

Canine hemangiosarcoma originates from hematopoietic precursors with potential for endothelial differentiation

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Objective. Two competing hypotheses can be formulated regarding the origin of canine hemangiosarcoma (HSA). One states HSA originates from differentiated vascular endothelial cells that undergo mutations which endow them with malignant potential. The other states HSA originates from transformed hemangioblastic stem cells. This study was designed to begin to distinguish between these possibilities, as well as to test if flow cytometry was sufficiently sensitive to detect malignant cells in blood samples from dogs with HSA.

Methods. We used multiparameter flow cytometry to examine expression of cell-surface determinants associated with hematopoietic precursors (c-kit, CD34, CD133, CD45) or with lineage-committed cells (CD3, CD11b, CD14, CD21, CD105, CD146, $\alpha_v\beta_3$ -integrin) in HSA cell lines and in blood samples from healthy dogs or dogs with HSA.

Results. The data show that HSA cells coexpress surface markers associated with hematopoietic precursors and with commitment to endothelial lineage, providing a means to identify their presence in circulation and distinguish them from normal or malignant white blood cells. The percentage of cells that coexpressed these markers ranged from 0.5 to 1.25% for HSA dogs, and was less than 0.3% for unaffected dogs or dogs with HSA that had the tumors removed within 48 hours prior to obtaining samples.

Conclusions. The results place the ontogeny of HSA with multipotential bone marrow-derived stem cells whose progeny arrest differentiation at the hemangioblast or angioblast stage. In addition, these expression patterns may assist to confirm an HSA diagnosis, monitor minimal residual disease, and detect the disease in early stages. © 2006 International Society for Experimental Hematology. Published by Elsevier Inc.

Hemangiosarcoma (HSA) is a common, aggressive malignant neoplasm of dogs that arises from vascular endothelial cells; however, its precise origins remain incompletely understood. Two competing hypotheses can be formulated regarding the ontogeny of HSA. One states that HSA originates from differentiated cells in the endothelial lining of blood vessels (i.e., cells that have matured to or past the angioblast stage) that undergo mutations which endow them with malignant potential [1]. This assumes that any cell that can proliferate is equally susceptible to transformation and retains the capacity for self-renewal. In this case,

tumor development would be a stochastic process driven by selection of mutations that favor proliferation and survival [2]. The competing hypothesis states that HSA originates from incompletely differentiated, bone marrow-derived, multipotential stem cells that are near or at the stage of endothelial commitment (i.e., hemangioblasts) [3]. Recent studies of leukemias, brain tumors, mesenchymal tumors, and mammary cancer support the concept of “cancer stem cells” [2,4–7], and when considered in light of the finding that early endothelial precursor cells migrate from the bone marrow to peripheral vessels and may be largely responsible for physiologic and pathologic angiogenesis [3,8], it increases the plausibility that transformation of such stem cells is responsible for HSA.

The mobilization of angiogenic progenitor cells in the bone marrow is mediated by matrix metalloproteinase-9,

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kit ligand, and vascular endothelial growth factor (VEGF) [9]. VEGF binding to VEGF receptor-2 (VEGFR-2) mediates a maturation cascade of endothelial commitment progressing from hemangioblast to angioblast to early and late endothelial precursor cells (EPC) [3,9]. Hemangioblasts are multipotent hematopoietic stem cells defined by the expression of CD133, VEGFR-2, and possibly CD34 and c-kit; they represent a small population with proliferative potential capable to give rise to late endothelial outgrowth [3,9]. EPC are virtually undetectable in the circulation of normal individuals except by the most sensitive methods or by prolonged expansion in culture [9].

The course of hemangioblast differentiation remains incompletely understood: it has been proposed that these cells go through stages where they express the common leukocyte antigen CD45 and the myeloid antigen CD14, which are downregulated in differentiated endothelial cells [9–12]; however, other investigators believe that monocytoid CD14⁺ cells that give rise to EPC represent a lineage that is distinct from bone marrow hemangioblasts [13]. There is consensus, however, that mature endothelial cells commonly express VEGFR, CD31, CD105, CD146, and Factor VIII-related antigen (vWF antigen). Both CD31 and vWF antigen are endothelial markers that are expressed in most cases of canine HSA [14,15]. CD146 (Mel-CAM) is an adhesion molecule present on endothelial cells throughout the vascular tree [16]. CD105 (endoglin) is a receptor for the transforming growth factors (TGF)- β 1 and - β 2, and is abundantly expressed on angiogenic endothelial cells [17]. In a previous study in our laboratory, stable expression

of CD146 and CD105 was confirmed in eight canine HSA cell lines after several passages [18]. Under conditions of activation that lead to proliferation, endothelial cells also upregulate $\alpha_v\beta_3$ -integrin (vitronectin receptor) [19,20].

