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Identification of drug-resistant subpopulations in canine hemangiosarcoma

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Abstract

Canine hemangiosarcoma is a rapidly progressive disease that is poorly responsive to conventional chemotherapy. Despite numerous attempts to advance treatment options and improve outcomes, drug resistance remains a hurdle to successful therapy. To address this problem, we used recently characterized progenitor cell populations derived from canine hemangiosarcoma cell lines and grown as non-adherent spheres to identify potential drug resistance mechanisms as well as drug-resistant cell populations. Cells from sphere-forming cultures displayed enhanced resistance to chemotherapy drugs, expansion of dye-excluding side populations and altered ATP-binding cassette (ABC) transporter expression. Invasion studies demonstrated variability between cell lines as well as between sphere and monolayer cell populations. Collectively, our results suggest that sphere cell populations contain distinct subpopulations of drug-resistant cells that utilize multiple mechanisms to evade cytotoxic drugs. Our approach represents a new tool for the study of drug resistance in hemangiosarcoma, which could alter approaches for treating this disease.

Keywords

ABC transporter; canine; drug resistance; hemangiosarcoma; side population

Background

Canine hemangiosarcoma is a common and highly metastatic cancer in dogs. As doxorubicin remains the cornerstone of adjuvant chemotherapy for this disease, there is minimal clinical response in most dogs when compared with no chemotherapy.^{1–3} More recent attempts to improve upon the beneficial effects of doxorubicin by altering the delivery method^{4,5} or combining doxorubicin with other treatment modalities⁶ have failed to increase survival times. Improved treatment outcomes for dogs with hemangiosarcoma are unlikely to develop until we have a better understanding of the underlying drug resistance mechanisms as well as identify cell populations within tumors that contribute to resistance.

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Resistance to chemotherapy and targeted therapies is one of the main causes underlying cancer treatment failure,⁷ and recent studies suggest specific populations of highly tumorigenic cells, or cancer stem cells (CSCs), as one of the main culprits behind this occurrence.⁸ CSCs are defined for their ability to self-renew, to differentiate into non-stem cancer cells, and to exhibit high tumorigenicity upon injection into immunodeficient mice. They are also highly resistant to chemotherapeutic drugs.

The CSC model remains controversial since not all cancers conform to the original hierarchical model first demonstrated in acute myeloid leukemia.^{9,10} In its simplest depiction, the CSC model portrays highly tumorigenic CSCs as sitting at the apex of a cellular hierarchy and generating more differentiated and less tumorigenic non-stem progeny, implying that cancer cell 'stemness' is a property of a specific subpopulation of cells. More recent studies have caused researchers to revise this model as the hierarchical nature of CSCs turns out to be more dynamic. Evidence now exists that progenitor cells can reacquire self-renewal capacity through genetic mutations and epigenetic modifications.^{11–13} Studies have also demonstrated the acquisition of self-renewal capabilities by non-CSCs^{14–16} implying a greater plasticity within cancer cell populations and indicating bidirectional interconversions between CSC and non-CSC states. These advances stress the importance of identifying CSCs, their progenitors and the differentiated non-stem bulk tumor cell population; they also emphasize the difficulty in definitively identifying these populations.

One approach to enrich for cells with stem/progenitor properties involves the culture of cells under non-adherent growth conditions.¹⁷ This technique generates long-term cultures growing as spheres or spheroids and has been shown to lead to the enrichment of cell populations that display tumor-initiating capabilities.^{18–20} We recently utilized this approach with hemangiosarcoma cell lines as a means to enrich for tumor cell progenitors and to address the cellular ontogeny of hemangiosarcoma.²¹ Sphere cells exhibited genotypic, phenotypic and functional properties consistent with specific molecular subtypes that we observed in primary hemangiosarcomas, including the expression of endothelial progenitor and early hematopoietic differentiation markers. As our results suggested that canine hemangiosarcomas arise from multipotent progenitors, their classification as CSCs must await further studies including limiting dilution assays. At present, their identification provides a unique model to study the pathogenesis of hemangiosarcoma, and the opportunity to examine potential drug resistance mechanisms associated with progenitor populations.

