Evaluation of procoagulant tissue factor expression in canine hemangiosarcoma cell lines

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OBJECTIVE

To evaluate expression of procoagulant tissue factor (TF) by canine hemangiosarcoma cells in vitro.

SAMPLES

4 canine hemangiosarcoma cell lines (SB-HSA [mouse-passaged cutaneous tumor], Emma [primary metastatic brain tumor], and Frog and Dal-I [primary splenic tumors]) and 1 nonneoplastic canine endothelial cell line (CnAoEC).

PROCEDURES

TF mRNA and TF antigen expression were evaluated by quantitative real-time PCR assay and flow cytometry, respectively. Thrombin generation was measured in canine plasma and in coagulation factor–replete or specific coagulation factor–deficient human plasma by calibrated automated thrombography. Corn trypsin inhibitor and annexin V were used to examine contributions of contact activation and membrane-bound phosphatidylserine, respectively, to thrombin generation.

RESULTS

All cell lines expressed TF mRNA and antigen, with significantly greater expression of both products in SB-HSA and Emma cells than in CnAoEC. A greater percentage of SB-HSA cells expressed TF antigen, compared with other hemangiosarcoma cell lines. All hemangiosarcoma cell lines generated significantly more thrombin than did CnAoEC in canine or factor-replete human plasma. Thrombin generation induced by SB-HSA cells was significantly lower in factor VII-deficient plasma than in factor-replete plasma and was abolished in factor X-deficient plasma; residual thrombin generation in FVII-deficient plasma was abolished by incubation of cells with annexin V. Thrombin generation by SB-HSA cells was unaffected by the addition of corn trypsin inhibitor.

CONCLUSIONS AND CLINICAL RELEVANCE

Hemangiosarcoma cell lines expressed procoagulant TF in vitro. Further research is needed to determine whether TF can be used as a biomarker for hemostatic dysfunction in dogs with hemangiosarcoma. (*Am J Vet Res* 2017;78:69–79)

Hemangiosarcoma is a common, highly aggressive malignant tumor of endothelial origin in dogs.¹ Dogs with hemangiosarcoma often have concurrent hemostatic abnormalities, particularly DIC.^{2,3} In 1

study,² 12 of 24 (50%) dogs with hemangiosarcoma had laboratory evidence of DIC, which may increase mortality and morbidity rates in dogs with this tumor. Disseminated intravascular coagulation is defined as uncontrolled thrombin generation as a consequence of activation of coagulation.⁴ Tissue factor is the main initiator of coagulation; during normal hemostasis, endothelial cell injury exposes TF on fibroblasts in the perivascular space to coagulation factors in the blood. The extrinsic pathway involves FVII in plasma binding to TF, which then acts as a cofactor for FVII enzymatic activity. Once bound to TF, FVII autoactivates to FVIIa, and the TF-FVIIa complex can subsequently bind to and activate FX of the common pathway, leading to thrombin generation and eventual fibrin clot formation.⁵ The presence of negatively charged phospholipids such as phosphatidylserine (which is expressed on the outer cell membrane of activated cells) increases the activity of coagulation factor enzyme function, including that of TF-FVIIa,

ABBREVIATIONS

AUC	Area under the curve
CAT	Calibrated automated thrombography
CnAoEC	Canine aortic endothelial cells
Ct	Cycle threshold
CTI	Corn trypsin inhibitor
DIC	Disseminated intravascular coagulation
FVII	Coagulation factor VII
FVIIa	Activated coagulation factor VII
FVIII	Coagulation factor VIII
FIX	Coagulation factor IX
FX	Coagulation factor X
FXI	Coagulation factor XI
FXII	Coagulation factor XII
FXIIa	Activated coagulation factor XII
MFI	Median fluorescence intensity
qRT	Quantitative real-time
TF	Tissue factor

in accordance with the cell-based theory of coagulation.^{6,7} Tissue factor is not normally expressed on endothelial cells but can be upregulated by lipopolysaccharide and inflammatory cytokines in disease states such as sepsis, and it is considered the main trigger for initiation of DIC in these conditions.⁸⁻¹⁰

Thrombosis is a known sequela of malignant tumors in people¹¹; nearly all cancer patients have laboratory evidence of low-grade systemic coagulation activity with a subset having clinically evident hemostatic disorders, particularly thrombosis.¹² Prothrombotic conditions such as thromboembolism and DIC increase the morbidity and mortality rates of cancer patients.^{13,14} Angiosarcoma, the human equivalent of hemangiosarcoma, is a highly metastatic tumor associated with a poor prognosis.¹⁵ Similar to the clinical situation for dogs with hemangiosarcoma, results of a retrospective study¹⁶ of human patients with angiosarcoma found that a substantial proportion (7/42 [17%]) had DIC, whereas another investigation¹⁷ identified DIC in a much smaller proportion of patients with other types of solid tumors (76/1,177 [7%]). Aberrant expression or overexpression of TF is thought to be among the main mechanisms by which tumors inappropriately activate hemostatic pathways to promote coagulopathies such as DIC.¹⁸ Tumor-expressed TF is implicated in the etiopathogenesis of coagulopathies in other cancer types, including those of epithelial and mesenchymal origin; high TF expression in human cancer is recognized as a marker of thrombotic risk¹⁹ and is associated with higher histologic grade and a poorer prognosis in patients with pancreatic and breast cancer, respectively.^{20,21}