Our goal was to distinguish between the two alternative possibilities of HSA ontogeny. Specifically, we surmised that the expression of markers associated with bone marrow progenitor cells could distinguish EPC or EPC-like cells (hemangioblasts) from mature endothelial cells. Previously we demonstrated that HSA cells represented a “primitive” lineage based on expression of c-kit [18]. Here, we confirm that HSA originates from bone marrow progenitors at various stages of hemangioblastic differentiation. In addition to their implications for a naturally occurring and accessible model to study the biology of cancer stem cells, these findings offer added insights into the pathogenesis of canine HSA, they can be used for diagnostic purposes, and they may be prognostically significant.

Materials and methods

Cell lines and tumor samples

HSA cell lines DD-1, Dal-4, CHAD-G4.1, CHAD-B7.4, Mocha-HSA, and Frog-HSA were derived and maintained as described [1,18]; Jurkat cells were also maintained as described previously [21]. Blood samples from 35 dogs, including 17 with biopsy-confirmed HSA, 16 that did not have HSA (9 healthy, 3 with leukemia, 3 with splenic hematoma, 1 with splenic nodular hyperplasia), and 2 with undiagnosed hemorrhagic effusions (pericardial, abdominal) were obtained with owner consent through

Table 1. Antibodies, source, and antigen specificities

Target CD antigen	Clone	Source and isotype	Antigen specificity	Vendor
CD3	CA17.2A12	Mouse IgG1	Canine CD3	Serotec ¹
CD5	YKIX322.3	Rat IgG2a	Canine CD5	Serotec
CD45	YKIX716.13	Rat IgG2b	Canine CD45	Serotec
CD11b	CA16.3E10	Mouse IgG1	Canine CD11b	Serotec
B cells	CA2.1D6	Mouse IgG1	Canine CD21 ²	Serotec
CD14	TuK4	Mouse IgG2a	Human CD14 ³	Serotec
CD34	2E9	Mouse IgG1	Canine CD34	BD Bioscience ⁴
c-kit (CD117)	YB5.B8	Mouse IgG1	Human c-kit ⁵	BD Bioscience
CD133	13A4	Rat IgG1	Murine CD133	eBioscience ⁴
CD146	PIH12	Mouse IgG1	Human CD146 ⁶	Chemicon ⁷
$\alpha_v\beta_3$ -integrin (CD51/61)	LM609	Mouse IgG1	Human CD51/61 ⁸	Chemicon
CD105	8E11	Mouse IgM	Human CD105 ⁹	Southern Biotechnology Associates ¹⁰

¹Raleigh, NC.

²Precise identity of target antigen as CD21 has not been verified. Reported cross-reactivity by vendor includes horse and cat CD21 homologues.

³Reported cross-reactivity by vendor includes dog CD14.

⁴San Diego, CA.

⁵Reported cross-reactivity by vendor includes dog c-Kit.

⁶Reported cross-reactivity by vendor includes dog CD146.

⁷Temecula, CA.

⁸Reported cross-reactivity by vendor includes dog $\alpha_v\beta_3$ -integrin.

⁹Reported to cross-react with dog CD105 [16].

¹⁰Birmingham, AL.

a protocol reviewed by the Institutional Animal Care and Use Committee and the Institutional Review Board of AMC Cancer Center.

Flow cytometry and immunofluorescence

Flow cytometric staining and immunofluorescence were done as described [18,22]. Adherent cells were detached using 1 mM EDTA or Accutase (Innovative Cell Technologies, San Diego, CA, USA). Preservation of surface antigens (and thus signal strength) was better with EDTA, but overall cellular viability was higher with Accutase. Cell lines were stained with primary antibodies described in Table 1, conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE), or labeled with Zenon probes (Alexa Fluor 488, Alexa-Fluor 647, or Alexa-Fluor APC, Invitrogen-Molecular Probes, Carlsbad, CA, USA), or with irrelevant, isotype-matched antibody controls and analyzed using Coulter Cytomics FC500 (Beckman Coulter, Fullerton, CA, USA) or FACSCalibur (BD Immunocytometry Systems, San Jose, CA, USA) flow cytometers. We used a series of iterative steps to identify circulating EPC or HSA cells in peripheral blood. First, we used single-color staining to define background levels for each antibody and to verify that the relative numbers of leukocytes ($CD21^+$ B cells, $CD3^+$ and $CD5^+$ T cells, $CD14^+$ monocytes, and $CD11b^+$ granulocytes) in our samples were within previously reported reference intervals [23–25]. Next, we used antibody combinations for two-color staining. Color compensation was adjusted using BD Biosciences CompBeads. Populations that stained positive for one or more of three markers associated with bone marrow progenitor cells (c-kit, CD34, CD133) and for a marker associated with endothelial cells (CD146) and/or *proliferating* endothelial cells ($\alpha_v\beta_3$ -integrin) were “back-gated” to two-dimensional light scatter dot plots to define the flow cytometric light scatter parameters of HSA cells vs normal leukocytes. This procedure was used for all the samples included in the analyses; in addition, we further

