^a$ and asialoagalacto (GlcNAc-terminating) forms.

Immunomodulatory activity of different AGP preparations was tested in vitro by measuring their effect on the proliferative response of human lymphocytes stimulated by PHA, and by determining their influence on the production of IL-1, IL-2, IL-6, and TNF in the stimulated cells. nAGP was less active compared to cancer or fetal AGP in the proliferation test, but more active in affecting cytokine production. Some AGP glycoforms had opposite immunomodulatory effects.

A new approach was developed in order to clarify the role of carbohydrate chains in the biological activity of AGP. A pool of N-linked oligosaccharide chains were attached to a soluble polyacrylamide matrix. This 'pseudoglycoprotein' was similar to AGP in its molecular weight; in its relative amounts of tetra-, tri-, and bi-antennary chains; and in the content of mono-, di-, tri-, and tetra-sialylated-oligosaccharides. This pseudo-AGP displayed a similar activity to its parent AGP in the biological tests.

Analytical flow cytometry of leukocyte subpopulation from human peripheral blood showed that monocytes and granulocytes but not lymphocytes were the main targets for the binding of AGP and pseudo-AGP. This binding was inhibited by synthetic glycoconjugates containing mannose or sialic acid. The binding curve data suggested that there are two monocyte and granulocyte populations. These may have different carbohydrate specificities.

All the evidence provided by these studies indicate that it is the carbohydrate chains on AGP that are important in modulating the immune system and not the AGP molecule itself.

Keywords: $\alpha_1$-acid glycoprotein, IL-1, IL-2, IL-6, immunomodulation, lectins, leukocytes, oligosaccharides, TNF, neo-glycoconjugates.

Introduction

$\alpha_1$-Acid glycoprotein (AGP) belongs to a group of acute phase proteins that may play a role in the modulation of the immune system in response to stress, along with many other functions [1, 2]. AGP concentration in healthy humans is 0.2–1 mg ml$^{-1}$, whereas during disease or injury (neoplasia, rheumatoid arthritis, burns) it can increase two to three times [3]. In a number of studies [4–7] it has been shown that the acute phase response is not limited to an increase in AGP concentration, but the structure of its carbohydrate chains also changes, in particular, the branching, the degree of sialylation, and the number of terminal Le$^a$ and SiaLe$^a$. Furthermore, glycoforms of AGP differ in their ability to affect lymphocyte proliferation in culture [8]. AGP has five N-glycosylation sites, which results in a high degree of heterogeneity in its carbohydrate structure. Occurrence of several AGP glycoforms on the one hand, and the variability of physiological effects of AGP on the other hand, further complicate any study of its mechanisms of action.

In our previous work [9–16], we studied the structure of the carbohydrate chains of different types of AGP, the structure of carbohydrate chains of the glycoforms of AGP
in normal and pathological states, the immunomodulatory role of the carbohydrate chains of AGP, and the binding of the different AGP forms to leukocytes. An attempt is made in this review to summarize these previous studies and correlate the carbohydrate structure of AGP with biological activity.

Is the peptide core of AGP a modulator or a carrier?

It is not clear from the literature the relative contributions of the carbohydrate chains and peptide core of AGP to its biological activity. It has been previously shown that AGP glycans suppress the proliferative response of lymphocytes [2, 8]. However, there is other evidence [17] that the modulation of IL-1 and TNF production by AGP is due to the peptide portion of the molecule. As there are a range of possible types of interaction it is difficult to obtain a clear-cut answer. These interactions could involve: (1) two-site binding to the cellular target, that involves glycan and protein units that are spatially separated; (2) a glycopeptide that is the interacting ligand; (3) a polypeptide that does not come into direct contact with the target, but presents one or several carbohydrate chains in the correct way; (4) binding that is mediated by a cluster of carbohydrate chains, but their mutual arrangement is not of great importance. Selective cleavage of the carbohydrate or protein component does not help one to choose between these situations. Therefore, the following approach [13, 18] was developed to try to discriminate these possibilities. This approach involved the transfer of oligosaccharide chains from AGP onto an inert polyacrylamide carrier. N-glycan chains of AGP were cleaved off; an amino group was added to a glucosamine residue at the reducing end of the chains, followed by the attachment of the aminated glycans to the polyacrylamide. The high yield from this process provides a synthetic molecule on which the number of carbohydrate chains is similar to that found on the natural AGP; the mild conditions used in the modification and attachment procedures keep the sialic acid intact, and the selection of the particular polymeric carrier permits the synthesis of a conjugate having an average molecular weight similar to that of AGP, ie 40 kDa [13].

