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Decreased sialylation of the acute phase protein $\alpha_1$-acid glycoprotein in feline infectious peritonitis (FIP)

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Abstract

Feline infectious peritonitis (FIP) is an immune-mediated disease of domestic and exotic felides infected with feline coronavirus. FIP is characterized by the overexpression of an acute phase protein, the $\alpha_1$-acid glycoprotein (AGP). In humans, AGP is a heavily glycosylated protein that undergoes several modifications of its glycan moiety during acute and chronic inflammatory pathologies. We studied the changes in AGP glycosylation in the course of FIP. Specifically, we focussed our attention on the degree of sialylation, fucosylation and branching. This study presents a purification method for feline AGP (fAGP) from serum, using an ion exchange chromatography strategy. The glycosylation pattern was analyzed in detail by means of interaction of purified fAGP with specific lectins. In particular, *Sambucus nigra* agglutinin I and *Maackia amurensis* agglutinin lectins were used to detect sialic acid residues, *Aleuria aurantia* lectin was used to detect $\mathbf{l}$-fucose residues and *Concanavalin A* was used to evaluate the branching degree. By this method we showed that fAGP did not present any $\mathbf{l}$-fucose residues on its surface, and that its branching degree was very low, both in normal and in pathological conditions. In contrast, during FIP disease, fAGP underwent several modifications in the sialic acid content, including decreased expression of both $\alpha$(2–6)-linked and $\alpha$(2–3)-linked sialic acid (76 and 44%, respectively when compared to non-pathological feline AGP).

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1. Introduction

Feline infectious peritonitis (FIP) is an immune-mediated disease of domestic and exotic felides infected with feline coronavirus (FCoV) (Pedersen, 1995). FIP develops when cats are exposed to variants of FCoV that replicate within macrophages, thereby spreading the virus into target organs. The pathogenesis of FIP is complex and still not well understood. Two positive acute phase proteins, $\alpha_1$-acid glycoprotein (AGP) and haptoglobin (HP), have been reported to increase during FIP (Duthie et al., 1997). HP concentrations in FIP are approximately twice the maximum value of healthy cats. More interesting, however, is that levels of AGP increase from 0–1.5 to 4–8 g/l. The biological significance of AGP overexpression during FIP and its correlation with the...
pathogenesis of the disease are still unknown. AGP is a glycoprotein with a molecular weight ranging from 40 to 44 kDa, depending on the species. One of the most interesting characteristics of AGP is the extent of its carbohydrate moiety, which accounts for approximately the 45% of its molecular weight (Fournier et al., 2000). Glycans are N-linked in the form of five, or six in some species, complex-types, that could, theoretically, increase to more than $10^5$ different glycoforms. So far no more than 12–20 glycoforms of AGP have been detected in non-pathological human serum.

Human AGP is overexpressed during systemic inflammatory responses in the course of several pathologies (Hochepied et al., 2003) but its precise function is unknown: all the data reported so far would suggest an immunomodulatory activity. Specifically, AGP inhibits the neutrophils chemotaxis and activation (Vasson et al., 1994) and may be responsible for an increase in IL1 receptor antagonist secretion (Tilg et al., 1993). In vivo experiments further suggest that AGP may be involved in the induction of non-specific resistance to infection, for example by blocking the binding of HIV to the chemokine receptor CCR5 of human macrophages (Atemezem et al., 2001).

The oligosaccharides micro-heterogeneity of AGP is profoundly affected by pathologic conditions and different glycoforms can appear in plasma during systemic inflammation or diseases (De Graaf et al., 1993; Van Dijk et al., 1995). The alterations of the glycan moiety is believed to be important for several AGP immunomodulatory activities. For example, a reduction in the degree of branching of attached oligosaccharides has been correlated to a reduction of IL1 activity (Bories et al., 1990). A change in the amount of fucose residues was found to increase in some human inflammatory diseases, and fucosylated AGP that expresses the sialyl Lewis X (SLLeX) epitope could counteract the extravasation of leukocytes, SLLeX being the ligand of endothelial selectins (Brinkman-van der Linden et al., 1996; De Graaf et al., 1993). Finally, several defensive functions proposed for AGP can be associated to the sialic acid content: sialic acid is a very important component of membrane glycoproteins, and AGP may therefore act as a competitor for cell surface receptors, blocking the binding and the invasion of infectious agents (Friedman, 1983; Hochepied et al., 2000).

Therefore, AGP carbohydrate moiety modifications may represent a very important mechanism in the strategy of the inflammatory response.

High levels of AGP have been described in cat serum during FIP (Duthie et al., 1997). Due to the importance of the glycan moiety on the immunomodulatory activity of AGP, we targeted our attention to the post-translational processing of that protein during FIP infection.

