Acute phase protein levels in dogs with mast cell tumours and sarcomas

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Introduction

The acute phase protein response is part of a non-specific and complex host response to inflammation. It occurs shortly after tissue injury and may be induced by a range of different causes, including infectious, inflammatory, neoplastic, traumatic or immunological disease. It is mediated by pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor-alpha (TNFα) and interleukin 6 (IL-6) (Dinarello 1984, Heinrich and others 1990, Sheahan and others 2011) and is stimulated by the disease process (Murata and others 2004, Peterson and others 2004, Ceron and others 2005). The acute phase protein response is a critical part of the early response to insult or injury, and plays an important role in survival of the host during these initial stages.

Acute phase proteins (APP) demonstrate major, moderate and minor response levels, and these vary between species (Eckersall and Bell 2010). An APP with a major response is one that has a low concentration in healthy animals, but will show a dramatic rise (100-1000-fold) on stimulation with a peak between 24-48 hours and then a rapid decline. A moderate response APP will increase 5-10 fold following stimulation, can take 48-72 hours to peak and will decrease more slowly. A minor response APP will increase only slowly, and only up to twice it's resting level. In dogs, major response APPs include C-reactive protein (CRP) and serum amyloid A (SAA), while moderate responders include alpha-1 acid glycoprotein (AGP) and haptoglobin (Hp). Acute phase proteins can also be divided into positive and negative proteins. The positive APPs (Hp, CRP, caeruloplasmin, SAA, AGP, fibrinogen) increase in

response to release of pro-inflammatory cytokines such as IL-1, IL-6 TNF-alpha, while the negative APPs (albumin, transferrin) decrease in response to inflammation (Ceron and others 2005).

Although it has been conventionally believed that APPs were exclusively hepatocyte derived, there is now increasing evidence to support extra-hepatic generation in neoplastic and other disease states. In people, CRP has been shown to be of value in identifying metastatic disease from primary renal tumours (Ramankulov and others 2008) as well as showing promise for monitoring rejection of renal transplants (Jabs and others 2003). Serum CRP correlates with survival in colorectal cancer (Chung and Chang 2003, Gutfeld and others 2006) and oesophageal squamous cell carcinoma (Nozoe and others 2002) while SAA concentrations have been shown to correlate with cancer activity, stage and prognosis in gastric tumours (Chan and others 2006, Malle and others 2009). Recent immunohistochemical studies in people with oesophageal carcinoma suggest that tumour tissue may itself elaborate APP (Nozoe and others 2002) with a poorer survival and outcome associated with tumours that elaborated higher levels of CRP. A similar association has been seen between AGP and colorectal tumours and ovarian carcinoma (Elg and others 1997, Croce and others 2005).

In veterinary patients, elevated levels of AGP have been identified in dogs with a range of tumours with AGP localisation to liver and splenic tissue in one study (Yuki and others 2011). Another study found higher levels of AGP in dogs with non-specific tumours of grade III-IV based on the WHO Tumour Node Metastasis (TNM) scale (Itoh and others 2009) and elevated serum AGP has been documented in non-specific tumour-bearing cats (Selting and others 2000) Elevated CRP levels have been documented in both dogs and cats with lymphoma (Tecles and others 2005, Mischke and others 2007) and serum CRP may be used as an indicator of complete remission status in dogs with multicentric lymphoma (Nielsen and others 2005). Elevated levels of CRP, Hp and SAA have been identified in dogs with mammary tumours, with significant increases over normal being seen in the presence of metastatic disease, primary tumours greater than 5cm in diameter and those with ulceration (Planellas and others 2009, Tecles and others 2009).