Pseudo-AGP differs from ‘normal’ AGP only in two respects: (i) it has no protein portion and (ii) the carbohydrate chains are not located in the same relative positions. As shown below, pseudo-AGP completely retains its ability to modulate the proliferative response of lymphocytes to a mitogen, to activate mononuclear cells, and to modulate TNF production by monocytes. According to flow cytometric analysis, pseudo-AGP binds to blood cells in a similar way to ‘normal’ AGP. These experiments show that, firstly, only carbohydrate chains of AGP are responsible for its biological effects, and secondly, the precise localization of the glycan chains on the carrier is not important for causing these effects.

Analysis of the structure of various types of AGP and their glycoforms

Previous methodologies

The general structure of AGP N-glycans is well known [1], it has sialylated bi-, tri-, and tetra-antennary chains of the ‘complex’ type. Some of the chains are often fucosylated at the peripheral GlcNAc residue, in this case, Le^a and/or SiaLe^a antigens are formed. A high degree of heterogeneity, however, still remains even after desialylation, this being partly due to chains missing both sialic acid and galactose, addition of fucose and the presence of a different number of branches. As AGP is an acute phase protein, it is interesting to study the structure of its carbohydrate chains in various disease states and at various stages of development, for example, AGP produced during embryonic development. Due to the high heterogeneity of glycan chains, the separation and complete determination of these structures for each sample would seem to be rather a time-consuming and unnecessary task. So, a method of oligosaccharide mapping was developed [19] that included: (1) the separation of fluorescent-labelled glycans using HPLC (see Table 1); (2) comparison with labelled oligosaccharides of known structure; (3) exoglycosidase digestion of labelled glycans followed by repetition of the HPLC analysis. Introduction of the fluorescent label (7-amino-4-methylcoumarin) gave us the possibility of increasing the sensitivity of the oligosaccharide analysis and improving the chromatographic separation. This approach which was analogous to 4-aminopyridine labelling [20], permitted the rapid comparison of the ‘fingerprints’ of glycoproteins that were structurally close, and the detection and quantitation of glycans. The methodologies that we previously used are summarized in the legend of Table 1, and the data are presented for the separation of different oligosaccharides and elucidation of the oligosaccharide composition of nAGP. In rare cases when a standard for a particular structure was not available, the glycan structure was confirmed using standard procedures (eg mass spectrometry).

Separation of AGP glycoforms

Affinity chromatography on ConA-Sepharose [9, 21] on nAGP revealed four glycoforms: A, unbound; B, delayed on the column; C, ConA-bound (eluted by methyl mannoside); D, strongly bound (eluted by buffer pH 3.5). The proportions of the different forms were 50:39:10:1. This molecular weight decreased from A to D (44 → 39.5 kDa), and their carbohydrate content decreased from 40.5 to 34% respectively, see Table 2. ConA possessed maximal affinity for biantennary chains and the glycoforms were separated according to the content of this glycoform. The unbound form A had only tri- and tetraantennary chains, whereas some biantennary chains were present in the other glycoforms, along with tri- and tetraantennary ones, ie form B contained one chain, form C — two, and the most strongly bound form...
The oligosaccharide composition of AGP is given as the number of moles of each structure detected in 100 mol of the oligosaccharide mixture. D - three biantennary chains. The general structure of the bi-, tri-, and tetra-antennary chains is shown on Figure 1.

