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Peripheral blood biomarkers of solid tumor angiogenesis in dogs: A polychromatic flow cytometry pilot study

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Abstract

A subset of peripheral blood hematopoietic stem and progenitor cells of bone marrow origin is elevated in humans with solid cancers before treatment and declines with therapy. This biomarker of angiogenesis is not specific to tumor type and has great potential in the objective assessment of treatment response in clinical trials. This pilot study was designed to develop a biomarker of neoangiogenesis in dogs for the diagnosis of cancer, the measurement of treatment response, and the provision of objective data in clinical trials. Polychromatic flow cytometry was used to quantify two subsets of circulating hematopoietic stem and progenitor cells in dogs with spontaneous solid tumors before ($n = 8$) and after ($n = 3$) treatment, and normal controls ($n = 6$).

Pro-angiogenic peripheral blood cells of bone marrow origin were detected in all eight cases and the six normal controls; however, there was no statistically significant difference between the two groups. Interestingly, an apparent decline in pro-angiogenic cells was observed after treatment. Bone marrow derived hematopoietic cells appear to contribute to tumor angiogenesis in dogs, as has been previously reported in humans. While the methodology for pro-angiogenic cell quantification in a small number of dogs in the current study did not result in a significant difference from normal controls, an optimized canine polychromatic flow cytometry protocol holds great promise in the development of a canine cancer model and for the objective measurements of treatment response in clinical trials.

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Conflict of interest statement

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Keywords

Dog; Cancer; Biomarker; Angiogenesis; Polychromatic flow cytometry

Introduction

Peripheral blood (PB) biomarkers of tumor burden and neoangiogenesis are currently limited in canine oncology. The discovery and validation of canine PB biomarkers could provide a valuable approach to monitor tumor progression and therapeutic response. Recently, studies have described a population of circulating hematopoietic progenitor cells in humans that was previously thought to be homogenous, but is in fact heterogeneous, containing two populations with distinct phenotypic and functional characteristics (Estes et al., 2010a; Estes et al., 2010b). The ratio of these two subsets of circulating hematopoietic stem and progenitor cells (CHSPCs) correlates to various stages of angiogenesis (Estes et al., 2010a).

The pro-angiogenic subset of CHSPCs are CD31⁺CD34^{bright}CD45^{dim}AC133⁺ (ProCHSPC) and have been shown to contribute to angiogenesis in solid tumors, whereas the non-angiogenic subset of CHSPCs (CD31⁺CD34^{bright}CD45^{dim}AC133⁻, NonCHSPC) fail to increase tumor size when injected into tumor bearing mice (Estes et al., 2010b). Each subset of the CHSPCs contained cells of different hematopoietic lineages (Estes et al., 2010b). The ProCHSPCs are predominantly myeloid progenitors whereas the NonCHSPCs are lymphoid progenitors (Estes et al., 2010b). Interestingly, the ratio between the ProCHSPCs and NonCHSPCs is increased in pediatric solid tumor patients compared to healthy controls (Estes et al., 2010b; Pradhan et al., 2011). Furthermore, following successful chemotherapy and/or radiation treatment, the ProCHSPC:NonCHSPC ratio returns to the normal range (Pradhan et al., 2011).

Spontaneous tumors in dogs are emerging as a useful model for human cancer, including cancer vasculature (Paoloni and Khanna, 2008; Pang and Argyle, 2009; Paoloni et al., 2009). Angiogenesis in canine solid tumors mimics that found in human solid malignancies such as fibrosarcoma and breast cancer (Al-Dissi et al., 2009; Clemente et al., 2010). Features shared between human and canine tumors include angiogenic factors such as vascular endothelial growth factor (VEGF) and VEGF receptors 1 and 2, the process of vasculogenic mimicry, and angiogenesis genetics (Al-Dissi et al., 2009; Clemente et al., 2010). The dog models correlate with human malignancies in regards to the genetic and molecular biology of the cancer stem cells within the tumor itself (Pang and Argyle, 2009). This parallel raises the interesting question of whether CHSPCs exist in dogs, can be phenotypically characterized, and function similarly between the two species.