Because APPs are by their nature non-specific, with marked variation in their response to tissue damage and inflammation, measurement of several APPs in tandem is likely to be more useful than looking at a single value in isolation. It has been suggested that APP profiles that use at least one major, one moderate and one negative APP may optimise clinical use (Ceron and others 2005). For the purpose of this study we evaluated Serum Amyloid A (SAA), C-reactive protein (CRP), Haptoglobin (Hp), and Alpha 1-Acid

Glycoprotein (AGP) in client-owned dogs referred for surgical and medical treatment of cutaneous mast cell tumours (MCT) and ~~soft tissue~~ sarcomas. The results were then assessed to see whether specific increases within the profile were associated with the presence of the tumour and the grade.

Materials and Methods

Client-owned dogs referred to the authors' institution for treatment of cutaneous MCTs (n=20) and sarcomas (n=21) were prospectively enrolled in the study. Cases were only enrolled in the study if blood sampling formed part of the clinical investigation and/or treatment, and where residual blood was available after diagnostic sampling which would otherwise have been disposed of as clinical waste. Criteria for inclusion in the study were that the patient was not currently being treated with steroids, did not have a recent history of infectious or inflammatory disease other than the tumour, a definitive histological diagnosis was available for the tumour and a full staging procedure was completed prior to surgery using standard oncological protocols to identify metastatic disease where present. Following surgical resection each tumour was submitted for full histological evaluation and grading to include assessment of the margins of excision. MCTs were further divided based on the system described by Patnaik and others (1984); there were 5 tumours which met the criteria for grade 1 (the lowest grade) and 15 grade 2. None met the criteria for grade 3 (the most severe grade). A more recent system described by Kiupel and others (2010) classifies MCTs as either high grade or low grade; ~~high-grade MCTs as those the presence of any one of the following criteria: at least 7 mitotic figures in 10 high-power fields (hpf); at least 3 multinucleated (3 or more nuclei) cells in 10 hpf; at least 3 bizarre nuclei in 10 hpf; karyomegaly (ie, nuclear diameters of at least 10% of neoplastic cells vary by at least two-fold). Fields with the highest mitotic activity or with the highest degree of anisokaryosis are used to assess the different parameters.~~ a~~A~~lthough this newer system has the advantage of greater simplicity it was not applied retrospectively to the cases in this study.

The sarcoma group included soft tissue sarcoma (4), fibrosarcoma (2), haemangiosarcoma (5), chondrosarcoma (2), osteosarcoma (7), histiocytic sarcoma (1) and poorly differentiated sarcoma (2). Although the variety in histological type in this group meant that a single grading system such as that proposed by Dennis and co-workers in 2010 for soft tissue sarcoma could not be used, each tumour was assigned a subjective grade by the pathologist who assessed the slides, based on well-established histological markers of malignancy such as mitotic index and degree of differentiation.

Blood samples were collected from either the jugular vein or cephalic vein at the time of presentation and before any invasive procedures, and residual serum was stored temporarily at -4^{o}C and then at -20^{o}C degrees centigrade until assayed. The APP levels were determined as previously described in detail by Lowrie and others (2009 a and b) as well as Eckersall and co-workers (1991, 1999). In brief, the CRP levels were determined by immunoturbidometric assay and Hp by means of haemoglobin binding capacity assay. SAA was measured with a commercial canine ELISA kit (TriDelta Development, Dublin, Ireland) and AGP was measured with a commercial radial immunodiffusion assay (J-Path Inc, Tokyo, Japan).

Normal values used by the laboratory for each APP that formed part of the profile were based on samples previously collected from healthy dogs (CRP n=51, HP n=54, SAA n=39, AGP n=37).

Statistical analysis

All comparisons using continuous data were checked for normality and equality of variances and appropriate statistical tests were employed. In order to test the hypothesis that APP concentrations in dogs with MCT or sarcoma were significantly different from normal reference ranges for each APP, we calculated a student's t test (operationalised as a two-sample Welch's test for samples of unequal sizes and variances) of whether the means of two independent, normally distributed populations were equal.