In the present study we used the lectin binding specificity for carbohydrates in order to gain insight into some major (branching) and minor (sialic acid content) glycan microheterogeneity of feline AGP (fAGP) purified from FIP affected cats. In particular, the aim of this study was to (a) setup a protocol for the purification of fAGP from cat serum, (b) evaluate the normal and pathological expression on the surface of fAGP of sialic acid and fucose and (c) evaluate the possible changing of the degree of branching of oligosaccharides chains.

2. Materials and methods

2.1. Collection of samples

Serum samples (0.5–1 ml) were obtained from 16 clinically healthy cats (10 males and 6 females) used as controls for fAGP purification and for the determination of the non-pathological glycosylation pattern of fAGP. Since analysis of the glycosylation patterns of the individual non-pathological fAGP samples revealed no differences, fAGP from 16 healthy cats were pooled and used throughout the experiment as the control.

Samples were then collected from 24 cats affected by FIP (14 males and 10 females), as diagnosed following post mortem examination and immunohistochemical detection of FCoV within the lesions, and from 8 FCoV exposed cats (4 males and 4 females), i.e. cats that cohabited with FIP affected cats but did not develop the disease. All the cats used in these experiments were negative for feline immunodeficiency virus and for feline leukemia virus.

2.2. Purification of fAGP from pathologic and non-pathologic sera

fAGP was isolated by conventional HPLC ion exchange chromatography avoiding desialylation and
structural degradation. Cat serum (0.5–1 ml of each sample) was dialyzed overnight against the buffer used for the initial chromatography (10 mM citrate–phosphate buffer, pH 4.0). The serum was centrifuged at 13,000 × g for 5 min, to remove proteins not soluble at pH 4.0, and the supernatant was applied to an HiTrap Q Sepharose strong anionic exchange column (Amersham Biosciences), equilibrated with the starting buffer. The column was washed from the starting buffer until no absorbance was recorded at 280 nm, and the protein was eluted in 100 mM citrate–phosphate buffer, pH 4.0.

The fraction containing fAGP (approximately 2 ml) was concentrated with Centricon 10 (Millipore) to 400 μl, and directly loaded onto an HiTrap SP Sepharose strong cationic exchange column (Amersham Biosciences), equilibrated in 100 mM citrate–phosphate buffer, pH 4.0. In these conditions fAGP is not retained by the column and is therefore eluted in void volume.

2.3. Electrophoresis and Western blotting

Purified fAGP (2 μg each lane) was subjected to SDS-PAGE in a discontinuous pH system (Laemmli, 1970) using a Miniprotean II apparatus (Bio-Rad) with a 4% stacking gel and a 12% separating gel. Gel were stained with Coomassie blue, or electroblotted onto nitrocellulose. Total proteins were visualized by staining the gels for 1 h according to the Bio-Safe™ Coomassie method (Bio-Rad).

Western blotting was performed with a mini trans blot electrophoresis cell (Bio-Rad) onto nitrocellulose. fAGP was detected with an anti-fAGP polyclonal antibody raised in sheep that was generously provided by Dr. Duthie (University of Glasgow) and an alkaline phosphatase-conjugated goat anti-sheep secondary antibody. The blots were developed using the Amplified AP Immun-Blot Kit (Bio-Rad).

2.4. Lectin staining

Four lectins were used in this study. The digoxigenin-conjugated lectins Sambucus nigra agglutinin (SNAI) (Roche) and Maackia amurensis agglutinin (MAA) (Roche) bind sialic acid residues in specific linkages. SNAI is specific for sialic acid α(2–6)-linked to galactose, and MAA recognizes sialic acid α(2–3)-linked to galactose. These two lectins were used at a concentration of 2 and 5 μg/ml, respectively. The biotin-conjugated lectin from Aleuria aurantia (AAL) (Vector Laboratories) shows affinity for α(1–6), α(1–2)- and α(1–3)-linked fucose. Concanavalin A (Vector Laboratories) binds to the diantennary glycans, and with lower affinity to tri- and tetra-antennary glycans. These two lectins have also been widely utilized to evaluate the presence of fucosylation and SLeX (AAL) and the degree of branching (ConA) on glycoproteins surface. These lectins were used at concentrations of 8 μg/ml (AAL) and 1 μg/ml (ConA).

Glycan analysis of fAGP that reacts with digoxigenin-conjugated lectins was performed following the Glycan differentiation kit (Roche), according to the method of Haselbeck et al. (1990). Digoxigenin-labeled lectins were identified with an alkaline phosphatase conjugated sheep anti-digoxigenin immunoglobulin.