modified the protocol for the last seven samples to exclude leukocytes by using antibodies against CD5, CD11b, and CD21 labeled with FITC and/or Alexa Fluor 488 to establish a “dump gate,” and to detect EPC in the remaining population by dual-staining with antibodies against c-kit, CD34, or CD133 along with antibodies against $\alpha_v\beta_3$ -integrin or CD146. At least 100,000 cells were analyzed for each antibody pair to ensure statistical validity for rare-event determination. A patent is pending for the assays to diagnose angiosarcomas and hemangiosarcomas in samples from humans and animals, respectively.

Statistics

Descriptive statistics were used to characterize each subgroup in the population. Nonparametric analyses (Kruskal-Wallis one-way analyses of variance with pairwise comparisons, using Dunn’s method adjusted for three pairwise comparisons using a bonferroni adjusted *p* value of 0.01667) were used to determine if any two groups were significantly different from each other. All analyses were conducted using SAS Version 9.1 (SAS Institute, Cary, NC, USA).

Results

To define the ontogeny of HSA and to test the alternative hypothesis that this tumor arises from hemangioblasts, we examined the expression of markers that would distinguish these cells from other lineages of hematopoietic differentiation and from mature, differentiated leukocytes and vascular endothelial cells. We used flow cytometry and immunofluorescence microscopy to examine expression of surface proteins restricted to bone marrow precursor cells (e.g., c-kit, CD34, CD133) and proteins that define lineage commitment (CD3, CD21, CD11b, CD105, CD146,

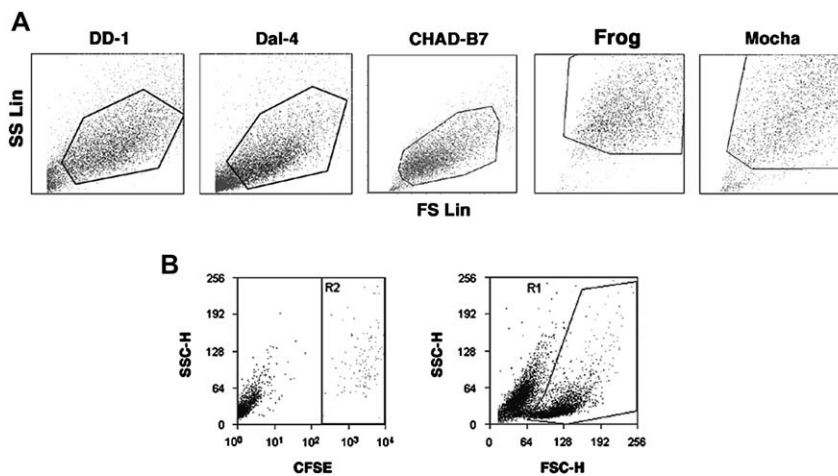


Figure 1. Light scatter profiles of HSA cell lines. (A) Two-dimensional light scatter dot plots (FS vs SS) for five HSA cell lines analyzed using the Coulter Cytomics FC500. Each axis has 1024 channels (0–1023). Events excluded from the gated regions represent dead cells and debris, or cell aggregates. The cell lines Frog-HSA and Mocha-HSA are very early passage lines that show the pleomorphic features of the cells when they are first established in culture. DD-1, Dal-4, and CHAD-B7 cells represent established lines that tend to be less pleomorphic. (B) Dal-4 cells were labeled with CFSE and mixed with human Jurkat T leukemia cells at a ratio of 1 Dal-4 cell for every 100 Jurkat cells. Cells were analyzed using the FACSCalibur; the left dot plot shows right angle side scatter (SSC-H) in 256 channels as a function of CFSE staining. On the right dot plot, CFSE⁺ cells were “backgated” onto the light scatter (FSC-H vs SSC-H) dot plots to show the relative distribution of HSA cells as compared to mononuclear lymphoblasts.

$\alpha_v\beta_3$ -integrin) in six HSA cell lines. We also evaluated expression of CD14 and CD45, both of which are expressed by hemangioblastic cells at various stages of lineage commitment and differentiation [3,26,27]. Cultured HSA cells were moderately pleomorphic, generating diffuse light scatter signatures (Fig. 1). The forward angle light scatter (FS) of HSA cells was larger than large mononuclear leukocytes and granulocytes; the right angle (side) light scatter (SS) was larger than that of mononuclear leukocytes and similar to that of granulocytes. In fact, when we labeled Dal-4 HSA cells with CFSE and mixed with Jurkat lymphoma cells at ratios ranging from 1:1 to 1:100, the cells were distinguishable from each other (Fig. 1).

