

Canine malignant hemangiosarcoma as a model of primitive angiogenic endothelium

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Hemangiosarcoma (HSA) is a common untreatable cancer of dogs that resembles human angiosarcoma. Detailed studies of these diseases have been historically hindered by the paucity of suitable reagents. Here, we show that expression of CD117 (c-Kit) can distinguish primitive (malignant) from mature (benign) proliferative endothelial lesions, and we describe eight independent cell lines derived from canine HSA explants. Endothelial origin was confirmed by sustained expression of surface CD105 (endoglin), CD146 (MUC18), and CD51/CD61 ($\alpha_v\beta_3$ integrin). The cell lines showed anchorage-independent growth and were motile and invasive when cultured on a basement membrane matrix. They required endothelial growth factors for growth and survival, and they could be induced to form tubular structures resembling blood vessels when cultured under low calcium conditions. The formation of vessel-like structures was blocked by nicotine, and restored by FK506, suggesting that 'nuclear factor of activated T cells' activity prevents differentiation of these cells. In summary, these cell lines represent a unique and novel resource to improve our understanding of endothelial cell biology in general and canine HSA in particular.

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Canine hemangiosarcoma (HSA) arises from transformed vascular endothelial cells, most commonly in the spleen, right atrium, or subcutis. The ontogeny of HSA cells can be confirmed by the expression of selected endothelial markers such as CD31 and Factor VIII-related antigen (vWF).^{1,2} Local infiltration and systemic metastases are the common growth patterns and metastatic sites are widespread, with lung and liver being the most frequently affected organs.^{3,4} Morbidity and mortality is often due to acute internal hemorrhage secondary to tumor rupture. Despite surgery and intensive chemotherapy, the median survival time for dogs diagnosed with HSA is little more than 6

months.^{5–8} This naturally occurring disease of dogs resembles human angiosarcoma. Angiosarcomas are uncommon soft tissue sarcomas that can arise in a variety of locations, including liver, spleen, skin, breast, and endocrine organs.^{9–11} Among these tumors, specific subtypes are associated with a variety of risk factors, such as occupational exposure to vinyl chloride for hepatic angiosarcoma,^{12,13} radiation therapy for mammary angiosarcoma,^{14–19} HIV-1 infection in the case of Kaposi sarcoma,²⁰ and heritable defects in the VHL tumor suppressor gene in human infantile angiosarcomas and hemangiomas.²¹ Exposure to ionizing radiation seems to be a shared risk factor for development of HSA in dogs.²² As is true for canine HSA, treatment of human angiosarcomas can be challenging and is frequently unrewarding.^{9,11,23}

In part, the paucity of effective treatments for endothelial cancers is due to the lack of appropriate reagents to study these diseases.^{24–29} Thus, our objective was to establish and characterize a cohort

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of canine HSA cell lines that could be used to explore the molecular and cellular features of this disease and that could serve as a resource to explore comparative aspects of canine and human endothelial tumors for therapeutic development.

Materials and methods

Establishment of Cell Lines

Viable samples were obtained from 11 family-owned pet dogs with primary (spleen, right atrium) or metastatic (liver, mesentery) lesions with gross features of visceral HSA (Table 1). Participation in the study required the dogs' owners to sign an informed consent form indicating they understood the goals and procedures for this study. Protocols and procedures were reviewed by the Institutional Review Board and Institutional Animal Care and Use Committee of AMC Cancer Research Center. Tumor explants were collected from surgical biopsy samples under sterile conditions into Dulbeccos's modified Eagle medium (Life Technologies, Bethesda, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml). Tissue was rinsed in sterile phosphate-buffered saline solution (PBS) to remove residual blood and divided into three portions that were (1) fixed in 10% neutral buffered formalin, (2) snap frozen, and (3) minced and digested enzymatically in 0.25% trypsin and 1 mM EDTA to establish cell cultures. Debris was removed from the samples using 120–200 µm mesh filters and cells were divided equally into six-well plates at a density of $0.3\text{--}2.5 \times 10^6/\text{ml}$ in 3 ml of F12K media (ATCC, Manassas, VA, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA), endothelial growth

supplements (BD Biosciences, San Jose, CA, USA), and 100 000 IU/ml of high molecular weight heparin (Sigma Chemical Co., St Louis, MO, USA). Cells were cultured overnight and residual blood cells were removed by gentle aspiration. In most cases, the adherent cells expanded into heterogeneous populations that consisted of large, polygonal or 'flag'-shaped cells, rounded cells, and elongated spindle cells that resembled fibroblasts or stromal cells. The cells were then subcultured into six-well plates at a density of $0.5 \times 10^6/\text{ml}$. In general, sublines expanded into homogeneous populations, and only those consisting of large, polygonal, or rounded cells survived beyond three passages. Cells were fed three times weekly and passaged when they reached ~80% confluence. Samples also were included from one dog with cutaneous HSA where the tumor was implanted subcutaneously into an immunodeficient mouse to select cells that could form tumors *in vivo*.³⁰ The cells were maintained in culture using the same conditions described above.