The goals of this study were to further characterize the properties of the progenitor (sphere) cell population and to identify potential drug resistance mechanisms. Using functional assays and surface marker expression, we show that sphere cells possess drug resistance properties, expansion of side populations and higher ATP-binding cassette (ABC) transporter expression. In addition, sphere cells from one cell line were more highly invasive than the corresponding monolayer. These results provide an additional tool to study drug resistance in canine hemangiosarcoma. Further refinement and characterization of this population will allow us to more strategically design treatments that target the bulk differentiated cells as well as those with drug-resistant potential.

Methods

Cell culture and sphere formation

The hemangiosarcoma cell lines, SB-HSA (SB), Frog and Emma were cultured as monolayers^{22–24} or under conditions favoring non-adherent sphere formation from these cell lines as described.^{21,25} Cells were maintained in a 37 °C incubator with a 5% CO₂ atmosphere and given medium with fresh growth factors every 2 to 3 days. Spheres were dissociated enzymatically once per week into single-cell suspensions for culture maintenance.

DyeCycle® Violet dye-efflux (side population) assay

Monolayer cells were removed from the cell culture flasks by trypsin, washed with phosphate buffered saline (PBS) and filtered. Sphere cells were collected by gravity, washed with PBS and incubated with Accutase® (Life Technologies, Grand Island, NY, USA) at room temperature for 10 min with intermittent mixing to dissociate the spheres. The sphere cells were dissociated further by pipetting up and down approximately 30–50 times using a 200-μL pipette tip. Sphere cells were filtered and washed with PBS. The viability and cell numbers for the monolayer and sphere cell populations were determined using a Countess® automated cell counter (Life Technologies), and the validation of single-cell suspensions was determined visually. Single-cell suspensions of monolayer and sphere cells were incubated in the presence or absence of 10 μM verapamil for 15 min at 37 °C. DyeCycle Violet (DCV) (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 10 μM, and the cells were incubated for an additional 60 min at 37 °C with intermittent mixing. Cells were washed, filtered and maintained at 4 °C for analysis. Propidium iodide was added to each sample immediately before collection to exclude dead cells from analysis, and the side populations were gated based on the verapamil controls. DCV emission was detected using 450/50 nm band-pass (blue) and 650 nm long-pass (red) filters in response to excitation by a violet diode laser (405 nm) using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software.

Flow cytometry

Single-cell suspensions of monolayer and sphere cells were generated as described above. The primary antibodies used were anti-CD243-PE (ABCB1, clone UIC2) and anti-CD338-PE (ABCG2, clone 5D3) (eBioscience, San Diego, CA, USA). For detection of cell surface markers, single-cell suspensions were washed with 200 μL of flow cytometry staining buffer [PBS containing 2% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA) and 2 mM EDTA (Sigma, St. Louis, MO, USA)]. Fc receptors were blocked with normal mouse or rat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) depending upon the host species of primary antibody used. Cells were stained with 1 test volume (according to the manufacturer's recommendations) of primary antibodies for 30 min on ice, and then washed twice in staining buffer. Samples were filtered before analysis and 7-Aminoactinomycin D (7-AAD) was added to each sample before collection to exclude dead cells from analysis. Fifty thousand events per sample were collected on a BD FACS Calibur (BD Biosciences). The results were analyzed using FlowJo software. Antibodies were

validated for cross-reactivity to the canine ABC transporters by immunoblotting and immunohistochemistry.

Cytotoxicity and cell viability assay

Filtered, single-cell suspensions of monolayer and sphere cells were generated as described above for the side population assay. Cell viability was determined using the colorimetric Cell Titer 96[®] Aqueous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega, Madison, WI, USA) to measure cellular responses to different cytotoxic drug conditions. Each condition was performed in triplicate using 10 000 cells per well in 100 μ L of culture medium. Cells were exposed to increasing concentrations of paclitaxel (TEVA Pharmaceuticals, North Wales, PA, USA) or doxorubicin (Bedford Laboratories, Bedford, OH, USA) for 72 h in 37 °C, 5% CO₂. MTS reagent was added at a ratio of 1:10 and incubated for additional 1–2 h. Absorbance (A_{490}) was measured using a Wallac Victor2 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA). Cell viability was expressed as the percentage of the A_{490} of drug-treated cells relative to untreated cells according to the following equation: % Viability = $100 \times (A_{490} \text{ drug-treated} / A_{490} \text{ untreated})$. The IC₅₀ for each drug was determined by fitting the relative viability of the cells to the drug concentration by using a dose–response model in the Prism program from GraphPad Software (San Diego, CA, USA).