Figure 2 shows one-dimensional flow cytometry dot plots for two representative HSA cell lines, DD-1 and Dal-4, which stained positive for c-kit, CD133, CD14, CD45, $\alpha_v\beta_3$ -integrin, and CD146. Staining for CD34 was positive in Dal-4 cells and in early-passage DD-1 cells, but expression of this antigen was lost after few passages (see below).

Cultured HSA cells floated on a discontinuous gradient of Ficoll-Hypaque with similar buoyant density as other blood mononuclear cells. Immunofluorescence staining for the cell lines was consistent with flow cytometry data as well as with previous results from our laboratory (data not shown and [18]). Each cell line expressed c-kit, CD133, $\alpha_v\beta_3$ -integrin, CD105, and CD146; expression of CD34, CD45, and CD14 was variable, and none expressed CD3, CD21, or CD11b. It is especially noteworthy that under conditions of logarithmic growth certain subpopulations in the cultures lacked expression of CD133, CD105, and CD146 (for example, see Fig. 2 and ref. [18]), and the density of receptor expression was also variable. We had previously reported expression of CD105, CD146, and $\alpha_v\beta_3$ -integrin in these lines [18], and a recent report shows HSA cells express VEGFR-2 [28]. While other hematopoietic tumors (leukemias, mast cell tumors, and multiple myeloma) can express one or more of these markers [29–32], the pattern of c-kit/CD34/CD133 and $\alpha_v\beta_3$ -integrin (or CD146) coexpression with no detectable leukocyte-specific markers (CD3, CD21, or CD11b) seems to be uniquely associated with HSA. The levels of expression for CD45, CD34, and CD105 increased in DD-1 and CHAD-B7.4 cells when they were cultured in the presence of endothelial growth factors as compared to basal media. In addition, when lines were maintained in culture for extended periods of time (more than 10–15 passages), they had a tendency to downregulate expression of CD133, c-kit, CD34, and CD105. Various non-mutually exclusive possibilities could account for these changes: 1) expression of these proteins is unnecessary in tissue culture; 2) the lines are genetically unstable and “drift”; or 3) “stem cells” in the populations are lost at the expense of differentiated progeny [18].

HSA cells are found in the blood vessel lining and are thus in intimate contact with the peripheral circulation. It

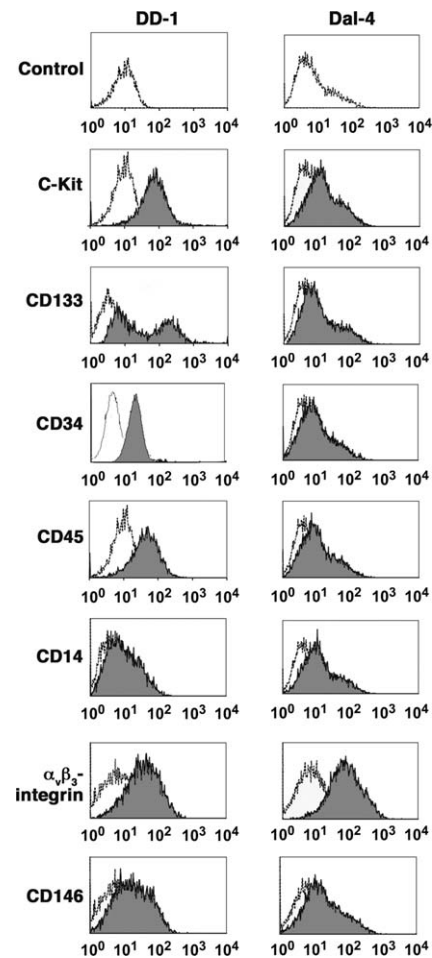


Figure 2. Expression of cell-surface determinants in canine HSA cell lines. One-dimensional overlay histograms show canine HSA cells stained with the indicated antibodies and analyzed by flow cytometry (gray). X-axes represent logarithmic fluorescence intensity and y-axes represent cell numbers (5000 to 10,000 events analyzed). Histograms are overlaid on corresponding negative controls using irrelevant antibodies (white). Data shown for DD-1 cells are from 3 experiments and represent more than 7 experiments done. Data for Dal-4 cells represent more than 4 experiments done. Variability in antigen expression by these cells is described in the text.

has been postulated that many tumors shed cells into the microenvironment [33]; thus, the frequency of hemangioblasts or EPC-like cells might be increased in the blood of dogs with HSA. We used samples from a healthy dog, 3 dogs with HSA, 2 dogs with leukemia, and a dog with splenic nodular hyperplasia to determine the feasibility to distinguish putative HSA cells from other bone marrow-derived cells like mature proliferating endothelial cells, leukocytes, or mast cells. After lysing red blood cells, we stained the samples with antibodies to CD45, c-kit, and $\alpha_v\beta_3$ -integrin. A population of cells was present in the dogs with HSA, but not in the others, with similar light scatter properties to cultured HSA cells, higher autofluorescence than the rest of the white blood cells, and detectable levels of CD45, c-kit, and $\alpha_v\beta_3$ -integrin (Fig. 3).