High heterogeneity is a characteristic feature of AGP carbohydrate chains; most of the structural variants in Figure 1 can be detected by chromatography. The number of glycans that could be separated using chromatography exceeded 50; after desialylation the number of variants considerably decreased to 16; half of them were represented by tetra-antennary glycans, including 10% having the repeated structure Galβ1-4GlcNAc. The proportions of asialo-, mono-, di-, tri-, and tetra-sialylated glycans of nAGP were 0.5:6.5:39:49:9, respectively. Approximately every fifth tri- and tetra-antennary glycans were terminated by Leβ or possibly SiaLeα structure.

As shown below, however, the biological effects of these glycoforms were considerably different; moreover, they caused opposite effects on the immune system.

### Human AGPs from different sources

Besides AGP from healthy donors (nAGP), the carbohydrate chains of AGP from the serum of breast cancer patients (cAGP), ascitic fluid of stomach cancer patients (sAGP), and the fetal blood removed during abortion (fAGP) were studied. These four glycoproteins had similar amino acid composition, very close molecular weights (44 ± 0.5 kDa), but they differed in the range of isoelectric points that they gave (see Table 3). The amino acid composition of fAGP was identical to nAGP; terminal amino acid could not be determined. Immunochemically fAGP was not different from nAGP. The greatest difference between fAGP and the other AGPs was in the structure of the carbohydrate chains; nAGP, cAGP and sAGP all carried solely N-linked chains (five chains per molecule), whereas fAGP carried three N- and three O-linked chains [22].

Localization of the O-chains on the peptide core was not established, but their structure was determined using mass spectrometry of methylated alditol acetates. The major chain in fAGP was a typical O-linked glycan [10]:

\[
\text{Galβ1-4GlcNAcβ1-3(6)}
\]  

\[
\text{GalNAcβ1-6(3)}
\]

Moreover, a structural motif unusual for the other AGPs was also found in fAGP N-linked chains, ie glycans having (multiple) 5–8 lactosamine units. Presence of the poly-lactosamine units was confirmed by the data obtained with HF-solvolysis. These units were only found in trace quantities in nAGP [22]. About one third of the fAGP N-glycans were present in this form [10]. The content of N-linked chains with a single lactosamine unit was slightly different in fAGP from that of nAGP, and coincided with the content in sAGP. Considerable differences in glycosylation patterns of fetal and normal glycoproteins have also been demonstrated in previous work by Durand and coworkers [23].
Table 2. Glycoforms of $\alpha_1$-acid glycoproteins from serum of normal donors (nAGP) and ascitic fluid of cancer patients (sAGP).

<table>
<thead>
<tr>
<th>Glycoform</th>
<th>Content</th>
<th>Chain type (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield (%)</td>
<td>$M_r$ (kDa)</td>
</tr>
<tr>
<td>nAGP-A</td>
<td>50</td>
<td>44.0</td>
</tr>
<tr>
<td>nAGP-B</td>
<td>39</td>
<td>42.0</td>
</tr>
<tr>
<td>nAGP-C</td>
<td>10</td>
<td>41.2</td>
</tr>
<tr>
<td>nAGP-D</td>
<td>1</td>
<td>39.5</td>
</tr>
<tr>
<td>sAGP-A</td>
<td>42</td>
<td>44.5</td>
</tr>
<tr>
<td>sAGP-B</td>
<td>35</td>
<td>42.5</td>
</tr>
<tr>
<td>sAGP-C</td>
<td>20</td>
<td>41.5</td>
</tr>
<tr>
<td>sAGP-D</td>
<td>3</td>
<td>39.0</td>
</tr>
</tbody>
</table>

Glycoproteins were isolated and separated into their glycoforms as previously described [11].