Chemicals and Reagents

Tissue culture materials were obtained from VWR Scientific (South Plainfield, NJ, USA) and chemicals from Sigma, unless otherwise specified. Human umbilical vein endothelial cells (HUVEC) were obtained from the ATCC and cultured in supplemented FK12 media following the ATCC recommendations. Anti-CD31 (PECAM-1) antibody (clone JC/70A) was from Dako Cytomation, (Carpinteria, CA, USA), anti-CD117 (c-Kit) antibody was from Cell Marque (Hot Springs, AR, USA), anti-CD51/CD61 ($\alpha_v\beta_3$ integrin) antibody (mAb 1976) and anti-CD146 (MUC18, S-endo) antibody (mAb 16985) were from Chemicon (Temecula, CA, USA), anti-CD105 (endoglin) antibody (clone 8E11) was from

Table 1 Origin and phenotype of samples and cell lines

Dog ID	Gender/breed	Histologic diagnosis (site of cell line origin)	CD31 expression	CD117 expression	Cell line
Dog 1	Male Dalmatian	Splenic HSA (spleen)	+	+	Dal-4
Dog 2	Male Golden Retriever/Great Pyrenees mix	Splenic HSA (spleen)	+	+	DD-1
Dog 3	Male Golden Retriever	Splenic HSA with extensive necrosis (N/A)	ND	ND	None established
Dog 4	Male Golden Retriever	Splenic HSA (spleen)	+	+	CHAD-G4.1
Dog 5	Female Golden Retriever	Splenic HSA with mesenteric metastasis (spleen)	+	+	CHAD-G6.4
Dog 6	Male Border Collie	Atrial HSA (right atrium)	+	+	CHAD-B7.4
Dog 7	Male Golden Retriever	Splenic HSA with vascular emboli (spleen)	+	+	CHAD-G8.2
Dog 8	Male Portuguese Water Dog	Splenic HSA with liver metastases (liver)	+	+	CHAD-P9.5
Dog 9	Male German Shepherd Dog	Cutaneous HSA (subcutis)	+	ND	SB-HSA
Dog 10	Male Standard Poodle	Splenic hematoma (N/A)	—	—	None established
Dog 11	Female Labrador Retriever	Splenic hematoma (N/A)	+	—	None established
Dog 12	Female Golden Retriever	Splenic hematoma (N/A)	+	—	None established

N/A, not applicable; ND, not done.

Southern Biotechnology Associates (Birmingham, AL, USA), and the control anti-TNP antibody (IgG_{2a}- κ) was from BD Biosciences. Sterile EDTA was used in culture at a concentration of 0.1 mM, nicotine tartrate salt was dissolved in HEPES-buffered saline (HBS) and used in culture at a concentration of 10 μ M, and FK506 (Calbiochem, San Diego, CA, USA) was dissolved in 100% ethanol and used in culture at a concentration of 100 ng/ml. All compounds were diluted at least 1000-fold in cell cultures. The addition of sterile water, HBS, or ethanol at 0.1% did not affect any of the parameters measured.

Pathology and Immunohistochemistry

Sections from each explant were fixed in 10% neutral buffered formalin for 24 h. Diagnoses were made from routine H&E stained slides using standard histopathologic criteria. HSAs were characterized by poorly demarcated and nonencapsulated proliferation of atypical ovoid to spindylloid cells. The cells proliferated as solid sheets, but often broke apart to form rudimentary and tortuous vascular channels. The cells were also markedly invasive into adjacent parenchyma. Individual cells were characterized by scant to moderate eosinophilic cytoplasm, and moderately pleomorphic, euchromatic nuclei with medium-sized nucleoli. There usually were one to two mitotic figures per high power field, and there was significant organizing hemorrhage in the adjacent, noninvolved parenchyma. Splenic hematoma specimens were characterized by poorly demarcated and nonencapsulated hemorrhage throughout the splenic parenchyma. There was early peripheral proliferation of well-differentiated fibroblasts (fibroblastic organization), accompanied by significant hemosiderin deposition. Within the lesion, there was variable proliferation of stromal fibroblasts, fibrohistiocytic cells, and vascular endothelial cells. The adjacent noninvolved splenic parenchyma was usually normal. It is reported that splenic hematomas often form adjacent to regions of nodular lymphoid hyperplasia, which may be a predisposing factor for this lesion.³¹ Expression of CD31 was examined as described;² for the detection of CD117, sections were subjected to microwave antigen retrieval in a buffer of 1 mM EDTA, pH 8.0, and the antibody was used at a 1:50 dilution. The anti-CD117 antibody was validated in one of our laboratories (JW) using sections from CD117-positive mast cell tumors (generously provided by Dr Emily Walder, Venice, CA, USA).

Flow Cytometry

Adherent cells were washed with PBS to remove residual serum, incubated in 1 mM EDTA for 5 min, and detached by gentle scraping with a sterile

rubber policeman. Staining buffer consisted of PBS with 0.5% bovine serum and 0.1% sodium azide. One million cells were incubated with anti-CD51/CD61 antibody (2 μ g/ml), anti-CD105 antibody (1:5000 dilution), or anti-CD146 antibody (2 μ g/ml) for 30 min at 4°C. An anti-TNP antibody was used as a control for nonspecific binding. Cells were then washed four times in staining buffer and incubated with a goat-anti-mouse IgG/IgM antibody conjugated to fluorescein isothiocyanate (FITC, Pharmingen, San Diego, CA, USA) for 20 min. Cells were again washed four times in staining buffer and then fixed in 2.5% neutral buffered formalin. Samples were kept at 4°C protected from light until analysis using a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Hialeah, FL, USA) running the Expo 32 software package. For ploidy analysis, cells were fixed in 80% ethanol and stained using propidium iodide as described.³² Analysis was carried out using the ModFit LT Software Package (Verity Software, Topsham, MA, USA).