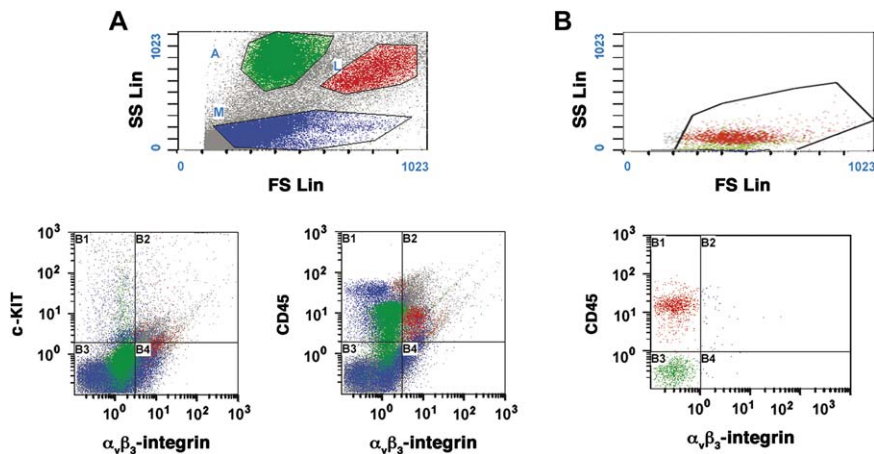


Figure 3. Distribution of cells expressing endothelial markers. The relative distribution of cells expressing CD45, c-kit, and $\alpha_v\beta_3$ -integrin was determined in samples from a dog with HSA (A) and a dog with CLL (B). In (A), gates were drawn in the light scatter dot plots to delineate the granulocyte regions (green), the mononuclear cell region (blue), and the region that would contain putative HSA cells (red). The gating scheme was then used to determine the expression of c-kit, CD45, and $\alpha_v\beta_3$ -integrin in cells included in each of those regions. Note that only the cells in the “red” region consistently show expression of c-kit, CD45, and $\alpha_v\beta_3$ -integrin. The results also show that there is overlap among the regions, as some cells that express these markers distribute to the mononuclear region. Monocytes can be distinguished from HSA cells by the high levels of CD45 expression without $\alpha_v\beta_3$ -integrin expression. In (B), the cells represented a relatively monomorphic population based on light scatter properties. These cells did not express c-kit, CD34, or $\alpha_v\beta_3$ -integrin, so gating was done based on CD45 expression. Note the discrete populations that express CD45 (probably normal mononuclear cells) and those that do not (probably CLL cells).

We then analyzed samples from the next 28 dogs recruited into the study for coexpression of a panel of markers that would identify circulating EPC-like cells. Two of the 28 dogs had undiagnosed hemorrhagic effusions and were excluded from analysis (each of these had 0.2% EPC-like cells). The remaining dogs were grouped into “No HSA” ($n = 12$), “HSA” ($n = 10$), and “HSA where blood samples were obtained <48 hr after splenectomy to excise the tumors” ($n = 4$) for analysis. In the unaffected dog group, 8 were healthy, 3 had splenic hematoma, and 1 had leukemia. The percentage of cells that coexpressed one or more markers of immature bone marrow precursors (c-kit, CD34, CD133) and $\alpha_v\beta_3$ -integrin ranged between 0.5 and 1.25% for dogs with HSA, and was less than 0.3% for unaffected dogs and for dogs with HSA that had their tumors removed within 48 hours prior to obtaining samples. A test of normality (Shapiro-Wilk test) showed that group 1 was not consistent with a normally distributed random variable, exhibiting substantial positive skewness. Groups 2 and 3 both showed some skewness: Group 2 positive skewness and Group 3 negative skewness, but this skewness was not statistically significant in either group. Analysis of variance showed that Group 2 was significantly different from Groups 1 and 3 (Fig. 4). The distribution for each group, including the medians, the 25th and 75th percentiles, and the interquartile range, are shown in Table 2.