PB biomarkers of cancer, tumor burden and angiogenesis would be extremely beneficial in the veterinary field, where they might complement more expensive and sometimes invasive measures of tumor burden. The currently available diagnostic modalities include radiography, endoscopy, or advanced imaging under anesthesia (computed tomography, magnetic resonance imaging, positron emission tomography) which permit objective tumor measurements during clinical trials and facilitate the assessment of treatment response. Currently there are limited PB biomarkers available for the diagnosis and objective measurement of treatment response of cancer in dogs (Henry, 2010). Biomarkers of canine angiogenesis are currently in the early stages of development, with PB levels of VEGF being related to outcome for dogs with a variety of cancers (Wergin et al., 2006; Thamm et al., 2008) and a decline in circulating endothelial cells being observed in dogs that responded to antiangiogenic therapy (Rusk et al., 2006).

The aim of this pilot study was to develop a biomarker of canine neoangiogenesis that would be of potential benefit for the diagnosis of cancer, the measurement of treatment response, and provision of objective data in clinical trials. Our hypothesis was that the ProCHSPC:NonCHSPC ratio would be increased in dogs with cancer compared to normal controls. Secondly, we hypothesized that the ProCHSPC:NonCHSPC ratio would be reduced following the initiation of therapy.

Materials and methods

Dogs

Inclusion criteria were adult dogs with solid malignancies undergoing definitive therapies (excisional surgery, radiation therapy and/or chemotherapy). Patients with hemangiosarcoma were excluded from this pilot study to prevent potential confusion with hemangioblasts from which the tumor originates, since they are detectable in the PB by flow cytometry (Lamerato-Kozicki et al., 2006). Eight dogs with malignant solid tumors were prospectively enrolled in this study. Each dog had a definitive diagnosis of solid malignancy based on histopathology. To avoid the influence of post-operative wound healing on angiogenesis, patients suspected to have a solid malignancy and scheduled for excisional surgery were enrolled in the study and had pre-therapeutic samples drawn prior to surgery, and only maintained in the study if histopathology confirmed a solid malignancy. Dogs previously diagnosed with a solid malignancy via punch biopsy were also included. PB draws, as well as diagnostic procedures and tumor staging, were performed at the Veterinary Teaching Hospital, Purdue College of Veterinary Medicine. All protocols and procedures were approved by the Purdue Animal Care and Use Committee (protocol 10-006). Written informed consent was obtained from all owners.

Isolation of mononuclear cells

PB samples (6–9 mL) were collected in EDTA at 1–3 time points for each dog. A pre-treatment sample (all dogs), and one or two post-treatment samples (3 dogs) were collected. At least 21 days elapsed after any stimulus of angiogenesis including surgery, incisional biopsy or trauma. There was also at least 21 days between sampling of the same dog. PB samples (6–9 mL) were also collected from six control dogs, defined as normal controls based on history and physical examination. Mononuclear cells (MNCs) were isolated using Ficoll (GE Healthcare) and were centrifuged at 700 *g* for 30 min at room temperature. Following separation, the cells were washed with 2% fetal bovine serum (FBS) in phosphate buffered saline (PBS). In addition to collection of PB in EDTA, MNC isolation was compared to blood collected in the Cell Preparation Tube (CPT) Vacutainer system by centrifuging at 1600 *g* for 30 min at room temperature, and the recovery of MNC was much lower.

Immunophenotyping

MNCs were incubated with Fc Block (Miltenyi) for 10 min on ice to prevent non-specific binding of antibodies. To assess surface antigens of the isolated MNCs, CD34 Phycoerythrin PE and CD14 Pacific Blue (BD Biosciences), CD133 Allophycocyanin (APC) (eBioscience), CD45 Fluorescein isothiocyanate (FITC) (AbD Serotec) and LIVE/DEAD (Invitrogen) were added to ascertain the previously identified circulating progenitor cell populations in human PB (Estes et al., 2010a; Estes et al., 2010b). All antibodies were titrated based on the mean fluorescence intensity with fluorochrome conjugate coupling to specific antigens optimized for the four-antibody plus viability marker panel (Baumgarth and Roederer, 2000; Mahnke and Roederer, 2007; Tung et al., 2007; Estes et al., 2010a).