In order to test the hypothesis that APP concentrations were significantly different in dogs with different grades of tumour Mann-Whitney tests were used. In order to test the hypothesis that dogs with APP concentrations above the ~~normal~~ reference range for each APP were associated with MCT or sarcoma grade Chi-square tests or Fishers exact tests were used, where appropriate.

Results

Hypothesis 1: APP concentrations in dogs with MCT or sarcoma are significantly different from the ~~normal~~ reference ranges for each APP (table 1.).

CRP concentration was statistically significantly greater than reference range CRP in dogs with MCT (p-value <~~=~~ 0.00) and sarcoma (p-value = 0.02). Hp concentration was significantly greater than reference range Hp in dogs with sarcoma (p-value <~~=~~ 0.00), but not

Comment [KP1]: The individual who provided statistical advice has indicated that they think it is more appropriate to maintain the text p<0.00 rather than saying p<0.01 as per the reviewers request. We will leave this to an editorial decision.

those with MCT (p-value = 0.58). Surprisingly, the SAA concentration was significantly lower than reference range SAA in dogs with MCT (p-value <= 0.00), but not significantly different from the ~~normal~~ reference range in dogs with sarcoma (p=0.22). The AGP concentration was significantly greater than reference range AGP in dogs with sarcoma (p-value < 0.00), and also with MCT (p-value = 0.02).

Hypothesis 2: APP concentrations are significantly different in dogs with different grades of MCT or sarcoma (table 2.).

SAA concentration was statistically significantly greater in dogs with grade 2 sarcoma when compared to dogs with grade 1 sarcoma (p-value =0.02), however the SAA concentrations were not significantly higher than ~~normal values~~reference ranges in either grade. There were no statistically significant differences in any of the other APPs when compared to tumour grade. ~~although sample size was low which could have affected the findings or lack of statistical significance~~

Hypothesis 3: Dogs with APP concentrations above the ~~normal~~ reference range for each APP are associated with MCT or sarcoma grade.

None of the positive APPs were statistically significantly associated with sarcoma or MCT grade in dogs with elevated APP. ~~however the majority of grade 2 sarcoma cases fell into the positive category for CRP and Hp with values above the normal reference range (see Table 3) and both grade 1 and grade 2 sarcoma had more cases falling in the SAA negative group with values below the normal reference range.~~

Discussion

Patients with neoplastic disease often have altered immune status, such that a greater response than normal can be expected to challenges to the immune system (Duthie and others 1997, Kishi and others 1999). The proportion of T helper 2 cells that produce cytokines including interleukins 4, 6 and 10 are increased in tumour bearing patients and this suppresses cellular immunity (Blay and others 1993, Oka and others 1996, Berghella and

1998). Tumour cells will produce large amounts of active-type transforming growth factor beta, cytokines including interleukins 6 and 10, and prostaglandin E2, which again suppress cellular immunity (Takiuchi and others 1992, Oka and others 1994, 1995 and 1996, Berghella and others 1998). These alterations to immune status in patients with neoplastic disease and the resulting changes in the APP component of the inflammatory response are largely what underpin the potential use of APP profiles to assess tumour burden and prognosis (Itoh and others 2009).