Figure 5 shows representative data from a dog with a splenic hematoma (Dog 1, 0.03% EPC) and a dog with splenic HSA (Dog 18, 0.97% EPC). In this example, cells that were positive for CD133 and $\alpha_v\beta_3$ -integrin were back-gated to two-dimensional light scatter dot plots, and

the percentage of positive cells within the HSA cell gate was determined. The frequency of EPC-like cells in dogs with HSA (and no surgery prior to obtaining the sample) was significantly different from the other groups; similarly, working on the assumption that EPC in the circulation are rare events that follow a Poisson distribution (assuming as high as 1 in 100 events), the results shown confirm the increased frequency of EPC in HSA compared to dogs with no HSA and dogs with prior surgery ($t = 2.22$); specifically, the probability to observe a minimum of 5 EPC-like cells as a random occurrence for every 1000 events analyzed is $\sim 0.004\%$.

Previously, we showed that expression of c-kit in splenic endothelium is restricted to HSA, with no detectable expression in normal spleen or in splenic hematomas [18]. For this study, we were able to compare the phenotype of circulating and cultured HSA cells in Dog 21. Both expressed c-kit, $\alpha_v\beta_3$ -integrin, CD133, and CD146 (Fig. 6). Finally, we also tested hemorrhagic effusions from 3 dogs with HSA (13, 20, and 21). Each of these contained greater than 0.8% EPC-like cells with similar phenotypes to those detected in peripheral blood samples (Fig. 6).

Two healthy dogs (Dogs 9 and 10), 2 with splenic hematoma (Dogs 11, 12), and 3 with HSA, including 1 with a tumor in the right atrium that was untreated (Dog 22) and 2 in the group that had surgery in the previous 48 hours (Dogs 25 and 26) were included in the analyses using a leukocyte “dump gate.” Figure 7 illustrates this process with samples from Dog 9 and Dog 22. Although statistical analyses cannot be applied to this small number of cases, the frequency of cells obtained by this method was similar to that

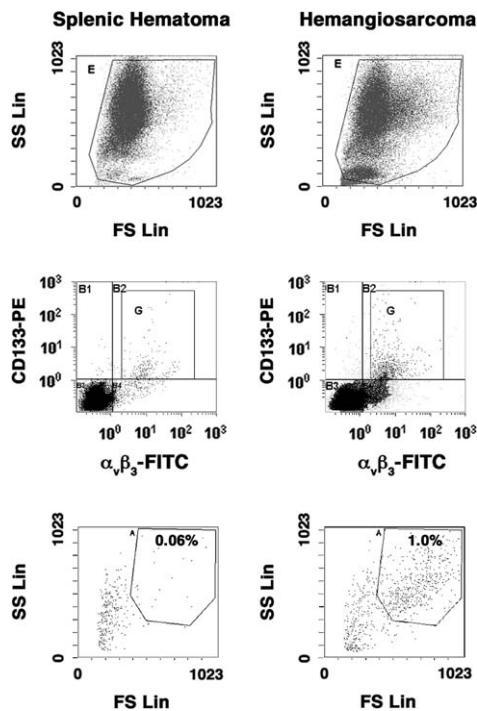


Figure 4. Coexpression of markers of hematopoietic precursors and proliferating endothelial cells in blood from dogs with or without HSA. Peripheral blood was stained and analyzed as described in Materials and Methods. The left column shows data from a dog with biopsy-confirmed splenic hematoma, and the right column shows data from a dog with biopsy-confirmed HSA. The top panels show two-dimensional light scatter dot plots (FS vs SS) for all cells analyzed. The middle panels show two-dimensional dot plots for FITC logarithmic fluorescence intensity on the x-axis and PE logarithmic fluorescence intensity on the y-axis, documenting expression of $\alpha_v\beta_3$ -integrin vs CD133. The bottom panels show the results of “back-gating” $\alpha_v\beta_3$ -integrin/CD133 double-positive cells onto the light scatter dot plots, excluding all negative (nonstaining) events.

obtained without the “dump gate” for unaffected dogs and dogs with prior surgery (<0.01%), and for the affected dog with no surgery (0.5%). The major advantage of applying this method was the simpler interpretation due to reduced background noise.

Table 2. Descriptive statistics for EPC in the circulation of dogs in the study groups^a

	Group 1 (No HSA)	Group 2 (HSA) ^b	Group 3 (HSA; prior surgery)
Median	0.07	0.64	0.14
25th percentile	0.03	0.81	0.09
75th percentile	0.21	1.01	0.18
Interquartile range	0.18	0.20	0.09

^aValues denote percent of nucleated cells detectable by flow cytometry in peripheral blood samples, and they are shown graphically in Figure 5.

^bGroup 2 is significantly different from Groups 1 and 3 ($p < 0.01$ for non-ferroni adjusted pairwise comparisons using Kruskal-Wallis one-way analysis of variance).