Immunofluorescence

Expression of CD51/CD61, CD105, and CD146 was examined in live cells grown in 24-well plates (BD Discovery Systems). Adherent cells were washed three times with staining buffer and incubated with 2 μ g of anti-CD51/CD61 antibody, a 1:100 dilution of anti-CD105 antibody, or 2 μ g of anti-CD146 antibody in 200 μ l of staining buffer at 4°C for 30 min. Cells were washed again three times with staining buffer and incubated with goat anti-mouse IgG/IgM antibody conjugated to FITC (1:2500 dilution) in 200 μ l of staining buffer for 30 min. Cells were washed three times with staining buffer. After the last wash, 500 μ l of staining buffer was added to the wells, and cells were examined using an Olympus IX71 inverted microscope with an Olympus Microfire S79809 cooled digital camera (Scientific Instrument Company, Aurora, CO, USA). Transmitted light images under phase contrast were captured using a gain of 1.0 and exposures ranging from 12 to 60 ms. Fluorescent images were captured using a gain setting of 4.0 and exposures ranging from 2.6 to 4 s. Brightness and contrast for each image were optimized using Photoshop 7.0 (Adobe, San Jose, CA, USA).

Anchorage-Independent Growth and Motility in Basement Membrane Matrix Media

Semi-solid medium was prepared by mixing supplemented F12 K media with 0.6% Agar Noble (1:2, v:v) to make a bottom agar (1 ml/well in 24-well plates). After 30 min at room temperature, 7.5×10^4 HSA cells were gently overlaid on the bottom agar, along with 1 ml of top agar, consisting of 50% F12 K and 50% 0.6 Agar Noble. Freshly thawed Matrigel Brand Basement Membrane Matrix (Collaborative

Biomedical Products, Bedford, MA, USA) was mixed with supplemented F12K media (1:1, v:v) while maintaining the temperature below 8°C. The medium was gently layered into 24-well plates (1ml/well) and allowed to solidify at 37°C for >2h. HSA cells (7.5×10^4 in 1ml of supplemented F12K media) were gently overlaid on the solid Matrigel and allowed to remain undisturbed in culture at 37°C for at least 24h before the plates were removed for microscopic examination. Cell growth, colony formation, and invasion were determined by daily examination under an Olympus IX71 inverted microscope. Transmitted light images under phase contrast were captured using a gain of 1.0 and exposures ranging from 12–60 ms.

Proliferation

Cell proliferation was measured using the CyQuant Cell Proliferation Assay Kit (Invitrogen/Molecular Probes, Eugene, OR, USA). Standard curves were developed using Dal-4 cells grown for 24h. The dynamic range of this assay covered four orders of magnitude (linear from 200 cells/well to 50 000 cells/well). Replicate samples of 3000 cells were cultured in 200 μ l (15 000 cells/ml) and frozen at 24 h intervals. CyQuant GR fluorescence was measured following the manufacturer's instructions.

Vascular Endothelial Growth Factor Expression

Expression of vascular endothelial growth factor-A (VEGF) mRNA was examined using reverse transcriptase-polymerase chain reaction (RT-PCR) as described.³⁰ Cells were grown in EBM-2 media (Clontech, Palo Alto, CA, USA) containing 10% FBS, but without endothelial growth supplements. After 6 h of culture, RNA was isolated using RNA Wiz (Ambion, Austin, TX, USA). Quantitative assessment of steady state levels of VEGF message was performed by Northern blotting, where 10 μ g of RNA was separated electrophoretically on 1% agarose/5% formaldehyde denaturing gels, and the canine-specific VEGF amplification product generated for the RT-PCR reaction was used as a probe. Cell-free culture supernatants were harvested by centrifugation followed by removal of cellular debris using 0.2 μ m filters. VEGF was measured in the cell-free supernatants using the human VEGF Quantikine kit (R&D Systems, Minneapolis, MN, USA) as described.³³

Results

Isolation of Morphologically Homogeneous Cell Lines from HSA Explants

Surgical biopsies were collected from 10 dogs with splenic masses, one dog with a right atrial mass, and one dog with a subcutaneous mass, which were grossly consistent with HSA. Diagnosis was con-

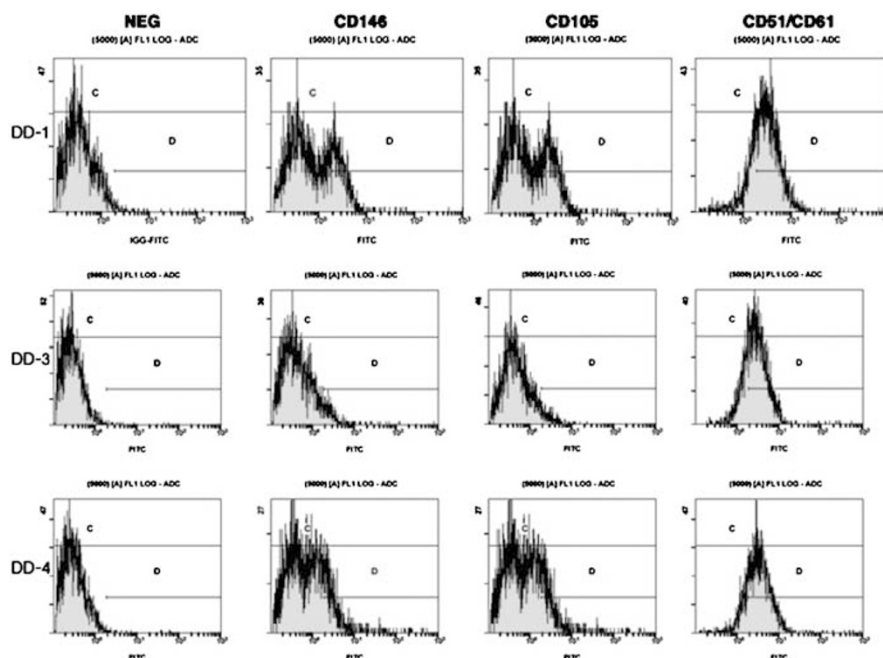


Figure 1 Expression of endothelial antigens by canine HSA sublines. The explant from Dog 2 was minced and filtered through a 200 μ m mesh to remove debris and obtain a single-cell suspension. This single-cell suspension was divided into six wells in a six-well plate and grown to near confluence (80–90%) in FK12 media with endothelial growth supplement. Cells from each well were then subcultured into individual wells on new six-well plates and grown to near confluence. Cells from selected populations among these (DD-1, DD-3, DD-4) were then stained using antibodies directed to CD146, CD105, and CD51/CD61 and examined flow cytometrically to identify lines representing endothelial cells.