CRP was the first APP to be identified, and it is the major human APP (Eckersall and Bell 2010). Serum CRP has been identified as a useful prognostic factor in several clinical studies of colorectal neoplasia in humans (Nozoe and others 1998 and 2000, McMillan and others 2003, Canna and others 2004, Crozier and others 2006, Gunter and others 2006). It was found to have the potential as an independent marker to predict early disease recurrence in stage I and II colorectal patients in one study, with the investigators concluding that it was superior to the carcinoembryonic antigen (CEA)-independent prognostic marker it was judged against (Koike and others 2008). In patients with oesophageal squamous cell carcinoma CRP levels are prognostic for survival, particularly the level of CRP expression within tumour tissue (Nozoe and others 2002). CRP may also be useful for prognosis in renal squamous cell carcinoma where it correlates with disease free interval (Casamassima and others 2005). In men with androgen-independent prostatic cancer, elevated CRP levels are strongly predictive of poor survival (Beer and others 2008). There are many known possible causes of elevated CRP in dogs, including babesiosis, leptospirosis, leishmaniasis, parvovirus and E.coli endotoxaemia arthritis, lymphoma and inflammatory bowel disease (Jergens and others 2003, Ceron and others 2005, Nielsen and others 2007, Lowrie and others 2009a); and a range of neoplastic diseases although specific correlations have only be described so far for mammary tumours and lymphoma (Mischke and others 2007, Planellas and others 2009, Tecles and others 2009,Yuki and others 2011). Cases in the MCT group in our study had significantly elevated CRP levels compared to the normal range while cases in the sarcoma group had elevated levels of CRP, Hp and AGP. Fujimoto and others (2003) documented previously that canine mast cell activation is linked to CRP with mast cell migration in response to CRP being both chemokinetic and chemotactic. The authors postulated that CRP may play a role in migration of mast cells to areas of inflammation during the acute phase response. One of the main biologic functions of CRP lies in its ability to identify damaged or abnormal cells, and then mediate their elimination through recruitment of complement and phagocytic cells. Whether the elevated CRP in cases with MCTs reflects a response to neoplastic-induced inflammation, or potentially a role

in tumorigenesis is unknown however it intuitively seems more likely that CRP is involved through the neoplasia-induced inflammatory response. Large, rapidly growing tumours are more likely to contain areas of ischemia and/or to develop ulceration, both of which will incite a non-specific inflammatory response to tissue damage and tumour necrosis (Planellas and others 2009, Tecles and others 2009).

It has been shown that human patients with advanced malignancies who have AGP glycoforms containing highly fucosylated triantennary and tetra-antennary sugar chains for long periods after surgery are likely to have a poor prognosis although there was no correlation with tumour size or metastatic disease (Hashimoto and others 2004). These authors also documented in a previous study that levels of serum alpha (1,3)-fucosyltransferase, the enzyme response for fucosylation of AGP, is elevated in patients with highly malignant or metastatic disease and a separate study suggested that tumour tissue may itself be the source of this raise (Asao and others 2001). There is currently little or no information available regarding AGP in dogs with neoplastic disease, and to the authors knowledge no studies have looked at the potential role of different glycoforms of AGP in dogs with tumours. In our cases the AGP level was significantly different to normal in dogs with both MCT and sarcoma. Our findings suggest that measuring AGP may be useful in dogs with these tumours, although the numbers were too small to evaluate whether there is a direct link with tumour size, grade and extent of disease.

There is only one subtype of Hp known in dogs, compared to three in humans, and there are at least two identifiable structural differences between human and canine Hp. Fucosylated Hp has been identified as a marker of disease in human pancreatic cancer and as a glioblastoma-specific serum marker and expression of haptoglobin has been found to predict recurrence in human head and neck cancer (Ching-Chih and others 2010, Kumar and others 2010, Miyoshi and others 2010). Glycosylation patterns are known to vary between different disease states in dogs (Andersson and others 1998) and a study by Andersson and Sivelius in 2001 identified a higher incidence of Hp glycosylation in both auto-immune disease and neoplasia of lymphoreticular or myeloproliferative origin. There are numerous studies documenting elevated Hp in cattle, pigs, sheep and horses with non-neoplastic diseases such as babesiosis and mastitis, but very little information regarding Hp in small animal populations, and in particular very little regarding Hp in specific neoplastic conditions. To the authors knowledge this is the first study looking at Hp levels in dogs with solid tumours of soft tissue origin. Similar to the findings for AGP, we found that Hp levels were elevated in dogs with sarcoma but not in those with MCT. Interestingly, Hp has been shown to bind to

human mast cell line HMC-1, inhibiting up to 40% of the spontaneous growth of the cells (El-Ghmati and others 2002). Whether this is related to the findings here that there is no increase in Hp in dogs with MCT is an intriguing question and worthy of further study.

