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Expression patterns in feline blood and tissues of α_1 -acid glycoprotein (AGP) and of an AGP-related protein (AGPrP)

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Abstract α_1 -Acid glycoprotein (AGP) is an acute-phase protein (APP) that modulates immune responses, probably – at least in humans – owing to the modification of its glycosylation pattern. On this perspective, feline AGP can be a useful comparative model, as it has different concentrations in cats susceptible or resistant to some disease. As a preliminary approach to the study of feline AGP (fAGP) we have purified this protein from feline serum by HPLC using human AGP (hAGP) as a model. Immunoblotting with a polyclonal antibody against fAGP and with a monoclonal antibody against hAGP was performed on serum from healthy cats, from cats exposed to feline coronavirus (FCoV) infection and from cats with purulent inflammations, such as feline infectious peritonitis (FIP), feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV). Immunohistochemistry on tissues from healthy cats and from cats with different diseases (FIP, FIV, FeLV, locally extensive inflammation) was also performed with the same antibodies. Both hAGP and fAGP have been purified to homogeneity as determined by SDS-PAGE. fAGP did not react with the anti-hAGP antibody which, in contrast, detected in feline serum a low MW protein

that we called fAGP-related protein (fAGPrP). This protein was underexpressed in cats with FeLV and FIP. Both fAGP and fAGPrP were immunohistochemically detected in plasma and hepatocytes with a stronger intensity in cats with FIP and some inflammatory conditions. Moreover, fAGPrP was detected in the cytoplasm of tissue cells, most likely identifiable with plasma cells. These cells were rarely detectable in cats with FIV and FeLV, and numerous in cats with FIP and with locally extensive inflammation. In conclusion, purified fAGP has physicochemical characteristics similar to those of hAGP, but does not cross-react with anti-hAGP antibodies. In contrast, the anti-hAGP detected an AGP-related protein whose blood concentration and tissue distribution was not related to that of fAGP. Moreover, both fAGP and fAGPrP were differently expressed in cats with pathologic conditions compared to controls. Further study of these proteins by analysing their structural characteristics is required.

Keywords α_1 -Acid glycoprotein (AGP) · AGP-related protein (AGPrP) · FIV · FeLV · FIP · Inflammation

Abbreviations AGP α_1 -acid glycoprotein · APP acute-phase protein · FCoV feline coronavirus · FeLV feline leukemia virus · FIP feline infectious peritonitis · FIV feline immunodeficiency virus

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Introduction

α_1 -Acid glycoprotein (AGP) is a major positive acute-phase protein (APP) in many species. AGP belongs to the lipocalin family, a group of extracellular binding proteins specific for small hydrophobic molecules. Together with α_1 -microglobulin and glycodelin, it forms the immunocalin subfamily (Logdberg and Wester 2000). Human AGP (hAGP) contains 183 aminoacids and is characterised by low molecular weight (41–43 kDa), high solubility, very low pH (2.8–3.8) and a high percentage of

carbohydrates (45%). Its glycosylation pattern is very variable (12–20 glycoforms in humans), depending on the physiological or pathological conditions, such as pregnancy, inflammation or cancer (Biou et al. 1991; Kim and Varky 1997).

The function of AGP has not been completely defined: an immunomodulatory and anti-inflammatory role has been suggested (Williams et al. 1997). It can downregulate neutrophil responsiveness, stimulate IL-1R antagonist secretion by macrophages (Vasson et al. 1994; Bories et al. 1990), inhibit platelet aggregation and lymphocyte proliferation and modulate the production of anti-inflammatory cytokines by peripheral blood leucocytes (Costello et al. 1979). These activities are correlated to the carbohydrate moiety of AGP (Shiyan and Bovin 1997). In particular, the rate of sialylation has been proved to be protective in inflammation and in HIV infection (Mackiewicz and Mackiewicz 1995; Rabehi et al. 1995; Williams et al. 1997).