firmed using standard histologic criteria and expression of CD31 and CD117 (Table 1). Two dogs (Dog 5 and Dog 8) had gross metastases to the mesentery and the liver, respectively, and one dog (Dog 7) had evidence of tumor emboli within splenic blood vessels. One sample (Dog 3) had extensive necrosis and was excluded from further analyses. CD31 expression was detectable in endothelial cells from each HSA sample and two of three benign hematomas (poorly demarcated areas of hemorrhage with proliferation of nonmalignant stromal fibroblasts, fibrohistiocytic cells, and vascular endothelial cells). In contrast, CD117 expression in endothelial cells was detectable exclusively in HSA samples and not in benign hematomas. Expression of CD117 was also detectable in endothelial cells from three additional archival HSA samples that were not included in this study because fresh tissue was not available. Viable cell lines capable of growing >20 passages were established from eight of nine HSA explants. On the other hand, cells derived from benign hematomas grew slowly in culture, if at all, and did not survive beyond three passages. Initially, cells isolated from the explants were moderately pleomorphic, likely because the cultures included both endothelial and stromal cells. To obtain homogeneous samples, cells were subcultured once and each derivative subline was analyzed for expression of endothelial cell antigens CD146, CD105, and CD51/CD61 (Figure 1). Sublines that showed consistent expression of at least two of these antigens were selected for further study. The cells in each subderivative line were morphologically homogeneous; however, there were notable differences among the different lines. Representative phase contrast photomicrographs from each line are shown in Figure 2. Dal-4 and CHAD-B7.4 cells showed spindled to polygonal morphology; DD-1, CHAD-G4.1, and CHAD-G6.4 cells showed round to square to polygonal morphology; CHAD-P9.5 cells showed spindled morphology; and SB-HSA cells were small with prominent, thin cytoplasmic dendrites. Every cell line had euchromatic nuclei with prominent nucleoli. In addition, each cell line except SB-HSA showed domed granular perinuclear zones with extensive flattening in the distal cytoplasmic regions. The cells also showed clear cytoplasmic zones that were highly refractile. Similar vacuolar structures were present extracellularly, suggesting they might represent secretory vesicles. Apoptotic cells (detached cells or those showing condensed nuclear chromatin) were seen infrequently (<1%) in logarithmically growing cultures, but were more common (>20%) in cells that were allowed to reach confluence. The cells also showed fastidious growth properties. Cultures showed reduced growth or complete growth arrest when cultured without endothelial growth supplements despite expression of endogenous VEGF (see below). Although some of the lines could survive up to two passages in the presence of

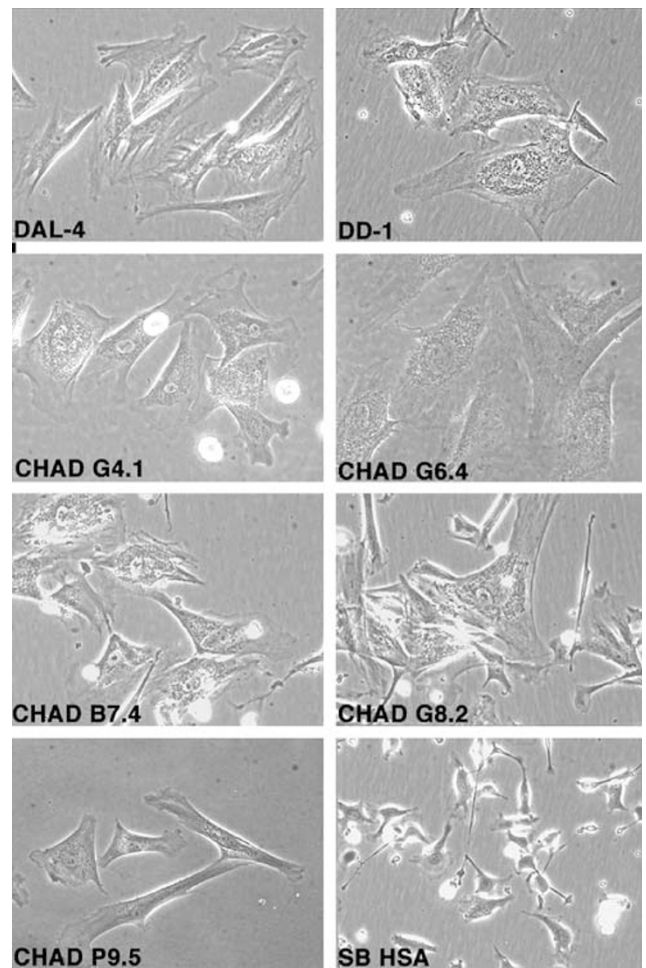


Figure 2 Morphologic appearance of canine HSA cell lines. Cell lines were maintained in culture for at least five passages and photographed under phase contrast ($\times 320$ magnification) under conditions of logarithmic growth (day 2 after passage). Images were acquired using an Olympus Microfire digital camera and assembled using Photoshop 7.0.

serum without endothelial growth supplements, they became pleomorphic and eventually died. Although the cells grew in various endothelial media formulations, they thrived in F12K media supplemented with endothelial growth factors.