SAA has been shown to increase in a wide range of human malignancies, including gastric, nasopharyngeal, colorectal and breast cancer, as well as in mouse models for cancer research (Biran and others 1986, Chan and others 2006). Gutfeld and co-workers (2006) investigated the expression of SAA at various stages of human colon carcinoma progression and found that expression increased gradually as cell progressed through dysplasia to neoplasia. RT-PCR analysis confirmed the expression of the SAA1 and SAA4 genes in colon carcinomas, expression that was barely detectable in normal colon tissues. These findings led the authors to postulate that SAA production may play a role in colonic tumorigenesis. A role in cancer progression has been suggested as a result of the apparent relationship between SAA levels and development of metastatic disease from solid tumours (Ramankulov and others 2008). Elevation of this APP in dogs with known malignancy would seem logical; however this was not what we found. Interestingly, and unexpectedly, the SAA levels in dogs with MCT were significantly lower than the reference range, but those patients with sarcomas had no significant difference when compared with the normal range. Sample size was low however which could have affected the findings or lack of statistical significance

It is difficult to explain this apparent reversal of what would normally be expected with a major APP in the presence of neoplastic disease. SAA is not affected by the presence of haemolysis, lipaemia or bilirubinaemia in blood samples; it is not influenced by sex or age, and is not prone to significant circadian variations (Ceron and others 2005).

Conclusions:

The numbers in our groups were small which compromises the validity of statistical evaluation so our results must be interpreted with caution. However some interesting relationships have emerged from the initial evaluation. Our findings suggest that specific combinations of serum APPs for MCTs and sarcomas may be useful as a screening test for the presence of disease. For patients with MCTs, CRP and AGP levels would be expected to increase, with a concurrent drop in SAA levels. In sarcoma patients CRP, AGP and Hp can all be expected to increase. These initial results need to be evaluated in larger numbers of cases with naturally occurring disease to validate the findings. Further areas for

investigation include assessment of correlation between APP levels and presence of metastatic disease, and whether the APP values alter following surgery as has been documented in people.

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Table 1. APP values for MCT and SARCOMA compared to a "normal" control group (i.e. normal range) using two-tailed P-values i.e. alternative hypothesis is that mean APP values for affected individuals are significantly different from those of normal intervals (significance set at $p<0.05$)

APP	Normal mean (SD)	MCT mean (SD) N= 20	**Two-sample T-test p-value**	SARCOMA mean (SD) N=21	**Two-sample T-test p-value**
CRP	1.97 (1.34)	10.41 (7.43)	**<0.00**	**26.21 (42.01)**	**0.02**
HAPTO	1.59 (0.57)	1.83 (1.90)	0.58	**2.99 (1.77)**	**<0.00**
SAA	2.88 (0.34)	1.67 (1.34)	**<0.00**	6.06 (11.10)	0.22
AGP	0.26 (0.11)	0.53 (0.45)	**0.02**	**0.65 (0.26)**	**<0.00**

Table 2. Mann-Whitney tests for comparison of the APP values obtained and the grade of the MCT or Sarcoma (significance set at $p<0.05$)

APP	MCT grade 1 (median) N=5	MCT grade 2 (median) N=15	Mann-Whitney p-value	Sarcoma grade 1 (median) N=7	Sarcoma grade 2 (median) N=14	Mann-Whitney p-value
CRP	9.91	12.49	0.97	9.21	13.28	0.14

HAPTO	2.39	0.90	0.24	1.68	3.27	0.08
SAA	1.06	1.47	0.63	0.80	2.04	**0.02**
AGP	0.69	0.31	0.32	0.45	0.68	0.74

Comment [KP2]: The reviewer stated that Table 3 was no longer necessary, but this was already removed before submitting the revised manuscript.

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