Polychromatic flow cytometry (PFC) acquisition and analysis

Stained fixed MNC samples were acquired on a BD LSRII flow cytometer (BD Biosciences) fitted with a 405-nm violet laser, 488 nm-blue laser, and a 633-nm red laser. At least 100,000 events were collected for each sample. Data were acquired without compensation and exported as FCS 3.0 files. Compensation of the samples and analysis of the populations was performed in Flowjo v.8.7.3 (Treestar) using bi-exponential display and was based on 'fluorescent minus one' gating controls to ensure the proper identification of true positive and negative events (Roederer, 2001; Parks et al., 2006).

Statistical analysis

Mean and median ProCHSPC:NonCHSPC ratios were calculated for the cases and the normal controls. Gaussian distribution of the data was confirmed using the Kolmogorov and Smirnov method. The mean values of the ProCHSPC:NonCHSPC ratios of the cancer cases and normal controls were compared with an unpaired *t* test with Welch correction. $P < 0.05$ was considered significant. A two-tailed power analysis was performed with a power of 80% and an α value of 5%.

Results

Eight dogs with solid malignancies including osteosarcoma ($n = 2$), transitional cell carcinoma ($n = 2$), squamous cell carcinoma ($n = 2$), multilobular tumor of bone ($n = 1$) and adenocarcinoma ($n = 1$) were enrolled in this pilot study. Post-therapy samples were drawn on the case of metastatic anal sac adenocarcinoma treated with palliative radiation therapy (twice), a urethral transitional cell carcinoma (TCC) being treated with chemotherapy (once), and a mandibular osteosarcoma treated with excisional surgery receiving post-operative carboplatin (once). Survival from the day of diagnosis was 154–214 days in five dogs, with three dogs still alive at 18–374 days (Table 1).

The Kolmogorov and Smirnov ProCHSPC:NonCHSPC ratios for the cancer cases (0.2707, $P = 0.09$) and the normal controls (0.2416, $P > 0.1$) indicated that both data sets were normally distributed.

The median ProCHSPC:NonCHSPC ratio for untreated dogs with solid malignancies was 0.71 (range, 0.59–1.39). The median ratio following treatment was 0.58 (range, 0.54–1.02). The median ProCHSPC:NonCHSPC ratio for normal controls was 0.72 (range, 0.68–0.85). The mean ProCHSPC:NonCHSPC ratio for the untreated cancer patients was 0.81, compared with a mean ratio of 0.74 for the normal controls (Fig. 1), with no significant difference between the mean ratios ($P = 0.52$). Power analysis indicated that 100 controls and 100 dogs with cancer would be required to generate statistically significant data.

Following initiation of therapy (chemotherapy [$n = 1$], palliative radiation [$n = 1$] or surgery with post-operative chemotherapy [$n = 1$]), the ProCHSPC:NonCHSPC ratio fell in all instances, with a median value of 0.58 (range, 0.54–1.02) (Fig. 2).

Discussion

In the current study, we report for the first time in dogs with a wide variety of malignant solid tumors the detection of two subsets of CHSPCs that have been previously described in humans (Estes et al., 2010b; Pradhan et al., 2011). Although in this pilot study the sample size was not sufficient for statistical significance, interesting trends were seen between normal and cancer bearing dogs. In particular, the decrease following treatment in the cancer cases may be promising as a marker of treatment response. Potentially, statistical significance could be reached with a larger sample size for specific types of cancer.

At the time of the study, CD31 was not commercially available for dogs, so we chose to use the modified definition for CHSPCs, which are CD34^{bright}CD45^{dim}CD133⁺ (ProCHSPC) and CD34^{bright}CD45^{dim}CD133⁻ (NonCHSPC). Monocytes were excluded using CD14 to prevent misidentification of the progenitor populations. Furthermore, the manufacturer's recommendations were to use CD133 (which had been tested on canine samples) over AC133 (Miltenyi Biotec), which had not been tested on canine samples.