Finally, to verify the stability of the cell lines, expression of CD51/61, CD105, and CD146 was reexamined in cells maintained in culture for five to eight additional passages. Figure 3 shows that expression of each antigen was retained by each cell line, although not every cell in each line expressed every antigen. The morphological features of the cells also remained consistent for at least 20 passages. Expression of CD31 and vWF was also examined in DD-1, Dal-4, and SB-HSA cells using immunocytochemistry. Each of the three cell lines expressed CD31, although the intensity of expression was variable. Expression of vWF was detectable in SB-HSA cells, at low levels in <10% of DD-1 cells, and not in Dal-4 cells. These data are

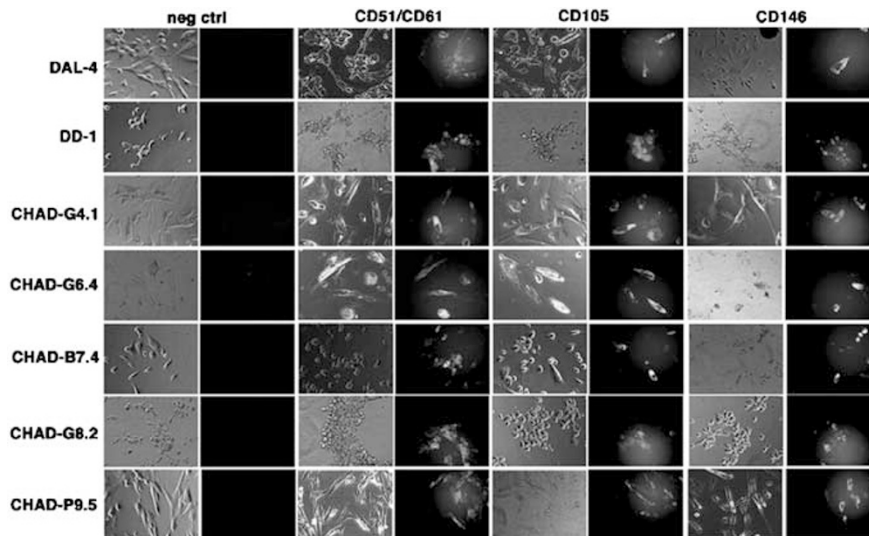


Figure 3 Expression of CD51/CD61, CD105, and CD146 in canine HSA cell lines after 5–8 passages. Cells were maintained in culture for at least five passages in the presence of endothelial growth supplements, after which they were grown in 24-well plates and stained for the endothelial antigens in the wells. Paired photomicrographs of the same field show the cells under phase contrast illumination (left) and the expression of each antigen under fluorescent illumination (right) at $\times 200$ magnification. Images were acquired using an Olympus Microfire digital camera and assembled using Photoshop 7.0.

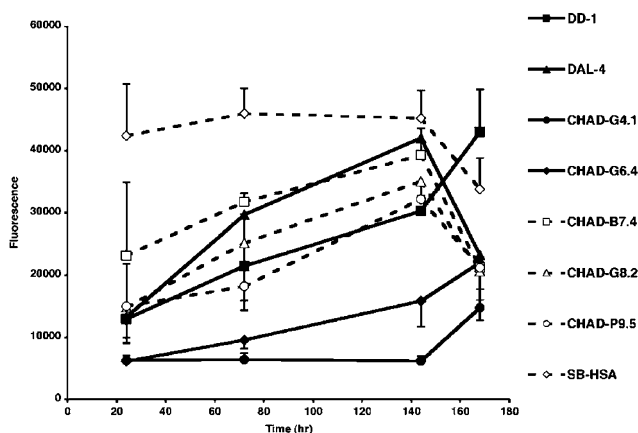


Figure 4 Proliferation of HSA cell lines. Replicate wells for each cell line containing 3000 cells in $200\mu\text{l}$ were cultured in 96-well plates, and plates were frozen at 24 h intervals. Proliferation was measured using the CyQuant assay. Data represent the CyQuant GR emission (measured at 495 nm) as a function of time (in h). The relative rate of proliferation for each cell line corresponded closely to the time required for cells to achieve confluence (3–7 days when seeded at 5×10^5 cells in 5 ml in 25 cm^2 tissue culture flasks).

consistent with previously published results that suggest vWF expression may occur in later stages of differentiation and may be restricted to specific subsets of endothelial cells,^{34–36} and with our observation that vWF expression in canine HSA appears to localize preferentially to formed vessels rather than to solid areas of malignant cells (JW, unpublished).

Growth Properties and Malignant Potential of HSA Cell Lines

The different cell lines showed variable growth properties as determined by proliferation, anchorage-independent growth, and motility on basement membrane matrix. CHAD-G4.1 cells showed the least rapid proliferation, with a slow steady rate of DNA synthesis that increased as the density of the cells in culture approached 60% (Figure 4). CHAD-G6.4, CHAD-P9.5, DD-1, CHAD-G8.2, DAL-4, and CHAD-B7.4 showed progressively greater levels of DNA synthesis, with doubling times ranging from 2 to 4 days. SB-HSA cells grew rapidly with a doubling time of ~ 20 – 24 h and maintained a consistently high steady state of DNA synthesis (Figure 4). DNA synthesis in all the HSA lines appeared to occur in defined, synchronous cycles, as it occasionally lagged from one day to the next (not shown). The reduced levels of DNA synthesis observed for each cell line except CHAD-G4.1 and CHAD G6.4 reflect exhaustion of growth factors from the media.