Cell invasion assay

A modified Boyden chamber cell invasion assay was performed using the Cultrex[®] 96 Well BME Invasion Assay (Trevigen, Gaithersburg, MD, USA). After overnight serum and growth factor starvation, single-cell suspensions of monolayer or sphere cells (1×10^5 cells in 100 μ L) were generated as described above, and the cells were added to the top of a polycarbonate transwell membrane coated with 0.5X basement membrane extract (BME). Cell culture medium containing 0 or 2% FBS was placed in the bottom chamber, and the plate was incubated at 37 °C in a 5% CO₂ atmosphere for 24 h. The membrane and the lower chambers were washed with wash buffer, and Cell Dissociation Solution containing Calcein-AM was added to the lower chamber. After 1 h incubation at 37 °C and 5% CO₂, the chambers were removed and the fluorescence in each well was determined using a Wallac Victor2 1420 Multilabel Counter with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. A standard curve was generated for both the monolayer and sphere cells. The number of cells that had invaded through the membrane was determined from a standard curve based on the fluorescence intensity of known cell numbers.

Protein extraction and immunoprecipitation

Total protein was extracted from approximately 1×10^6 cells by incubating in RIPA lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0, 10% glycerol) supplemented with fresh Halt[™] protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Pittsburgh, PA, USA) at 1:100 dilutions for 30 min on ice with periodic vortexing. The supernatant was collected by centrifugation at $20\,000 \times g$ for 10 min at 4 °C and the protein concentrations determined by BCA[™] protein assay (Thermo Fisher Scientific). Immunoprecipitation of ABCG2 protein from cell lysates was performed using approximately 500 μ g to 1 mg of

total protein per reaction, pre-cleared by incubating with equilibrated protein A/G/L magnetic beads (BioVision, Milpitas, CA, USA) to eliminate non-specific binding. Pre-cleared protein lysates were adjusted to 500 µL total volume and incubated with 5 µg of mouse anti-ABCG2 monoclonal antibody (clone 5D3; EMD Millipore, Billerica, MA, USA) overnight at 4 °C with constant mixing. The protein–antibody mix was then incubated with 25 µL of equilibrated protein A/G/L magnetic beads for 1 h at 4 °C with constant mixing to allow for precipitation. The beads were then washed three times with lysis buffer and eluted with IgG elution buffer (Thermo Fisher Scientific) containing 1X Laemmli sample buffer (Bio-Rad, Hercules, CA, USA).

Immunoblotting analysis

Immunoblotting analysis was performed as previously described.²⁶ Briefly, protein samples containing 1X Laemmli sample buffer were boiled for 5 min and loaded onto 4–15% gradient SDS-polyacrylamide gel for electrophoresis, transferred to a nitrocellulose membrane and blocked with TBST (20 mM Tris–HCl pH 7.4, 137 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk. Mouse anti-ABCG2 [BXP-21] (Abcam, Cambridge, MA, USA) primary antibody was added at a 1:500 dilution in blocking buffer and incubated for overnight at 4 °C with constant rocking. The membrane was then washed three times with TBST and followed by incubation with IRDye® 680RD donkey anti-mouse secondary antibody conjugated with infrared fluorescence dye (680 nm) (LI-COR Biosciences, Lincoln, NE, USA) at a 1:10 000 dilution in blocking buffer for 1 h at room temperature. The membrane was then washed, scanned and documented using Odyssey infrared imaging system (LI-COR Biosciences).