Sepharose analogous to nAGP), that were enriched in biantennary chains. This decrease in branching shown by the ConA separation was also confirmed by the percentage of individual chains with different branching in nAGP and sAGP (see Table 2). It was also shown previously that there was a difference in the sialylation patterns of sAGP and nAGP [24] and that the degree of fucosylation was higher in the cancer forms of AGP than in nAGP [12]. These extra fucose residues could be constituents of SiaLex$^a$ antigens, and one can speculate that if the cancer forms are enriched in SiaLex$^a$, then they could down-regulate selectin-mediated interactions in metastasis.

Comparison of individual glycoforms (A, B, C, D) from nAGP and sAGP permitted us to draw the following conclusions [14]. (1) the same glycoform from different AGP preparations was practically indistinguishable with respect to glycan composition, except for fucose content; (2) glycoforms from sAGP and cAGP were enriched in fucosylated oligosaccharides (total amount of fucose was two to three times higher), this included those structures with at least two fucose residues on the same glycan chain. (This was shown by treatment of the oligosaccharide with $\alpha$-fucosidase which resulted in species with HPLC mobility that corresponded to the loss of two monosaccharide residues; see Table 1); and (3) finally, mono-agalacto glycans (ie glycans having GlcNAc as a terminal residue), that were mainly tetraantennary chains, were present in sAGP and not in chains on nAGP.

The immunomodulating effects of AGP and its glycoforms

Proliferation of lymphocytes

Despite its high concentration in blood, AGP itself does not initiate lymphocyte proliferation, however, it is capable of modulating the action of other substances that cause proliferation, in particular mitogens [2]. A convenient
Table 3. Comparison of α1-acid glycoproteins from serum of normal donors (nAGP), serum of cancer patients (cAGP), ascitic fluid of cancer patients (sAGP) and abortion serum (fAGP).

<table>
<thead>
<tr>
<th></th>
<th>Mr (kDa)</th>
<th>pI</th>
<th>Sugar (wt%)</th>
<th>Neu5Ac (mol/mol AGP)</th>
<th>Fucose (mol/mol AGP)</th>
<th>Chains (no.)</th>
<th>Chain type (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nAGP</td>
<td>43.5</td>
<td>2.7–3.4</td>
<td>40</td>
<td>13</td>
<td>1.4</td>
<td>5</td>
<td>49</td>
</tr>
<tr>
<td>cAGP</td>
<td>44.0</td>
<td>2.7–3.1</td>
<td>41</td>
<td>15</td>
<td>3.8</td>
<td>5</td>
<td>44</td>
</tr>
<tr>
<td>sAGP</td>
<td>44.0</td>
<td>2.7–3.2</td>
<td>40.5</td>
<td>14</td>
<td>2.5</td>
<td>5</td>
<td>42</td>
</tr>
<tr>
<td>fAGP</td>
<td>44.0</td>
<td>2.8–3.0</td>
<td>44</td>
<td>13</td>
<td>3.0</td>
<td>3</td>
<td>44</td>
</tr>
</tbody>
</table>

Glycoproteins were isolated as previously described [11].

Experimental model for the evaluation of AGP modulating effects is to study lymphocyte proliferation caused by phytohaemagglutinin (PHA) [15]. It is important to note that similar results with AGP were obtained when lymphocyte proliferation was induced by treatment with anti-CD3 antibodies. Unseparated preparations of sAGP, cAGP, and fAGP, suppressed the proliferation of stimulated lymphocytes in the concentration range of 31–1000 μg ml⁻¹ – a range that covers physiological levels of AGP. The activity of the cancer and fetal forms was higher than that of nAGP [11, 15].