Glycan analysis of fAGP that reacts with biotin-conjugated lectins was performed following a peroxidase reaction using the Vectastain ABC Kit (Vector Laboratories) and developed using HRP Immun-Blot Assay Kit (Bio-Rad).

2.5. Other methods

The protein content was determined by direct spectrophotometric measurement at 280 nm and by the Coomassie blue dye binding method (Bradford, 1976). Densitometric analysis was performed using the Imagemaster 1D software (Amersham Biosciences). The membranes were processed with a scanner and subjected to computerized image analysis. On each membrane image, profile plots were obtained displaying the fAGP peak, and the volume of each peak was calculated by the software.

3. Results

3.1. Purification of serum fAGP

Fig. 1 reports the chromatographic procedure set up to purify fAGP. The fraction containing fAGP (Fig. 1a, fraction 2) from Q-Sepharose chromatography was loaded onto a SP-Sepharose chromatography column.
The eluted fraction (Fig. 1b, fraction 4) contains the purified fAGP. The purity was determined by SDS-PAGE (Fig. 1 insert, lane 4), which showed a single band on the gel after Coomassie Blue staining. The identification of the homogeneous band as fAGP was determined by Western Blotting using a polyclonal anti-fAGP antibody as primary antibody (Fig. 1 insert, WB, lanes 2 and 4).

3.2. Lectin binding and identification of carbohydrate moieties in healthy cats

Fig. 2a shows a representative glycosylation pattern of non-pathological fAGP. This glycosylation pattern was identical among all 16 control cats. In particular, the degree of sialylation was determined by the lectin-binding of fAGP with SNAI (sialic acid α(2–6)-linked to galactose) and MAA (sialic acid α(2–3)-linked to galactose), in lanes 1 and 2, respectively, the degree of fucosylation with AAL (lane 3) and the degree of branching with ConA (lane 4). fAGP strongly reacts with SNAI and, with minor affinity, with MAA. The reactivity of fAGP with SNAI and MAA has been presented as a density plot in Fig. 2b. The dendrogram displaying the average volume of density profile plots of non-pathological fAGP after reaction with SNAI and MAA is shown in Fig. 2c. The figure shows that the variability in non-infected cats is very low. On the contrary, fAGP glycoforms from healthy cats did not bind to the lectins AAL and ConA. The non-reactivity of fAGP with these two lectins therefore suggests that in non-pathological conditions the protein does not present any fucose residue on its surface, and that the glycoforms with one or more diantennary glycans are limited.
3.3. Lectin binding and identification of carbohydrate moieties in FIP affected cats

Fig. 3 reports the glycosylation patterns of fAGP purified from FIP affected cats: lane np represents pooled non-pathological fAGP, while pathological fAGP from the 24 FIP affected cats were loaded onto lanes 1–24. In all 24 cases, pathological fAGP did not react with AAL (Fig. 3c) or with ConA (Fig. 3d). This result suggested that fAGP expressed during FIP does not present any fucose residues on its surface, and its branching degree is not increased during disease.

Fig. 3a shows that there are several differences in the α(2–6) sialylation of fAGP among FIP affected cats. Several proteins, for example those in lanes 2, 3, 4, and 10, share an evident desialylation, that can be quantified by densitometry in 44, 42, 52 and 60%, respectively (data not reported) when compared to controls. On the contrary other proteins (lanes 7 and 8) exhibit an increase in the degree of sialylation (123 and 122%, respectively, data not reported). In other cats (lanes 5, 6, 12 and 14) the α(2–6) sialylation of fAGP is not modified during FIP. Histograms in Fig. 4 present the semi-quantitative evaluation to the peaks corresponding to fAGP profiles shown in Fig. 3. Using the lectin SNAI, the sialic acid α(2–6)-linked content of fAGP from FIP affected cats decreases to an average of 76% when compared to non-pathological fAGP. We can therefore conclude that the α(2–6) sialylation of fAGP is reduced during FIP. On the contrary, the reaction with MAA lectin (Fig. 3b), that preferentially binds α(2–3)-linked sialic acid residues, showed a marked difference in the binding between non-pathological and pathological fAGP. All except one (fAGP in lane 8) of the pathological fAGP purified in this experiment clearly showed a marked reduction in the reaction with MAA lectin and the decrease was very strong in several cats. Some proteins, for example those in lanes 2, 3, and 6, showed clear desialylation, quantified by densitometry in 11, 7, and 20%, respectively (data not reported) when compared with non-pathologic fAGP. This result strongly supports the hypothesis that there is a marked decrease of the sialic acid residues linked to galactose in position α(2–3). Using the lectin MAA, the sialic acid α(2–3)-linked content of pathological fAGP decreases to an average value of 44% when compared to non-pathological fAGP.
3.4. Lectin binding and identification of carbohydrate moieties in FIP exposed cats

We further examined whether any differences between non-pathological fAGP and fAGP from FIP-exposed cats could be observed (data not shown). As in the previous experiments, AAL and ConA did not show any reaction with AGP. On the contrary, SNAI and MAA exhibited strong reactions with fAGP. However, very few differences between the non-pathological and the pathological forms were observed (Fig. 4b). Moreover, the strong similarity of the electrophoretic mobility of all these proteins (data not presented) may suggest that fAGP from FIP exposed cats share the same oligosaccharide moiety with fAGP from healthy cats.