Immunohistochemistry

The HeyA8 MDR ovarian cancer cell line (provided by Anil Sood, University of Texas M. D. Anderson Cancer Center) was used as a positive control for ABCB1 expression. HeyA8 MDR and SB cells were removed from cell culture flasks using trypsin, washed in PBS and counted using a Countess automated cell counter. Cells were fixed in 10% neutral buffered formalin for approximately 3 h, centrifuged to pellet the cells and the cell pellet resuspended in 70% ethanol. Cells were embedded in paraffin and sectioned for staining. Antigen retrieval was performed using citrate buffer for 30 min. Sections were then treated with 0.3% hydrogen peroxide followed by blocking for 15 min with normal mouse serum. Cells were incubated with a 1:200 of anti-CD243 (eBio-science) for 30 min at room temperature followed by incubation with mouse Envision reagent (Dako, Carpinteria, CA, USA) for 30 min. Color development was performed using 3,3-diaminobenzidine tetra-hydrochloride (DAB) for 5 min. The slides were counterstained with hematoxylin and coverslipped for microscopic evaluation by a board certified veterinary pathologist (G. O'Sullivan, University of Minnesota). An isotype control was used to evaluate potential background staining. All immunohistochemical processing and staining was carried out through the Comparative Pathology Shared Resource, University of Minnesota, Twin Cities.

Statistical analysis

All *in vitro* assays were performed at least twice with duplicates or triplicates, as needed, in each experiment. Representative results are depicted in this report. Background values were

subtracted from the mean value of each sample where indicated. Data are presented as background adjusted mean values $\pm$ SD. Comparisons between monolayer and spheres were made using a Student's *t*-test. A *P* value of 0.05 or less was considered statistically significant.

Results

Sphere cell populations enriched from hemangiosarcoma cell lines display altered drug resistance

Sphere cells derived from other cancer cell lines have been shown to display resistance to a variety of chemotherapeutic agents.^{18–20} To determine if sphere cells derived from hemangiosarcoma cell lines displayed differential drug sensitivities to those of the original monolayer, we conducted a comparative assay using paclitaxel as well as doxorubicin, which is the chemotherapeutic agent commonly used in the treatment of hemangiosarcoma. The relative viabilities of the SB and Frog monolayer and sphere cells after treatment with paclitaxel and doxorubicin are presented in Fig. 1A–D. Compared to their monolayer counterparts, sphere cells demonstrate lower susceptibility to both anticancer agents, with the exception of Frog monolayer and sphere cells treated with doxorubicin (Fig. 1B). In this case, resistance levels determined for the Frog sphere and monolayer cells were similar. Both the Emma monolayer and sphere cells were extremely drug resistant with IC₅₀ values greater than 10 μ M (data not shown). Taken together, these results demonstrate that sphere cells are more drug resistant than their monolayer counterparts. However, we interpret our results with caution because some changes in resistance may be due to culture artifacts.

Sphere-cultured cells display expanded side populations and increased ABC transporter expression

As we noted that the hemangiosarcoma sphere cell populations were more drug resistant overall than their corresponding monolayer cells, we sought to identify drug resistance mechanisms and phenotypes that might contribute to this process. To do this, we used two complementary approaches: functional dye efflux or side population assays and ABC transporter expression. We focused our efforts on the drug transporters ABCB1 (P-glycoprotein) and ABCG2 (breast cancer resistance protein or BCRP) as these transporters have been associated with enhanced drug resistance in many different cancers.²⁷ In addition, we chose to examine these transporters because doxorubicin serves as a substrate for both the ABCB1 and ABCG2 transport pumps, while paclitaxel is more exclusively effluxed by ABCB1. We used DCV for the dye efflux assays as Hoescht 33342 dye appeared to reduce cell viability, and this problem was not observed with DCV. In addition, DCV dye efflux has been attributed to ABCB1²⁸ and ABCG2^{29,30} expression, making transporter expression a major determinant of the side population profile.