We have shown that untreated dogs with cancer display ProCHSPC:NonCHSPC ratios across a wide range (0.59–1.39), and that following treatment, the ProCHSPC:NonCHSPC ratio is decreased compared to pre-treatment. The values in untreated controls were grouped much more tightly (0.68–0.85). However, significant overlap was seen between untreated dogs with cancer and normal controls, and only 2/8 cases with cancer displayed a ProCHSPC:NonCHSPC ratio above the values detected in normal dogs.

In addition to its small sample size, a limitation of this study was the potential for false positive increases in the Pro:NonCHSPC ratio due to non-malignant conditions leading to angiogenesis, such as post-surgical wound healing. The potential for interventions leading to increases in the ratio was limited by only drawing blood at least 21 days after surgery. However, whether invasive procedures lead to increases in the ProCHSPC:NonCHSPC ratio and the duration of such elevations has not yet been studied in either dogs or humans.

The discovery of CHSPCs in solid tumor bearing dogs is important because in humans bone marrow derived circulating cells apparently contribute to angiogenesis (Schattelman and Awad, 2004; Stoll et al., 2003; Zhu et al., 2009), a finding not yet documented in the dog. Furthermore, the ranges of the populations containing both the ProCHSPCs and the NonCHSPCs subsets were much more variable in dogs with malignancies compared to normal controls.

In human pediatric solid malignancies, our group has previously shown that the ProCHSPC:NonCHSPC ratio is significantly elevated compared to normal age-matched controls (Pradhan et al., 2011). In this canine pilot study, the CHSPC subpopulations were quantitated using a slightly modified antigen panel based on previously published data in humans and on the commercial availability for canine cells. As the CHSPCs have been further characterized, we have found that the human markers do not fully correlate with the canine cell subtypes. Interestingly, recent studies utilizing flow cytometry have shown differences in myeloid and lymphoid cells in the diagnosis and successful treatment of canine malignancies (LeBlanc et al., 2010; Tominaga et al., 2010; Comazzi et al., 2011). In a preliminary flow cytometry assessment of therapy with the anti-angiogenic agent ABT-510, there was no significant change in circulating endothelial cells in 10 dogs that experienced early disease progression. In contrast, in three dogs that did respond to therapy, there was a significant decrease in total circulating endothelial cells characterized by decreases in CD106- and CD133-positive endothelial precursors, and an increase in Annexin V-positive apoptotic endothelial cells (Rusk et al., 2006). However, there was no comparison to normal controls in that study.

Other biomarkers of angiogenesis in solid tumor-bearing dogs have been previously studied, including homeobox proteins in canine vascular tumors (Kodama et al., 2009), VEGF and VEGF receptor in mammary adenocarcinomas (Al-Dissi et al., 2010), fibrosarcomas (Al-Dissi et al., 2009) and skin cancers (Al-Dissi et al., 2007), and the report of a significant relationship between VEGF and prognosis for meningioma (Platt et al., 2006). Notably, despite the importance of angiogenic markers in the solid tumors, VEGF and microvascular density were not prognostic factors for lymphoma (Wolfesberger et al., 2012), which was excluded from the current study along with all round cell tumors. Round cell tumors were

excluded from the current pilot study because the role of angiogenesis is still emerging in hematological malignancies (Orpana and Salven, 2002) and most studies have been based on solid tumors, indicating that round cell tumors may be best studied separately.

These studies all utilized tumor samples, while a significant relationship between treatment outcome and pre-treatment levels of a non-tumor specific PB biomarker has only been demonstrated in early studies of VEGF. Pre-treatment plasma VEGF was significantly associated with time to treatment failure for 60 dogs with a variety of tumors undergoing both low-dose and high-dose radiation therapy (Wergin et al., 2006), while lower serum VEGF was associated with a longer median disease free interval and median survival time in 22 dogs treated for osteosarcoma (Thamm et al., 2008).