Of particular interest in the lymphocyte proliferation studies was the effect of individual AGP glycoforms [15]. Form D that contained the maximal number of biantennary chains was the most active in inhibiting proliferation, whereas glycoforms C and B that were deficient in biantennary chains were less active (see Figure 2). However, biantennary chain content did not solely determine the glycoform activity, (i) form A that had no biantennary chains was second only to form D with respect to anti-proliferative activity; similar results were previously described by Pos et al. [8]. This increased inhibitory activity of form A is probably due to sialylation, because desialylation abolished the effect. (ii) Form B from sAGP increased the PHA-induced proliferation in low concentrations. In contrast, form B from nAGP was generally a suppressor of lymphocyte proliferation; (iii) the sAGP and cAGP that were characterized by increased fucosylation and the presence of asialoagalacto oligosaccharides, possessed higher immunosuppressive activity than nAGP [11].

The AGP action on PHA-induced lymphocyte proliferation was completely independent of the peptide part of the molecule. This was concluded because the effect obtained with pseudo-AGP was very similar to that observed for nAGP (see Figure 2) [13].

Influence on mononuclear activation
AGP also modulated phagocytic activity. AGP itself did not activate mononuclear cells but it modulated the activation of these cells initiated by phorbolmyristyl acetate (PMA) or opsonized zymosan in a dose-dependent manner (Shiyan SD, Mikhalchik EV, unpublished results). It is interesting to note that the effect of AGP glycoforms depended on the nature of the activating substance. Thus, during activation with PMA, the inhibitory effect of AGP decreased in proportion to the increase in the number of biantennary chains (ie from A to C), whereas during activation with zymosan an opposite effect was observed – form C was the most potent inhibitor. A similar action for AGP glycoforms has been previously observed during neutrophil activation by opsonized zymosan [25].

Influence on production of cytokines
These experiments were carried out in vitro because it gave us the possibility to study the direct interaction of AGP on cell populations. Glycoforms of AGP modulated the production of at least four cytokines: IL-1, IL-2, IL-6, and TNF.
TNF

LPS-induced production of TNF by mononuclear cells was changed by the addition of AGP and its glycoforms (Figure 3). All forms of nAGP acted as stimulators of TNF production, only high concentrations of nAGP and AGP-A suppressed the production [26]. On the other hand, glycoforms A and B of sAGP acted as suppressors (data not shown) and this finding explained why unseparated sAGP did not modulate TNF production, being only an inhibitor of the process at high concentrations. In contrast, glycoform sAGP-D acted in a similar way to glycoform nAGP-D, i.e., it was a potent stimulator, but only at high concentrations. Pseudo-AGP completely retained the ability to affect TNF production (Figure 3), which suggested that the peptide core of AGP had no major role in this effect. This is in contrast to the data from a previous study [17].

IL-1

The action of nAGP glycoforms on the production of IL-1 is similar but not identical to that of TNF (Figure 4). Glycoform A suppressed IL-1 production by mononuclear cells; the effect being much weaker than for TNF. Glycoforms B, C, and D however stimulated IL-1 production by monocytes [26]. In contrast, sAGP as well as its A, B, and C glycoforms did not suppress IL-1 production; only form D slightly stimulated cytokine production.

IL-6

The results from the modulation by AGP of IL-6 production in LPS-stimulated monocytes are shown in Figure 5. nAGP in low concentrations inhibited this process, whereas in high concentrations the effect was negligible. The sAGP preparation however had high inhibitory activity on IL-6 production, and the effect nearly followed a linear dose-dependent relationship (AL Pukhaksky, SD Shiyan, NV Bovin, unpublished results). No data is available on the effects of AGP glycoforms (A to D) on IL-6 induction and the effect of pseudo-AGP was similar to that of nAGP (data not shown).

IL-2

An infraccionated preparation of sAGP did not influence the in vitro production of IL-2 by human peripheral blood lymphocytes, however the glycoforms displayed some activity. Glycoform A (in high concentrations) was inhibitory, whereas form C (in low concentrations) stimulated IL-2 production (see Figure 6) [15].
Figure 6. Production of IL-2 (% from control) by human peripheral blood lymphocytes (10^6 cells per ml), stimulated by ConA (20 μg ml⁻¹) in the presence of sAGP and glycoforms A and C in different concentrations (μg ml⁻¹). IL-2 activity in supernatant was determined as in [15]. Standard deviation values are less than 10%.