Using DCV as our readout, we determined that all three cell lines contained an abundant side population when grown as monolayers (Fig. 2A,E,I) ranging from about 3% (Frog) up to approximately 16% (SB) of the total viable cells. All of the monolayer cell populations were highly sensitive to verapamil (Fig. 2B,F,J) as indicated by the selective elimination of the side population in each case. Verapamil is a broad inhibitor of ABC transporter activity,

but it primarily affects ABCB1.³¹ Compared to the monolayer cells, the sphere cell side population increased dramatically in both Frog and Emma cells from approximately 3% to over 30% in Frog and 5 to 21% in Emma. A concomitant increase was not observed between the monolayer and sphere cells in the SB line. This may be due to the method used to generate the SB cell line (derivation from a primary xenografted tumor) versus the method used to generate the Frog and Emma cell lines (direct culture of the primary tumor). We also noted small populations of cells in the lower left quadrant of the majority of the monolayer and sphere cell populations. These cells were viable based upon our gating strategy and the use of a viable indicator dye, but they either did not take up DCV or were able to efflux the dye completely, and they accounted for approximately 2% of the monolayer and up to 10% of the sphere cell populations. Interestingly, sphere cells from all three lines contained side population cells resistant to dye exclusion blocking by verapamil (Fig. 2C,G,K versus Fig. 2D,H,L), albeit to varying degrees. Although the side populations in both the SB and Emma sphere cells were reduced by the inhibitor, a change in the Frog side population was not observed. Because a change in verapamil sensitivity might indicate potential differences in ABC transporter expression between the side populations of the monolayer and sphere cells, we decided to determine the expression levels of ABCB1 and ABCG2 in these cells.

To do this, we first validated the ABCB1 and ABCG2 antibodies for cross-reactivity with canine cells. ABCG2 expression was assessed by immunoblotting using the canine SB, Frog and COSB monolayer cells to represent hemangiosarcoma. The human A549 lung cancer cell line was used as a positive control (Fig. 3A). A 70-kDa protein was identified in all four cell lines. The SB cell line was assessed for the expression of ABCB1 by immunohistochemistry, and the human ovarian cancer cell line, HeyA8 MDR, served as a positive control. ABCB1 expression has been noted in the plasma membrane as well as other cellular structures including the Golgi complex and the rough endoplasmic reticulum.³² Cytoplasmic staining was observed in the HeyA8 MDR (Fig. 3B) as well as the SB cell line (Fig. 3C), but not all cells in each line were positive for the marker, indicating heterogeneous expression of the transporter. On the basis of these results, we utilized these antibodies to characterize ABCB1 and ABCG2 cell surface expression levels in the monolayer and sphere cells by flow cytometry (Fig. 4).

Overall, we did not observe cell surface expression of ABCB1 or ABCG2 in the monolayer cells or the expression was very low. A notable exception was made for the Frog cell line where the expression of both transporters was observed in the monolayer cells. ABCB1 expression was increased or retained in sphere cells from the SB and Frog lines, and bimodal expression of the transporter was observed in both the SB and Frog sphere cell populations. The increased expression of ABCB1 by specific subpopulations informs to the heterogeneity within the sphere cell populations and may indicate the enrichment of more highly drug-resistant cells with the SB and Frog sphere cells. ABCB1 expression was not observed in the Emma monolayer or sphere cells. ABCG2 expression proved to be more variable and was not observed in the majority of the sphere cells.

Overall, our results indicated that sphere cells appeared to have increased expression of both verapamil-sensitive and -insensitive (as indicated by dye efflux) transporters, and one or more heretofore undefined verapamil-insensitive transporters may play a key role in drug

resistance in the sphere cells. Furthermore, the lack of ABCB1 and ABCG2 expression in both the Emma monolayer and sphere populations points to a different drug transporter or resistance mechanism in these cells. As the Emma sphere cell side population was partially reduced by verapamil (yet ABCB1 expression was not observed), it is possible that the same verapamil-insensitive transporter is expressed by the Emma sphere cells, and this transporter is responsible for the observed drug resistance. In addition, a verapamil-sensitive transporter also appears to play a role in drug resistance in the Emma monolayer and sphere cell populations since side populations are reduced in the presence of the drug, but ABCB1 expression is not detected.