Conclusion

Populations of bone marrow-derived CHSPCs may contribute to angiogenesis in dogs as in humans. These preliminary results indicate that the precise phenotypic CHSPC populations used in our study did not directly translate from human to dog. However, these cellular populations based on functional phenotype hold great promise as a PB biomarker of angiogenesis and tumor burden for dogs with solid tumor malignancies. Further studies are required to determine the precise canine specific antibodies for the optimal detection of CHSPCs. This biomarker could provide a novel diagnostic tool and improve monitoring of treatment response.

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Fig. 1. Pro-angiogenic to non-angiogenic circulating hematopoietic stem and progenitor cells (CHSPCs) polychromatic flow cytometry ratios (Pro:NonCHSPC ratios) in dogs with solid malignancies compared to normal controls. Box and whisker plots of the range (whiskers) and upper and lower quartiles (boxes).

Fig. 2.

Representative flow cytometry contour plots showing the decrease in pro-angiogenic to non-angiogenic circulating hematopoietic stem and progenitor cells (CHSPCs) polychromatic flow cytometry ratio (Pro:NonCHSPC) ratio following treatment in a tumor bearing dog. (a) Pro:NonCHSPC ratio (1.35) at diagnosis, (b) Pro:NonCHSPC ratio (1.00) at 6 months post-treatment.

Table 1

Polychromatic flow cytometry ratios of pro-angiogenic to non-angiogenic circulating hematopoietic stem and progenitor cells (CHSPCs) in normal controls and dogs with cancer

Dog	ProCHSPC (CD133⁺) % of Parent CHSPC population	NonCHSPC (CD133⁻) % of Parent CHSPC population	Pro:NonCHSPC ratio
Normal control 1	44.0	56.0	0.79
Normal control 2	45.8	53.8	0.85
Normal control 3	40.3	59.1	0.68
Normal control 4	40.9	59.1	0.69
Normal control 5	42.2	57.3	0.74
Normal control 6	40.9	58.6	0.70
Mean (range)	42.4 (40.3 – 45.8)	57.3 (53.8 – 59.1)	0.74 (0.68 – 0.85)
Osteosarcoma (osteoblastic)	47.3	51.8	0.91
Transitional cell carcinoma (high grade)	45.3	54.7	0.83
Multi-lobular tumor of bone	36.5	61.9	0.59
Transitional cell carcinoma	57.7	41.4	1.39
Squamous cell carcinoma:-lymphatic metastasis	41.1	58.0	0.71
Adenocarcinoma:- lymphatic metastasis	39.5	59.9	0.66
Squamous cell carcinoma	39.3	60.7	0.65
Osteosarcoma (periosteal)	40.9	58.3	0.70
Mean (range)	43.5 (36.5 – 57.7)	55.8 (41.4 – 61.9)	0.81 (0.59 – 1.39)

Table 2

Polychromatic flow cytometry ratios of pro-angiogenic to non-angiogenic circulating hematopoietic stem and progenitor cells (CHSPCs) in eight dogs with solid cancer

Diagnosis	Location	Therapy	Survival (Days)	Pro:NonCHSPC ratio		
				Diagnosis	Post-treatment sample 1	Post-treatment sample 2
Osteosarcoma (osteoblastic)	Proximal tibia	Surgery:-amputation	157	0.91		
Transitional cell carcinoma (high grade)	Prostate and trigone	Surgery:- debulking. Post-operative chemotherapy:- mitoxantrone	277 (alive)	0.83		
Multi-lobular tumor of bone	Dorsal skull	Surgery:- excision	374 (alive)	0.59		
Transitional cell carcinoma	Proximal urethra	Chemotherapy:- targeted vinblastine conjugate	214	1.39	1.02 Partial remission	
Squamous cell carcinoma:- lymphatic metastasis	Oropharynx	Radiation therapy:- palliative	158	0.71		
Adenocarcinoma:- lymphatic metastasis	Anal sac	Radiation therapy:- palliative	154	0.66	0.54 Stable disease	0.54 Progressive disease
Squamous cell carcinoma	Maxillary gingiva	Surgery:- excision	18 (alive)	0.65		
Osteosarcoma (periosteal)	Mandible	Surgery:- excision. Post- operative chemotherapy:- carboplatin.	180	0.70	0.61 Complete remission	