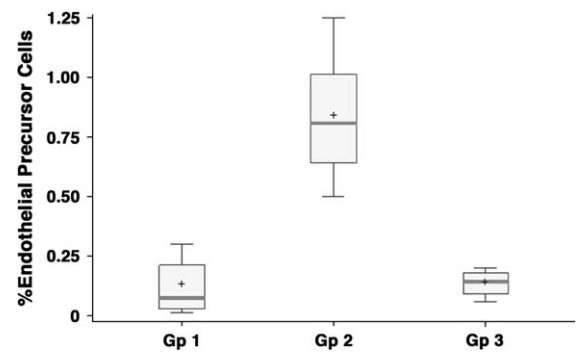


Figure 5. Dogs with HSA have significantly higher numbers of circulating EPC than unaffected dogs. Box-plots are used to provide a visual summary of the data and to identify outliers for endothelial precursors quantified as in Figure 3 for healthy dogs ($n = 12$, Group 1), dogs with HSA ($n = 10$, Group 2), and dogs with HSA that had their tumors removed within 48 hours prior to obtaining samples ($n = 4$, Group 3). The bottom and top edges of the boxes correspond to the sample 25th and 75th percentiles, and the box lengths represent one interquartile range. The center thick horizontal lines correspond to the sample medians and the “+” signs represent the means. The “whiskers” projecting out from the box extend as far as the data extend (outliers).

Discussion

Our goal was to verify the origin of HSA cells, but more importantly, to distinguish if these cells arise from mature (transformed) endothelial-lining cells or from hemangioblasts; that is, bone marrow-derived hematopoietic precursors with capacity for endothelial differentiation. Our data indicate that HSA originates from bone marrow precursors that arrest their differentiation at the hemangioblast to angioblast stage; these cells express c-kit, CD34, CD133, and in some cases CD45. Our sample population does not yet include enough dogs to determine if HSAs arising in different anatomic locations (spleen, heart, liver, skin) have consistently different phenotypes. A possible interpretation of the results is that, rather than reflecting bona fide arrest of differentiation at the hemangioblast to angioblast stage, the results reflect aberrant expression of lineage-associated proteins by the tumors. Although our results do not formally exclude this possibility, we believe it is unlikely that each tumor tested would reactivate similar expression profiles for these lineage markers in the absence of a preset genetic program (i.e., one that reflects their hematopoietic ontogeny).

It also is possible that hemangioblast-like cells in the circulation of dogs with HSA were normal bone marrow-derived EPC mobilized upon increased hematopoietic demand due to chronic bleeding, or secondary to cytokines elaborated by the tumors [18,34,35]. We can rule out the first possibility, since only 2 dogs in the study were anemic (hematocrit <35%) and none were leukopenic or thrombocytopenic. One of the anemic dogs had HSA and greater than 0.5% circulating EPC; the other had a splenic hematoma and less than 0.1% circulating EPC. This indicates anemia is not a major impetus that promotes release of

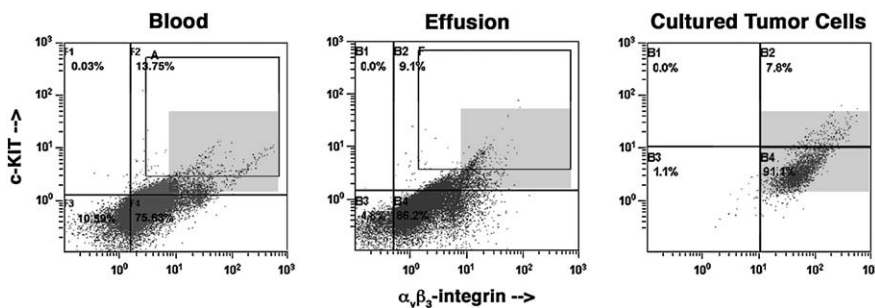


Figure 6. HSA cells in circulation and malignant effusions have similar properties to cultured HSA cells derived from the same tumor. Peripheral blood and abdominal effusion from Dog 21 were analyzed as described in Figure 4. A cell line (FROG-HSA) established from a surgical biopsy of this dog's tumor was subsequently analyzed using the same parameters. The differences in quadrant gating are due to autofluorescence, especially by the cultured cells in the FITC spectrum.