SB sphere cells display a higher invasive capacity

One feature of canine hemangiosarcoma is its highly aggressive nature and ability to rapidly metastasize.^{23,33} In addition, there is increasing evidence that CSCs possess a distinct potential for migration and invasion.^{34,35} We compared the *in vitro* invasive capacity of the sphere cell populations to those of the original monolayer using coated transwell migration chambers. Single-cell suspensions of monolayer and sphere cells were examined for their ability to invade through the BME layer over 24 h in the absence and presence of FBS (Fig. 5). SB monolayer and sphere cells showed increased invasion in response to FBS, and the sphere cells were significantly more invasive than their monolayer cell counterparts ($P < 0.01$). In contrast, increased invasive capacity was not observed in the Frog monolayer or sphere cells in the presence of FBS, and only the Emma monolayer cells showed increased invasion in response to the serum stimulus. Thus, the SB sphere cells possess a higher intrinsic invasive capacity, attributing a functional phenotype associated with tumor aggressiveness in this line.

Discussion

As hemangiosarcoma is one of the most common cancers diagnosed in pet dogs,³⁶ the standard-of-care for this disease has not advanced significantly over the past 20–30 years.³⁷ An improved understanding of the disease progression may provide clues to the development of more effective treatment strategies. We previously described an enriched tumor progenitor population derived from canine hemangiosarcoma cell lines grown under sphere-forming culture conditions.²¹ These cells showed co-expression of both early endothelial and hematopoietic progenitor cell markers indicating a common multipotent progenitor as a cell of origin. Here, we test the hypothesis that sphere cells possess functional and phenotypic properties associated with drug resistance and present the idea that sphere cells may provide a readily available tool for identifying drug-resistant populations as well as the study of drug resistance mechanisms.

A number of groups have demonstrated that cells within sphere populations are more chemoresistant than non-sphere cells,^{18–20} indicating that sphere formation may be a strategy to identify drug-resistant populations and associated drug resistance mechanisms. Resistance to cytotoxic agents by CSC and progenitor populations has been attributed to factors such as increased expression of drug transporters that mediate drug efflux, up-regulation of anti-apoptotic proteins, and increased efficiency of DNA repair.²⁷ We noted

that the sphere cell populations used in our studies were more resistant overall to chemotherapy agents. This prompted us to investigate potential mechanisms in sphere cells that might contribute to the resistance phenotype, specifically side population expansion and ABC transporter expression.

Goodell et al.³⁸ first described a small population of cells in mouse hematopoietic stem cells based on the cells' ability to efflux Hoechst 33342 dye. Cells capable of efflux were termed as the 'side population' and possessed an enriched clonogenic capacity, tumorigenicity, multipotency and long-term regenerating properties.³⁸ More importantly, side population cells were more chemoresistant. As we noted more variability in our side population assay and a potential decrease in cell viability when using Hoechst 33342, we chose to use DCV as a means to examine side populations in monolayer and sphere cells. Difficulty using Hoechst dye to examine side populations in canine cells, including hemangiosarcoma, has been described previously.³⁹

As we observed expanded side populations in the Frog and Emma sphere cell populations, the overall number of sphere cells in the SB side population remained relatively unchanged. Lack of expansion of the SB side population may reflect the method used to generate this line, which was created by subcutaneous serial passage of a primary hemangiosarcoma xenograft in immunocompromised mice.²² In contrast, the Frog and Emma cell lines were derived by direct placement of primary tumor cells into cell culture medium.²³ Direct xenograft implantation may have preserved or enriched for hemangiosarcoma progenitor cell populations as *in vivo* preservation of CSCs has been noted in other systems. For example, tumors established by direct implantation of resected primary glioblastoma multiforme from human patients and passaged in the flanks of immunocompromised mice preserved multipotency, invasive migration *ex vivo* and lethality *in vivo* of brain tumor stem cells when limited *in vivo* passages (<15) were performed. *In vivo* progenitor cell or CSC preservation also may explain the high invasive capacity of the SB sphere cells when compared to the sphere cells generated from the Frog and Emma cell lines. Thus, side population expansion and invasiveness may be contingent upon the hemangiosarcoma cell model employed. Hemangiosarcoma cell lines derived in a manner similar to the SB cell line may prove more informative for drug resistance and progenitor cell studies than cell line populations generated directly from cell culture.