In cats, AGP is one of the most examined APPs. It has been studied in different viral and neoplastic diseases (Duthie et al. 1997; Selting et al. 2000). In particular, the serum levels of AGP during feline infectious peritonitis (FIP) are very high (Duthie et al. 1997), but this increase is only transient in FCoV-exposed cats (Giordano et al. 2003). This could be due to the protective role of some AGP glycoforms similar to humans. Although diagnostic kits to quantitate feline AGP (fAGP) are commercially available, there is currently no information about fAGP sequence, structure and tissue distribution, or commercial anti-fAGP antibodies. We thus decided to design a research project focused on the structural characteristics of fAGP. In this paper we describe the results of the first step of this project, during which fAGP was purified from feline blood using hAGP purification protocol as a model. fAGP distribution in blood and tissues of healthy cats and of cats with different diseases was then assessed using a polyclonal anti-fAGP antibody. During the fAGP purification a second, different protein was identified. This was named fAGP-related protein (fAGPrP), only because it cross-reacts with a monoclonal antibody directed against hAGP. To date, it is not known what this small protein (about 29 kDa) is. Before studying the fAGPrP further, however, it was decided to evaluate its possible pathophysiological role by assessing its blood and tissue distribution and its relationship with fAGP.

Materials and methods

hAGP and fAGP purification

hAGP and fAGP were purified to homogeneity following the same procedure. Aliquots (1 ml) of serum from healthy cats and from human donors were brought to 0.01 M citrate-phosphate buffer (CPB), pH 4.0, by ultrafiltration with Centricon YM 10 (Millipore Co., Bedford, MA, USA). The solution was centrifuged at 12 000 g and the supernatant directly loaded onto a Q-Sepharose HiTrap column (Amersham Biosciences, Uppsala, Sweden) equilibrated in the same buffer. The column was washed with 20 ml of loading

buffer, and the proteins were eluted with 0.1 M CPB, pH 4.0. The fraction containing AGP was concentrated and loaded on to a second SP-Sepharose HiTrap column equilibrated in 0.1 M citrate-phosphate, pH 4.0. AGP is not retained on the column but eluted in the void volume.

Protein concentration was quantified using the BioRadTM Protein Assay (Bio-Rad, Hercules, CA, USA). Protein homogeneity was assessed by SDS-PAGE (Laemmli 1970). SDS-PAGE gels were directly stained with Coomassie blue.

Immunoblotting

Semiquantitative evaluations of AGP concentration were determined by immunoblotting on sera from healthy cats ($n = 3$), from cats living with FCoV shedders (FCoV-exposed cats, $n = 3$), and from cats with FIV ($n = 3$), FeLV ($n = 2$) FIP ($n = 2$), and purulent inflammations ($n = 3$) such as pyometra, pyothorax and purulent rhinoconjunctivitis.

Immunoblotting was performed on to nitrocellulose at 250 mA for 120 min. Nitrocellulose membranes were immunostained using, as primary antibodies, the following:

- Anti fAGP polyclonal antibody raised in sheep at the Glasgow Veterinary School by Professor David Eckersall using a fAGP purified from feline ascites with a procedure similar to the protocol used by us. The specificity of this antibody has been validated by the producers by an ELISA against fAGP. This antibody is routinely used at the Glasgow Veterinary School to determine the fAGP levels in cats with FIP (Duthie et al. 2002). The antibody was used in immunoblotting tests at a final dilution of 1:2000.
- Commercial anti-hAGP monoclonal antibody (Sigma, St Louis, MO, USA) recognising an epitope located in the 44 kDa subunit of denatured and reduced AGP. According to the manufacturers, this antibody should not react with other human acute-phase proteins or with AGP of different species other than human and baboon. The anti-hAGP antibody was also used at a final dilution of 1:2000.

Immunohistochemistry

Tissue samples were taken from two groups of cats:

Group A: controls: liver, spleen, pancreas, lymphnodes, kidney, large and small intestine, uterus, adrenal glands, thyroid, lung, muscle, heart, CNS and cutis were collected at necropsy from three cats without inflammatory disease.

Group B: tissue samples were collected at necropsy from cats with FIP ($n = 6$), FIV ($n = 2$), FeLV ($n = 2$), parvoviral enteritis ($n = 2$) and inhalation pneumonia ($n = 2$); moreover eight surgical biopsies from FIV- and FeLV-negative cats with lymphocytic plasmacytic gingivitis (LPG, $n = 4$), pyometra ($n = 2$) and lymphoplasmacytic enteritis (LPE, $n = 2$) were also included in this group.

Haematoxylin and eosin and toluidine blue stains were performed on sections prepared from formalin-fixed and paraffin-embedded samples.