Analysis of DNA content showed that CHAD-G4.1, CHAD-P9.5, and SB-HSA cell lines each contained aneuploid populations, including tetraploid and hypotetraploid cells that proliferated along with the diploid populations (Figure 5). The remaining cell lines had diploid DNA content. Cells recovered from a hematoma explant (Dog 10) were 100% diploid and largely in G0/G1 ($>90\%$), whereas the cell cycle distribution of HSA cell lines after 3 days in culture reflected active proliferation (70–80% of cells in G1 and 20–30% of cells in S/G2/M). Despite the fact that five lines had diploid

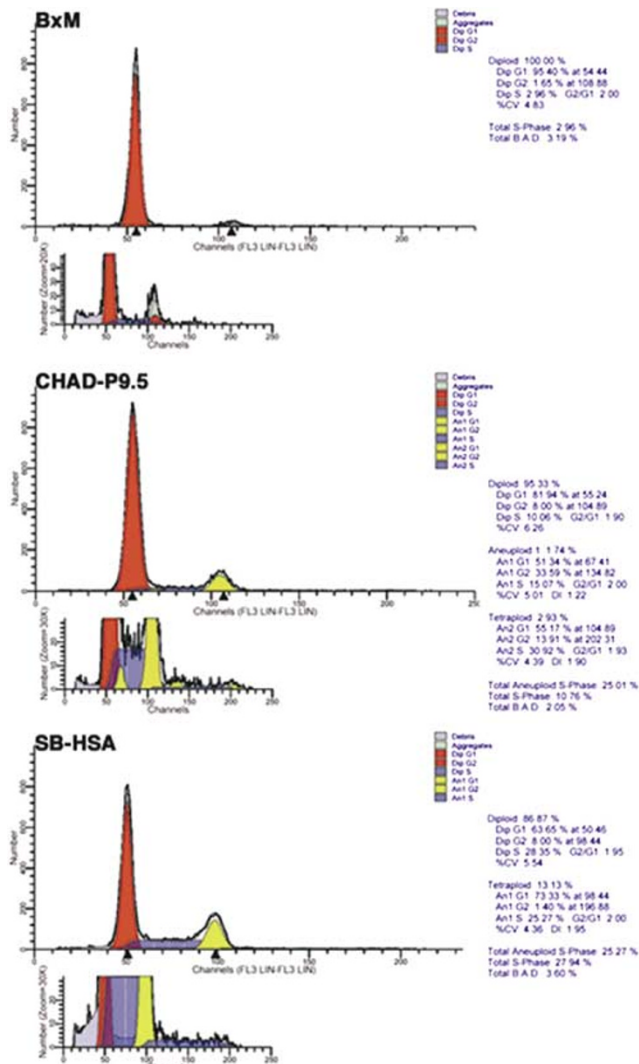


Figure 5 DNA content of representative HSA cell lines. Cells in the logarithmic growth phase were fixed in 80% EtOH, treated with RNase A (25 μ g/ml), stained with propidium iodide (25 μ g/ml), and analyzed flow cytometrically. Ploidy and cell cycle distribution were determined using best-fit analysis on the ModFit software. DNA histograms are shown for hematoma cells isolated from Dog 12 (BxM), CHAD-P9.5, and SB-HSA; a magnified view is shown below the main histogram for each line to show the S phase and aneuploid populations. In all 100% of the cells in the DD-1, DAL-4, CHAD-G6.4, CHAD-B7.4, and CHAD-G8.2 lines had diploid DNA content, but the G1 peaks accounted for 70–80% of the cells. Approximately 7.5% of cells in the CHAD-G4.1 line were tetraploid, excluding the diploid cells in G2/M.

DNA content, analysis of metaphase chromosomes from early passages (<3) showed that the HSA cells generally had complex and unstable karyotypes that included complete chromosome fusions, segmental deletions, and translocations (data not shown).

Anchorage-independent growth, motility, and invasion are common features of malignant cells. To examine anchorage-independent growth of HSA cells, 6×10^4 cells were cultured in 2 ml of soft agar

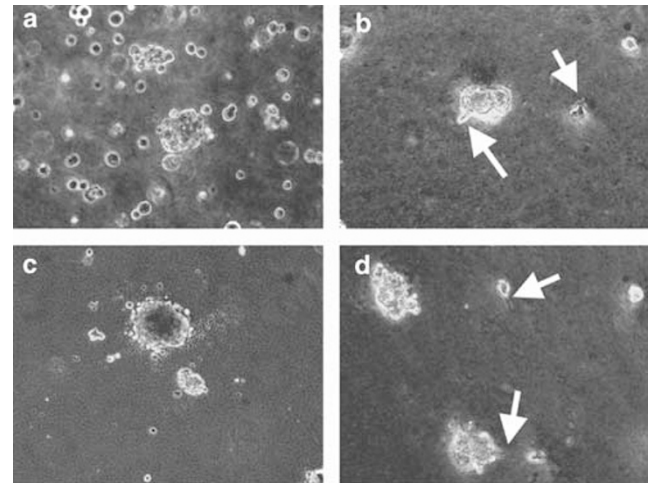


Figure 6 Growth of canine HSA cell lines in soft agar and Matrigel. HSA cells (7.5×10^4) were cultured in soft agar or solidified Matrigel containing endothelial growth supplements as described in Materials and methods. Cell growth was monitored daily for 5 days. Panels a and b show the appearance of DAL-4 and CHAD-G8.2 cells 48 and 120 h after plating on soft agar, respectively. Panels c and d show the microscopic appearance of CHAD-G4.1 and CHAD-B7.2 cells, respectively, after 5 days of growth on Matrigel. Note the dendrite extension in every dimension seen in the trailing cells in each colony, as well as in individualized cells (arrows).