4. Discussion

It has been reported that plasma acute phase protein AGP concentrations increase by two to five times during FIP (Duthie et al., 1997). In this study we have demonstrated several post-translational modifications of fAGP in the course of FIP. Specifically, the glycan moiety was analyzed by binding with specific lectins. The most evident post-translational modification during FIP is a decrease in the degree of sialylation. The function of fAGP has not been clearly defined, however most reported data suggest an anti-inflammatory and an immunomodulatory role. The heavy glycosylation level of AGP (45% of total MW) strongly suggests the involvement of the glycan moiety in these AGP functions.
The fAGP glycosylation pattern in FIP is surprisingly original, if compared to that reported in literature for other diseases. In human diseases, including rheumatoid arthritis and HIV, serum AGP exhibits several features, including an increase in the fucosylation levels and in the degree of branching level (Elliot et al., 1997).

We found that the branching degree and the fucosylation level of fAGP are not increased in FIP. It has been reported in mice that fucose epitopes are not exposed during prolonged inflammatory reaction, due to the absence of α3-fucosyltransferase in mouse liver (Havenaar et al., 1998). It is possible that cats may also lack this enzyme. Furthermore, our results clearly indicate that FIP affected cats exhibit a marked modification of sialic acid content such as a reduction (76%) of the sialic acid α(2–6)-linked to galactose, that was particularly marked in several subjects (4 out of 24). In other cats the number of epitopes of α(2–6) sialic acid were unchanged or increased. We therefore may conclude that the α(2–6) desialylation occurs on an individual basis and is likely correlated with the disease. We also observed a strong reduction (44% if compared to controls) of sialic acid α(2–3)-linked to galactose, from the surface of purified fAGP. This desialylation occurred, with varying degrees, in 22 out of 24 cats.

FCoV exposed cats apparently do not present any sialic acid, L-fucose or glycan branching modification. Our results suggest that it is probably the FIP, and not the simple infection with FCoV, that may cause the major glycan modification on the surface of AGP. At present, few data are available regarding biological differences between α(2–6) and α(2–3) linkage of sialic acid to galactose. Due to their negative electric charges and the terminal localization, sialic acid residues play a very important role in mediating intercellular recognition events. It is also thought that the sialylation of a glycoprotein exerts an antiproteolytic activity, de facto prolonging the life of the protein. Desialylation causes the molecule to be recognized by galactose specific lectins: this recognition targets the desialylated serum glycoproteins to hepatocytes for their removal from circulation (Lamari and Karamanos, 2002). Moreover, sialic acid is a component of receptors for cytokines, selectins and siglecs (sialic acid-binding immunoglobulin superfamily lectins) (Traving and Schauer, 1998).

The data presented here are not sufficient to suggest a role of fAGP in the pathogenesis of FIP. At present we can only speculate on the physiological and pathological role of the desialylation in the immunomodulatory function of AGP. Is the desialylation of AGP connected with a reduction of the half-life of AGP itself and, therefore, with a reduction of its immunomodulatory properties? The AGP interacts with monocytes, inducing an increase of the secretion of IL-1 receptor antagonist, and modulates the LPS-induced cytokine expression (Fournier et al., 2000). Does the modification of the glycan moiety alter the binding of pathological AGP to monocytes, thus increasing or decreasing the activation capability of these cells? FIP develops when cats are exposed to mutated FCoV that acquire the capability to replicate into macrophages (Stoddart and Scott, 1989), and therefore it is conceivable that the interaction of different AGP glycoforms may elicit different macrophage reactions.

In conclusion, we have reported in this study that a change in glycosylation of fAGP occurs during FIP. Since no information is currently available concerning glycosylation of fAGP in other feline pathologies (and very few are indeed available in humans and mice), it is at the moment impossible to propose fAGP glycosylation pattern as an additional specific marker for the diagnosis of this pathology. However, further studies of the carbohydrate moiety of fAGP in other disease states, and the evaluation of whether this protein is overexpressed or not, are warranted.

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