The reactivity and the dilution of the primary antibodies (see below) used in immunohistochemistry were previously assessed on cryostatic sections obtained from frozen samples of control cats. Immunohistochemistry was then performed on formalin-fixed and paraffin-embedded samples. Monoclonal anti-hAGP and polyclonal anti-fAGP antibodies (see above) were applied on serial sections at final dilutions of 1:5000 and 1:10 000, respectively. The following monoclonal antibodies were also applied on selected sections of intestine, lymph node and LPG: anti-myeloid cell antigens (MAC387 – DAKO, Glostrup, Denmark; 1:5000), anti-CD79a (DAKO, Glostrup, Denmark 1:1000), anti-feline IgA (Oxford Biomarketing, Oxford, UK; 1:40) and anti-feline IgM (Oxford Biomarketing, Oxford, UK; 1:20).

The Avidin-Biotin Complex (ABC) method with a commercially available kit (Vectastain Elite, Vector Labs Inc., Burlingame,

CA, USA) was used to detect the positive reaction, as previously described (Hsu et al. 1980), after inhibition of the endogenous peroxidase (H_2O_2 1% in methanol). Antigen unmasking was performed using microwave pretreatment (two cycles of 5 min in citrate-buffered solution, 0.01 M, pH 6.2) (Cattoretti et al. 1993), except for sections stained with the anti-IgA and the anti-IgM antibodies, which were trypsinised (25 min at 37 °C; 0.1% trypsin and calcium chloride 0.1% in distilled water, pH 7.8) (Waly et al. 2001). Diaminobenzidine served as the chromogen for the reaction and the slides were counterstained with Mayer's haematoxylin.

Results

AGP purification and immunoblotting

SDS-PAGE of homogeneous purified human (lane 1) and feline (lane 2) AGP is presented in Figure 1. The different electrophoretic mobility between fAGP and hAGP is probably due to a different glycosylation pattern, which is one of the characteristics of this family of proteins (Hochepied et al. 2003).

The homogeneity of the purification was evaluated after silver staining (data not presented) and Coomassie blue staining, which showed a single band in the gel. The identification of the homogeneous band at 51 kDa as AGP was carried out by Western blotting using a monoclonal anti-hAGP antibody and a polyclonal anti-fAGP. Anti-hAGP antibody strongly reacts with hAGP, both in human serum and in the homogeneous solution containing the purified hAGP (Fig. 2a, lanes 1 and 2, respectively). It does not react with fAGP, neither in feline serum nor with purified fAGP (Fig. 2a, lanes 3 and 4, respectively). Therefore, we concluded that monoclonal anti-hAGP does not cross-react with feline AGP.

Unexpectedly, anti-hAGP cross-reacted with a low molecular weight (29 kDa) human and feline protein: this protein can be observed in serum but is not present in purified fAGP solution. We called it 'AGP-related protein' (fAGPrP). We have no further structural information about fAGPrP: it can be detected in the whole sera of both human and feline, but it is apparently lost during the AGP purification procedure. The name of AGPrP is due to its cross-reactivity with both anti-human and antifeline antibodies.

Polyclonal anti-fAGP does react strongly with fAGP (Fig. 2b, lanes 3 and 4), but also cross-reacts with hAGP

Fig. 1 Purification of human AGP and feline AGP. SDS-PAGE of human and feline AGP-containing fractions after chromatography purification. Lane 1: 2 g of human AGP; lane 2: 2 g of feline AGP

(Fig. 2b, lanes 1 and 2). It also cross-reacts with a 66 kDa protein that exhibits an electrophoretic mobility and shape very similar to that of albumin. The strong binding capability of both proteins and the high glycosylation content of AGP may cause an incomplete denaturation of feline AGP during preparation of samples before SDS-PAGE, and therefore an amount of AGP may be delayed by interaction with albumin and comigrates during the electrophoretic run.

In order to rule out the possibility that anti-fAGP cross-reacts with albumin, feline albumin was purified by means of ionic exchange chromatography, Western blotted on to nitrocellulose, and the membrane was probed with anti-fAGP polyclonal antibody. The results (data not shown) confirm that anti-fAGP does not cross-react with feline albumin.

Immunoblotting on pathologic sera

In order to investigate the distribution of fAGP and of fAGPrP in different feline pathological conditions, immunoblotting was repeated on serum from cats with purulent inflammations, FIV, FeLV and FIP, and from

Fig. 2 Immunoblotting of hAGP and fAGP using as primary antibody the anti hAGP monoclonal antibody (a) or the anti fAGP polyclonal antibody (b). Lane 1: human serum (total protein: 4.5 g); lane 2: purified hAGP (total protein: 0.4 g); lane 3: feline serum (total protein: 4.5 g); lane 4: purified fAGP (total protein: 0.4 g)

Fig. 3 Semiquantitative evaluation of fAGP and fAGPrP by immunoblotting in sera from different pathologic conditions (PI, purulent inflammation)

FCoV-exposed cats. SDS-PAGE fractionated proteins were immunoblotted and nitrocellulose membranes were probed with anti-fAGP polyclonal antibody. Moreover, because the monoclonal antibody anti-hAGP cross-reacts strongly with fAGPrP, this antibody was used to evaluate the expression of that protein. Results are presented in Figure 3.