media containing endothelial growth supplements in 24-well plates. Motility and invasion on basement membrane matrix were analyzed under similar conditions using a commercial Matrigel™ formulation. Cells plated in soft agar remained individualized for the first 24 h. Within 48 h, small colonies were visible (Figure 6a) and these continued to expand for the 3 subsequent days (Figure 6b). Each cell line was capable of anchorage-independent growth, but few viable colonies were visible for CHAD-G7.4, CHAD-P9.5, and SB-HSA by day 5 of culture in soft agar. Cells plated in Matrigel showed similar behavior, except that the trailing cells in each colony as well as individualized cells showed dendrite extension in every dimension, consistent with a 'crawling' or motile phenotype (Figure 6c, d, arrows). Various factors control the angiogenic switch and vessel formation by endothelial cells. The transcription factors in the nuclear factor of activated T Cells (NFAT) family control various aspects of angiogenesis and metastasis, including VEGF production and invasion.^{37,38} To examine the role of NFAT factors in HSA cell motility, cell lines were cultured in 24-well plates in the absence of endothelial growth supplements and under conditions of low calcium (0.1 mM EDTA) with the calcineurin/NFAT antagonist FK506 (100 ng/ml). Untreated cells remained individualized in a monolayer (see Figures 2 and 3). In contrast, eight of 10 cell lines treated with EDTA and FK506 showed active migration after 4–6 h that led to the formation of elongated tubular structures with end buds that resembled blood vessels (Figure 7). Intriguingly, Dal-

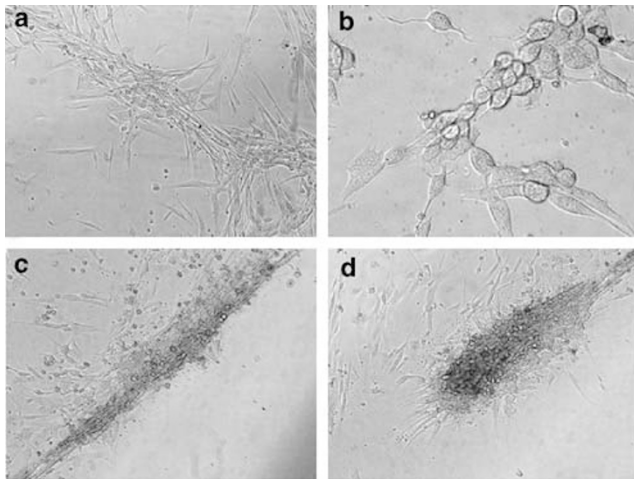


Figure 7 Aggregation of canine HSA cell lines into tubular structures. HSA cell lines were cultured under conditions of low calcium (0.1 mM EDTA) with FK506 (100 ng/ml) without endothelial growth supplements. Cells were examined microscopically after 4–6 h under phase contrast illumination. Panel a shows an example of DD-1 cells ($\times 100$ magnification) and panel b shows an example of DAL-4 cells ($\times 320$ magnification) to illustrate the active migration and aggregation of cells to form elongated structures. Panels c and d are photomicrographs of CHAD-G8.2 cells ($\times 100$ magnification) illustrating a vessel-like structure and an end bud, respectively.

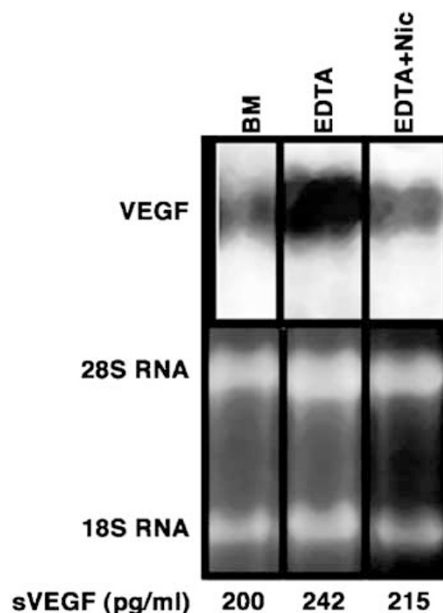


Figure 8 Increased VEGF expression in endothelial cells that form tubular structures. DD-1 cells were cultured in basal media (BM, without endothelial growth supplements), basal media under conditions of low calcium (0.1 mM EDTA), or basal media with EDTA and nicotine (10 μ M), an activator of NFAT transcription factors, for 6 h. Cell-free supernatants were collected by centrifugation and cellular debris was removed by filtration. The levels of VEGF in the culture supernatants (sVEGF) were determined using a commercial ELISA following the manufacturer's instructions. VEGF gene expression was examined by Northern blotting. Ribosomal RNA bands (28S and 18S) stained with ethidium bromide are shown as loading controls.

4 and DD-1 cells showed this aggregation phenomenon when cultured in EDTA alone, and this was associated with increased VEGF gene expression and VEGF in culture supernatants (Figure 8). Aggregation in these cells was inhibited if they were cultured with nicotine, which is an activator of NFAT (Frazer-Abel *et al*, manuscript submitted). This was associated with the concomitant reduction of VEGF message and protein back to basal levels, suggesting that modulation of endogenous VEGF (and probably other proangiogenic factors) could offer a strategy to induce terminal differentiation of these tumors.