In conclusion, it should be noted that the immunomodulatory effects of normal AGP and AGP from cancer patients can differ significantly. In addition the effects of individual glycoforms are also different; they can have the opposite effects. Sometimes the minor glycoform D was more active than the major glycoforms. It is supposed that these opposite actions of AGP glycoforms is a kind of immunological buffering that moderates abrupt changes in immune status. Other changes in glycosylation of AGP in disease may provide further evidence in this respect, eg the observed increase in synthesis of SiaLe⁴ antigen inhibiting selectin-mediated metastasis, and the synthesis of AGP that have oligosaccharide chains terminating in GlcNAc which could neutralize the action of rheumatoid factor. Therefore, if the opposite actions of glycoforms are taken into consideration, as well as the similar activity of natural and pseudo-AGPs in affecting cytokine production, it is clear that the carbohydrate chains of AGP have an important role in immunomodulation.

Binding of AGP to leukocytes

It is possible to propose several mechanisms for the action of AGP on cell: (i) a classical receptor-type interaction, membrane lectins can serve as receptors; (ii) an indirect interaction, via cytokines produced by other cells; (iii) a role in neutralizing the action of serum lectins. Before starting to search for an AGP receptor, if it exists, it is necessary to investigate the binding of various types of blood cells to AGP. These studies were based on a flow cytometry technique and used fluorescent-labelled AGP and pseudo-AGP. Peripheral blood lymphocytes as a component of non-separated leukocytes and as a separate fraction bound very weakly to nAGP [16], see Figure 7. On the other hand, when using a monocytes population about 80% of cells bound nAGP and using granulocytes population about 70% of the cells interacted [16]. These interactions were dose-dependent and they were mediated only by the oligosaccharide chains, because they were very similar for n-AGP and pseudo-AGP. There was also evidence for the involvement of oligosaccharide in this process from inhibition studies with various carbohydrate structures (data not shown). The binding of flu-AGP to monocytes and granulocytes followed a dose-dependent relationship and this was inhibited by unlabelled AGP, pseudo-AGP and some neoglycoconjugates. The most potent inhibitors were Manx-PAA, ManxGlcNAc₂-PAA, GlcNACβ₁₄GlcNAc-PAA, SiaLe⁴-PAA, Neu5AxC₅-PAA (where PAA is water soluble polyacrylamide, Mw 30 kDa); whereas albumin, Glc-PAA, and Gal-PAA were inactive. The binding of desialylated flu-AGP to cells was considerably weaker than the untreated AGP [16]. It seems that AGP can bind to, at least, two lectins on the surface of monocytes and granulocytes.

Concentration dependency of the flu-AGP interaction with monocytes measured by flow cytofluorometry made it possible to identify two types of binding sites, the first was mediated by 6800 sites and the second one by 1200 sites. The K_{aff} of the former was about 10⁶ 1 mol⁻¹. The binding data for granulocytes also indicated the presence of two sites (3500 and 1000). This bimodality of AGP binding, along with the data on the inhibition of the flu-AGP interaction with monocytes by sialylglycoconjugates and manno-glycoconjugates, suggest that at least two populations of monocytes expressing sialo-specific and manno-specific lectins are involved. It remains to be seen whether these lectins are ones that have been previously described [28–30] or new ones.
Acknowledgements

We would like to thank Dr. A.L. Pukhalsky for fruitful discussion about the manuscript and I.M. Belyanchikov for help in manuscript preparation.

References

31 (Editor: copies of papers 9 and 14, dealing in more detail with the carbohydrate analysis of AGP, are available from the publishers.)

Received 10 December 1996, accepted 21 March 1997