Compared to controls, fAGP was slightly underexpressed in cats with FIV and FeLV, unchanged in FCoV-exposed cats, and overexpressed in cats with FIP and with purulent inflammations. In contrast, fAGPrP

was underexpressed in cats with FIP and absent in one out of the two cats with FeLV.

Immunohistochemistry

In control cats, both the anti-fAGP and the anti-hAGP antibodies strongly stained plasma in small and medium vessels. Scattered to abundant positive granules were detected within hepatocytes. The anti-hAGP antibody also stained some large round or stellate cells with a small, round, central to eccentric nucleus and abundant strongly positive cytoplasm. Although positive cells were occasionally detectable in all the examined tissues, they were particularly abundant in the lamina propria of intestine and uterus and in lymphoid tissues, where they were detectable in sinuses and in the perifollicular areas (Fig. 4). These cells were negative to toluidine blue stain and did not stain with anti-CD79, anti-IgA, anti-IgM and MAC387 antibodies.

Intrahepatocytic and plasma positivity were also detectable in cats with diseases. The intensity of positivity was particularly high in cats with FIP and with

Fig. 4 Tissue distribution of fAGP and fAGPrP in control cats. A, Liver: intrahepatocytic granular positivity (anti-fAGP antibody, Mayer's haematoxylin counterstain, 400x); B, Kidney: diffuse positivity of plasma within small vessels (anti-fAGP antibody, Mayer's haematoxylin counterstain, 250x); C, Intestine: large round-shaped to stellate cells with abundant positive cytoplasm in the lamina propria (anti-hAGP antibody, Mayer's haematoxylin counterstain, 250x); D, Lymph node: perifollicular distribution of positive cells (anti-hAGP antibody, Mayer's haematoxylin counterstain, 100x)

Fig. 5 Tissue distribution of fAGP and fAGPrP in cats with pathologic conditions. A, Omentum, FIP: diffuse extracellular positivity, most likely due to plasma extravasation (anti-fAGP antibody, Mayer's haematoxylin counterstain, 250x); B, Kidney, FIP: endothelial positivity (anti-hAGP antibody, Mayer's haematoxylin counterstain, 250x); C, Lung, inhalation pneumonia: positive epithelial staining in a pulmonary area with acute interstitial inflammatory reaction and severe emphysema (anti-hAGP antibody, Mayer's haematoxylin counterstain, 100x); D, Lymphocytic plasmacytic gingivitis: positive cells are particularly abundant and scattered through the submucosa (anti-hAGP antibody, Mayer's haematoxylin counterstain, 400x); E, Lymph node, FIP: numerous positive cells, diffusely distributed through the follicular structures (anti-hAGP antibody, Mayer's haematoxylin counterstain, 100x). F, Lymph node, FeLV: rare positive perifollicular cells (anti-hAGP antibody, Mayer's haematoxylin counterstain, 250x)

LPG. In the same cats, a diffuse weak positivity close to pyogranulomatous and to lymphoplasmocytic foci, respectively, perhaps depending on plasma leakage from vessels, was also detectable. Moreover, the endothelium in the inflamed tissues and the alveolar epithelium stained positive with the anti-hAGP antibody. The number and the distribution of positive cells were very variable in the different pathological conditions: positive cells in tissues from cats with FeLV and with parvoviral enteritis were rare or absent. In contrast, a large number of positive cells were found in samples with pneumonia, pyometra and LPG. Moreover, in some cats with FIP the number of positive cells in perifollicular areas of lymph nodes decreased, whereas in others these cells

were particularly abundant and without a clear perfollicular distribution (Fig. 5).

Discussion

A rapid and straightforward purification procedure is the very first step to investigate the structure characteristics of a protein. To date, we have demonstrated that fAGP is a protein similar to hAGP but not cross-reacting with anti-hAGP antibodies. The primary structure of fAGP is unknown. So far the primary structure of AGP has been determined in human, rat, mouse, pig and chicken (Fournier et al. 2000). The degree of similarity among the AGP subfamily is very low. These data clearly indicate that AGP is not really well conserved between species; therefore, it is not really surprising that monoclonal anti-hAGP does not cross-react with fAGP.