Discussion

We describe the characteristics of eight independent endothelial cell lines derived from explants of canine HSA. Each cell line was derived from endothelial tumors (based on expression of CD31). In addition, our results suggest that expression of CD117 (c-Kit) may offer a sensitive way to distinguish benign canine hematoma (negative) from malignant canine HSA (positive), at least in the spleen. CD117 is the receptor for stem cell factor and is expressed by hematopoietic stem cells and by most primitive hematopoietic-derived cells.³⁹ A previous study showed that CD117 expression was detectable in human fetal capillary vessels, but not adult endothelial cells.⁴⁰ The same study showed high levels of CD117 expression in 56% (28/50) of human angiosarcomas, but the protein was undetectable in 10 epithelioid hemangioendotheliomas and 31 hemangiomas. This is consistent with our findings, and indicates that malignant canine HSA and human angiosarcoma originate from primitive, poorly differentiated endothelial cells that can be identified by CD117 expression, whereas human hemangiomas and the proliferative vascular cells seen in canine hematomas originate from mature, differentiated endothelial cells. This observation may offer not only a significant advancement to improve the diagnosis of canine HSA and human angiosarcoma, but also an appealing target for therapeutic development.

Our results show that HSA cell lines retain expression of endothelial-associated antigens including CD31, CD146, CD105, and CD51/CD61, and as such resemble HUVEC and bovine aortic endothelial cells (BAEC) that are commonly used for studies of angiogenesis.^{41–45} However, expression of these antigens was limited to subsets of cells within each HSA line, and some of the lines did not express vWF. This could reflect the incomplete differentiation of the cells or absence of environmental signals that upregulate vWF expression. Alternatively, it could also be due to differential regulation of expression during the cell cycle, and it could also reflect inherent instability within the cell lines. Cytogenetic analysis of these cells showed complex,

unstable karyotypes, and at least three cell lines showed evidence of aneuploidy. This is not unexpected, since genetic instability is a hallmark of most tumors,⁴⁶ and the populations in the tumor explants are likely to reflect extensive clonal evolution. Moreover, while this may raise a cautionary note regarding the use of these lines for preclinical development and for studies to elucidate the pathogenesis of canine HSA, HUVEC are hypodiploid (ATCC, technical details for CRL-1730, HUVEC-C), and in our experiments, expression of endothelial antigens in HUVEC was similarly variable and restricted to subsets within the population (not shown). The suitability of canine HSA cells as models to study tumor neoangiogenesis is further underscored by the similarities between the endothelial structures in these tumors and pericyte-poor, thin-walled 'mother vessels' that are formed during the early response of endothelial cells to elevated concentrations of VEGF, as well as during the process of tumor neoangiogenesis.⁴⁷ These mother vessels are leaky and can evolve into tortuous, poorly organized vessels that are predisposed to thrombosis, similar to what is seen in primary canine HSA.

The canine HSA cell lines we describe retain some features of normal growth, such as the requirement for endothelial growth factors. They also show contact inhibition and can be induced to form elongated tubular structures that resemble blood vessels, suggesting they are capable of differentiation. However, they also show characteristic features of malignant behavior, including anchorage-independent growth and three-dimensional motility on Matrigel. The capability to form vessels upon inactivation of calcium-dependent signaling pathways, including inhibition of NFAT, is especially intriguing. The activity of NFAT transcription factors is exquisitely regulated by alterations in intracellular calcium levels. These factors are expressed ubiquitously; NFATc1 and NFATc4 are highly expressed in the heart and endothelial cells.^{48,49} NFATc2 is the major form present in activated lymphocytes, and NFATc3 is the major form expressed in thymocytes, although both these proteins are also expressed outside of the immune system. The importance of NFAT proteins in endothelial cell growth and function was demonstrated by results from de la Pompa⁵⁰ and Graef³⁷ showing that NFATc1 deficiency resulted in embryonic lethality due to defects in cardiac morphogenesis, and mice deficient for calcineurin-B (which is required for NFAT activation) or doubly deficient for NFATc4 and NFATc3 were not viable due to vascular developmental abnormalities that included defective blood vessel assembly, despite (or possibly because of) increased production of VEGF and increased expression of the Flt-1 VEGF receptor. We have shown previously that nicotine is an activator of NFAT proteins (Frazer-Abel *et al*, manuscript submitted). Curiously, nicotine has been

shown to increase VEGF production and endothelial cell proliferation in various models of angiogenesis.^{51–54} The discrepancy between those results and the reduction in VEGF expression we show here may reflect differences in VEGF production and utilization between mature, differentiated endothelial cells and primitive HSA cells. Thus, elevated levels of VEGF may drive proliferation of mature endothelial cells, but they may induce differentiation of primitive endothelial cells. However, an alternative interpretation of the observation that nicotine prevents the formation of tube-like structures is that the apparent mitogenic effect of nicotine on endothelial cells may be, at least in part, due to a failure of differentiation, and that this effect is mediated by NFAT activation. This interpretation is also consistent with the recent observation that activation of NFATc2 (NFAT1) and NFAT5 was an important mediator of the invasive phenotype in human breast cancer cells,³⁸ and invasion required signaling through $\alpha_6\beta_4$ integrin. While the role of $\alpha_v\beta_3$ integrins (CD51/CD61) in blood vessel formation is well established,^{55,56} the possibility that signals delivered through CD51/CD61 and NFAT-dependent events may contribute to the invasive phenotype of neoangiogenic endothelial cells remains to be determined. Similarly, the possibility that exposure to environmental tobacco smoke may contribute to the pathogenesis of canine HSA should be carefully explored.

In summary, we describe eight cell lines derived from canine malignant endothelial tumors that offer a much needed resource to study the pathogenesis of canine HSA, provide desirable models to assess therapeutic strategies *in vitro* and *in vivo*, and offer a novel system for the study of endothelial biology and neoangiogenesis in cancer.

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