The percentages of cells contained in the observed side populations using DCV are somewhat high, which was unexpected. To our knowledge, ours is the first report of a side population analysis in hemangiosarcoma cell lines using DCV. A study using the SB line and Hoechst 33342 efflux analysis reported a side population of approximately 1%,³⁹ but we have not undertaken a rigorous comparison to determine differences between DCV and Hoeschst 33342. Anecdotaly, the use of Hoeschst 33342 to determine the side population in the Emma cell line showed an effluxing population of approximately 7% (D. Ito, unpublished observation). This number is in line with our results using DCV (~5%) in this line. Thus, larger side populations in hemangiosarcomas may be present, but a direct comparison of the methods will be needed to validate this idea.

Although expansion of the SB side population was not observed, the phenotype of the cells comprising the side population in the SB sphere cells differed from those of the monolayer as indicated by the altered sensitivity to verapamil and increases in ABCB1 and ABCG2 cell surface expression. As verapamil is more specific for ABCB1, it can inhibit ABCG2 activity at high concentrations.⁴⁰ Thus, increased expression of one or both of these transporters may account for the observed changes in drug resistance in the SB sphere cells. This argument provides a reasonable explanation for the increased chemoresistance observed in the SB sphere cells; however, it does not provide a sufficient explanation for the changes in efflux activity between the monolayer and sphere cell populations from the Frog and Emma cell lines in the context of their corresponding ABC transporter expression. DCV is effluxed by both ABCB1²⁸ and ABCG2.^{29,30} The Frog monolayer cells expressed low to moderate levels of both transporters, and the side population was completely diminished by verapamil treatment. However, the side population in Frog sphere cells increased dramatically (from 3 to ~30%), but these cells failed to respond to verapamil despite the overall increase in ABCB1 expression and a corresponding decrease in ABCG2. One interpretation of this result is that Frog sphere cells upregulate the expression of an unknown transporter capable of DCV efflux, but one that is insensitive to verapamil. Results using the Emma sphere cells also support the existence of this transporter as verapamil did not entirely mitigate dye efflux, yet expression of ABCB1 and ABCG2 was not observed. Attempts to identify the unknown ABC transporter(s) using microarray analysis failed to identify increased expression of one or more common ABC transporter across all sphere cell populations (J.-H. Kim and A. Frantz, unpublished observation).

As our results point to a role for ABCB1 and ABCG2 in drug resistance in hemangiosarcoma, inhibitors with higher potency and specificity for each transporter, such as tariquidar (ABCB1) and Fumitremorgin-C (ABCG2), may produce more definitive results. The identification and contribution of other transporters to drug resistance will be challenging due to the high degree of functional redundancy displayed by the ABC transporter family where more than half of the 49 ABC transporter family members have been suggested to play a role in this process.⁴¹

It is tempting to attribute increased ABC transporter expression to the expansion of the sphere cell side populations as well as drug resistance, but the association between these three entities may not be so simple. For example, side population cells isolated from the adrenocortical NCI-h295R cell line exhibited similar sensitivities to cytotoxic agents commonly used to treat adrenocortical carcinomas as non-side populations,⁴² indicating that drug resistance may not always associate with side population activity. Furthermore, both sorted side population and non-side population cells from several cancer cell lines have similar capacities for tumorigenicity and multipotency, indicating that functional side populations also may not be enriched for CSCs or CSC-like cells.⁴³ Isolation of side populations from both monolayer and sphere cells would allow for definitive assignment of ABCB1 and ABCG2 at the protein level to the side populations and assessment of drug resistance characteristics, and these studies would add vital information to the more descriptive work presented here. In addition, other mechanisms contributing to drug resistance (e.g. up-regulation of anti-apoptotic genes) also need to be considered.