Interestingly, the anti-hAGP antibody detected a small protein in human and feline serum. A computational analysis (<http://dove.embl-heidelberg.de/blast>) of human AGP, against which the antibody here used has been raised, did not find protein homologues to AGP other than those of the lipocalin family, except for some small proteins of bacterial origin and for a ribosomal protein. From this perspective it is not possible to know whether this protein is structurally related to AGP. Moreover, the protein disappears when AGP is purified to homogeneity, both in human and cat. On the other hand, we may not rule out the possibility that the antibody cross-reacts with a partially deglycosylated form of fAGP, which has no equivalent in human serum. Because the low isoelectric point of AGP is due largely to the glycan moiety of the protein, it is possible that a partial deglycosylated isoform of 29 kDa is not copurified with the completely glycosylated AGP.

Based on its cross-reactivity with the anti-hAGP antibody, we provisionally called this protein AGP-related protein (AGPrP). AGPrP purification and sequencing are required to clarify the nature of this protein. These procedures, however, are quite expensive and time-consuming. For these reasons it was decided to evaluate the expression of fAGPrP and its tissue distribution before engaging in the determination of the primary structure.

A semiquantitative analysis of the expression of fAGP and fAGPrP in serum from cats with different pathologies suggests that fAGPrP concentration is not related to that of fAGP: fAGPrP is underexpressed in cats with FIP, in which, by contrast, fAGP was overexpressed. The latter finding was in agreement with previous results obtained on the same cats using a commercially available radial immunodiffusion kit (Giordano et al. 2003). The underexpression of fAGPrP, if confirmed in a larger number of cats, might be a useful support in the diagnosis of FIP. Furthermore, the expression pattern recorded in blood from cats with

FIV, FeLV and purulent inflammations suggests a possible involvement of both fAGP and fAGPrP in these diseases.

The tissue distribution of fAGP was consistent with its role of acute-phase protein (APP): positivities were detectable within hepatocytes, where APP are produced, and in plasma (Ceciliani et al. 2002). Both these findings, however, must be carefully interpreted, because of the presence of a 66 kDa positive protein, most likely albumin. We ruled out experimentally that feline albumin cross-reacts with polyclonal anti-fAGP: it is probable that an aliquot of fAGP conjugates with albumin during SDS-PAGE of sera, and therefore the cross-reaction at 66 kDa might be considered as an electrophoretic artefact. As a further support to this hypothesis, fAGP resulted in overexpression in FIP and in inflammatory conditions usually characterised by decreased albumin (Pedersen 1995; Kaneko 1997).

The tissue distribution of fAGPrP does not help us to understand the nature of this protein: the intrahepatic localisation and the plasma positivity are typical of APP, suggesting that fAGPrP might be an AGP isoform. In contrast, no reports about the presence of AGP or of other APP on tissue cells are so far available, supporting the hypothesis of a protein different from AGP and characterised by the simultaneous cellular and plasma distributions. The morphology and the distribution pattern of positive cells in the intestine were very similar to those described by Waly et al. (2001) for IgA- or IgM-producing plasma cells. In the present paper, immunohistochemistry for CD79a, IgA and IgM was negative but samples were collected from a retrospective analysis of our archives, and this might have altered the preservation of membrane-bound antigens. The nature of fAGPrP-expressing cells needs to be further verified by the analysis of samples collected with a standardised fixation protocol. In contrast, the fixation procedure does not alter the ability to detect myeloid cells and mast cells by MAC387 antibody and toluidine blue stain, respectively, thus allowing us to exclude that positive cells were mast cells and macrophages.

The group of cats with pathologies was very heterogeneous: this does not allow for the exact definition of the role of those proteins in each disease. However, the changes in the distribution of positive cells observed in these cats suggest that AGPrP might play some role in the pathogenesis of the diseases. Based on these observations this protein will be further investigated.

In conclusion, this study allowed the purification of fAGP and the detection of another protein, fAGPrP, whose concentration in pathological conditions is different from that of fAGP. Both the proteins have a similar intrahepatic and plasmatic distributions, but fAGPrP is also detectable in tissue cells. All these findings suggest that these two proteins are structurally and functionally different, and that the cross-reactivity is probably due to the conservation of a very small epitope. The different expression pattern of these proteins

recorded in cats with pathological conditions compared to controls suggests the need to further investigate the structural characteristics of both fAGP and fAGPrP.

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