EPC into the circulation of dogs with HSA. We cannot completely exclude the possibility that VEGF and other cytokines released by the tumors mobilize EPC; however, there are at least two observations that make this unlikely. First, VEGF expression appears to be comparable in HSA and in splenic hematomas [1]. Nevertheless, HSA might release other cytokines that contribute to release of EPC from the bone marrow. Second, the EPC had distinct cell-surface phenotypes, but the patterns were not the same for each dog with HSA. That is, cells might coexpress CD34 and $\alpha_v\beta_3$ -integrin, c-kit and $\alpha_v\beta_3$ -integrin, CD133 and $\alpha_v\beta_3$ -integrin, or combinations thereof, but these patterns differed among the dogs. Although we were only able to compare the phenotype of circulating EPC to that of the primary tumor in one case (we did not have fresh tumor specimens from the other dogs tested), these showed predictable similarities. In contrast, we believe that normal bone marrow EPC mobilized by tumor cytokines should share similar phenotypes among all dogs, rather than the different phenotypes we observed in these cases. Finally, CD133⁺ cells represent the earliest identifiable multipotent cells in the hemangioblastic lineage, which is more consistent with release of tumor “stem cells” than with mobilization of bone marrow EPC. Experiments are in progress to enrich these populations by flow-sorting to assess their lineage differentiation potential in culture and to determine if they harbor typical mutations of the PTEN C-terminal domains or other gene expression profiles that may be peculiar to canine HSA [1].

Ultimately, whether the cells originate from bone marrow-derived stem cells that reside in the vascular endothelial lining or from bone marrow-derived stem cells that home to blood vessels after transformation still remains to be determined. Whichever the case, the nature of the tumor and the properties of the cells might offer an environment that favors entry of cells into the circulation. While this would help to explain the metastatic behavior of HSA, it also might provide the ability to develop a diagnostically useful assay that could be similarly adapted to detect

circulating malignant hemangioblasts in human patients at risk for developing angiosarcoma [18].

EPC in the circulation can increase under various physiologic or pathologic conditions in humans and mice. Physiologic conditions are mainly related to intense physical training. Pathologic conditions include stem cell mobilization after cytokine administration and vascular injury (including myocardial infarcts and early congestive heart failure, oxidative stress, and pulmonary hypertension) [9].

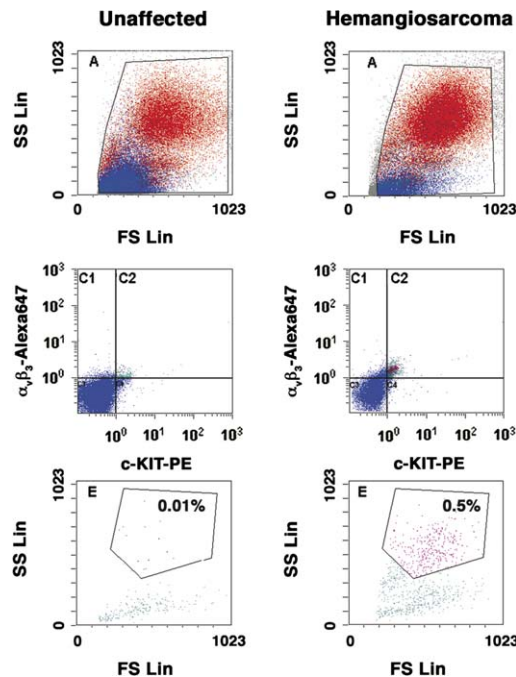


Figure 7. Coexpression of markers of hematopoietic precursors and proliferating endothelial cells in blood from dogs with or without HSA. Peripheral blood was analyzed as described in Figure 4, except that cells labeled with CD5, CD21, and CD11b-FITC (red population) were excluded from the analysis, and the middle panels show two-dimensional dot plots for PE logarithmic fluorescence intensity on the x-axis and Alexa-647 logarithmic fluorescence intensity on the y-axis, documenting expression of c-kit vs $\alpha_v\beta_3$ -integrin.

Nevertheless, even under pathologic conditions EPC in the circulation generally remain well below 0.5%. Unlike humans, risk factors that might elevate the number of circulating EPC would be rare in dogs, with intense conditioning or early congestive heart failure as the only common risk factors that might lead to increased numbers of EPC. Conversely, we believe that HSA lesions would shed EPC-like cells into the circulation continuously, meaning that a diagnostic procedure to detect EPC-like cells in excess of a normal reference interval would be a sensitive means to diagnose canine HSA (and possibly human angiosarcoma), even in early stages.

Our results suggest the assay might also be useful to 1) identify malignant cells in effusions, which may be predictive for the development of metastatic disease, 2) monitor remission and minimal residual disease (cells were undetectable in the 4 dogs that had their tumors removed within 24 hours), and 3) allow detection of the disease in early stages in at-risk patients in the prevention setting. The variable expression of CD45 and CD14 suggests that HSA cells might attain different stages of differentiation that affect response to therapy, although CD14 expression can be confounded by the overlapping morphology and light scatter of monocyte-derived cells, EPC-like cells, and possibly mesenchymal stem cells. Given the small number of dogs with HSA that achieve objective responses and the suitability of this disease to model responses of human patients with angiosarcoma [18], prospective trials to explore these questions are warranted.

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