The ultimate goal of this study was to develop a platform to identify and characterize drug-resistant populations from canine hemangiosarcoma cell lines as specific cell populations may be responsible for the development of treatment resistance and associated relapse. As cell lines may not display the entire parental tumor heterogeneity due to repeated passage, recent work from our group indicates retention of distinct molecular subtypes between primary hemangiosarcomas and derived hemangiosarcoma cell lines.²¹ The application of phenotypic and functional assays to cell lines as well as other defined populations derived from these lines (e.g. sphere cells) provides a tool to begin to dissect drug resistance mechanisms within hemangiosarcoma, and provides a starting point to identify similar mechanisms within primary tumors.

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Figure 1.

Sphere cells are differentially resistant to standard chemotherapy agents. Monolayer and sphere cells (5000 cells/well) were incubated in 96-well plates in the presence of increasing concentrations of either doxorubicin or paclitaxel for 72 h. The relative viability was assessed using a MTS assay. Sphere cells are represented by squares and monolayers by circles in all cases. Sphere cells from SB (A) were more resistant to doxorubicin than their monolayer cell counterparts (IC_{50} sphere cells, ~1000 nM; IC_{50} monolayer cells, <100 nM). Frog sphere cells (B) did not show a substantially altered resistance pattern when compared to Frog monolayer cells in the presence of doxorubicin (IC_{50} sphere cells, ~230 nM; IC_{50} monolayer cells, ~135 nM). SB sphere cells (C) were more resistant to paclitaxel than the monolayer cells (IC_{50} sphere cells, ~300 nM; IC_{50} monolayer cells, ~50 nM). Frog sphere cells were more resistant to paclitaxel (D) than the Frog monolayer cells (IC_{50} sphere cells, ~2500 nM; IC_{50} monolayer cells, ~330 nM). All conditions were performed in triplicate. Error bars represent standard deviation.

Figure 2.

Dye exclusion identifies a side population in hemangiosarcoma monolayer and sphere cells. Monolayer cells (A, E, I) show a small side population (SP) that is eliminated in the presence of the broad ABC transporter inhibitor verapamil (B, F, J). SPs are also present in the sphere cells (C, G, K) generated from the corresponding monolayer cell lines. The addition of verapamil to the sphere cells (D, H, L) reduces, but does not eliminate the sphere SPs in the SB and Emma cell lines (D, L). The SP in Frog sphere cells is not diminished in the presence of the inhibitor (H). Propidium iodide was added immediately before reading all samples in order to exclude dead cells from analysis. Dye retaining cells are indicated by DR.

Figure 3. ABCB1 and ABCG2 transporters are expressed by hemangiosarcoma cell lines. (A) The expression status of the ABCG2 transporters from the hemangiosarcoma cell lines, SB, Frog and COSB is shown by immunoblotting analysis. The lung cancer cell line, A549, was used as a positive control. Cell lysates were immunoprecipitated (IP) with clone 5D3 followed by immunoblotting (IB) with clone BXP-21. Immunohistochemistry was used to detect the expression of ABCB1 (brown staining) in the human ovarian cancer cell line, HeyA8 MDR (B), and the hemangiosarcoma cell line, SB (C).

Figure 4.

Alterations in ABC transporter expression are observed between hemangiosarcoma monolayer and sphere cells. ABCB1 and ABCG2 expression was examined in SB, Frog and Emma monolayer and sphere cell populations by flow cytometry. The isotype control for each experiment is shown as a shaded histogram. The data shown are representative of at least two experiments for each cell line and condition.

Figure 5.

SB sphere cells possess a higher invasion capacity. Single-cell suspensions of SB, Frog or Emma monolayer (white bars) and sphere cells (black bars) were tested for their ability to invade and migrate across a simulated basement membrane over 24 h using BME-coated transwells. Cell culture medium with or without 2% FBS was used to stimulate cell migration. The number of cells that had invaded through the membrane was determined from the standard curve prepared from monolayer or sphere cells based upon the relative fluorescence intensity of known cell numbers. Significant differences between the populations are indicated (* $P < 0.05$; ** $P < 0.01$). The percent invasion was determined by dividing the number of cells found in the lower chamber by the total number of cells added (1×10^5) to the upper chamber.