Hypercoagulability, thrombosis, and hemostatic dysfunction are clinical complications in dogs with various types of cancer.^{22,23} The pathogenesis of hypercoagulability in dogs with cancer is unknown. Functional TF expression *in vitro* has been identified in epithelial tumor cells and some mesenchymal tumor (including osteosarcoma and fibrosarcoma) cell lines from dogs.^{24,25} Two studies^{26,27} have shown that TF is expressed in canine gliomas and mammary tumors *in situ*. Higher TF expression was associated with higher-grade tumors, and there was evidence of coagulation activation, with intravascular thrombosis and intratumoral deposition of fibrin or fibrinogen, and fibrin or fibrinogen breakdown products, including D-dimer in the study²⁷ of gliomas. Similarly, dogs with mammary tumors that had high TF expression had evidence of hemostatic dysfunction, including DIC.²⁶ In an investigation in our laboratory, results of CAT revealed that TF-expressing canine mammary tumor cells generate thrombin *in vitro*, supporting a primary role for TF in hypercoagulability and thrombosis in dogs with mammary tumors.²⁵ It is not currently known whether TF is expressed and functionally procoagulant (ie, capable of generating thrombin) in dogs with hemangiosarcoma. The objective of the study reported here was to evaluate the expression of TF and generation of thrombin by canine hemangio-

sarcoma cells *in vitro*. We hypothesized that canine hemangiosarcoma cells would express TF and would have detectable TF-dependent procoagulant activity, causing activation of FX and generating thrombin in plasma. Because thrombin can also be generated through the intrinsic coagulation pathway (triggered by FXIIa, which initiates a cascade of enzymatic reactions culminating in FX activation),²⁸ a secondary aim was to assess the contributions of various coagulation factors of the intrinsic and extrinsic pathways, as well as TF and phosphatidylserine, to thrombin generation by hemangiosarcoma cells *in vitro*.

Materials and Methods

Samples and cell culture

A mouse-passaged (xenografted) cell line derived from a cutaneous hemangiosarcoma in a dog (SB-HSA)^{29,a} and cell lines from a metastatic brain hemangiosarcoma (Emma)^b and 2 primary splenic hemangiosarcomas (Frog and Dal-4)^b from dogs were used in the study. Hemangiosarcoma cell lines were cultured in Ham F-12K (Kaighn) medium^c supplemented with 10% (vol/vol) fetal bovine serum,^d endothelial cell growth supplement^e (0.05 mg/mL), HEPES^c (10 mM), heparin^f (0.01 mg/mL), penicillin^g (100 U/mL), streptomycin^g (100 µg/mL), and an additional antibacterial solution with antimycotic activity^h (100 µg/mL). Nonneoplastic canine endothelial cellsⁱ (used as controls) were cultured in canine endothelial cell growth medium.ⁱ All cells were grown in a humidified chamber with 5% CO₂ at 37°C. Cells were harvested from passages 32 to 36 (the earliest available) for SB-HSA, passages 4 to 8 for primary HSA cell lines (Emma, Frog, and Dal-4), and passages 2 to 5 for CnAoEC. Cells were incubated and detached with 0.25% trypsin-EDTA^f for 3 minutes and monitored for rounding before being lifted with gentle agitation and pipetting; trypsin was neutralized with growth medium without washing. Cells were used in the log phase of growth between 60% to 80% confluency. Cell viability was > 90% on the basis of trypan blue exclusion assays, and cells were counted by use of a hemacytometer.

qRT-PCR assay

Total RNA was isolated from harvested cells through the use of a commercial kit.^j Concentration and purity of total RNA were determined by UV measurements at wavelengths of 280 and 260 nm,^k and cDNA was synthesized from 1 µg of total RNA with a commercial kit including oligo(dT)₂₀ primers.^l The qRT-PCR assay was performed with a commercially available kit^m used according to the manufacturer's instructions and primers specific for canine TF that were designed to span the exon 2-3 junction of canine TF DNA (forward primer, 5'-AGTGGGAACCCAAACCCATC-3'; reverse primer, 5'-ATGGAGGCTCCCCAGAGTAG-3').ⁿ Canine ribosomal protein subunit 5, used as a housekeeping gene, was amplified with previous-

ly described primer sequences (forward primer, 5'-TCACTGGTGAGAACCCCT-3'; reverse primer, 5'-GTTCTCATCGTAGGGAGCAAG-3').^{30,n} Reaction conditions for qRT-PCR assay were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds, followed by 57°C for 1 minute.^o Amplification curves and Ct values were collected and analyzed with commercial software.^p Gene expression levels were calculated by means of the comparative Ct ($2^{-\Delta\Delta Ct}$) method, with canine TF mRNA expression normalized to expression of ribosomal protein subunit 5 mRNA. Data were represented as the mean $\pm$ SD of 3 separate RNA isolations for each cell line.

Flow cytometry

Detached cells (1×10^5 cells/reaction) were resuspended in PBS with 1% bovine serum albumin^d and 0.05% sodium azide (ie, reaction buffer) and were incubated with monoclonal mouse anti-dog TF antibody IgG (clone 133-2)³¹ or mouse IgG isotype control^q (each used at a final concentration of 20 μ g/mL) for 30 minutes on ice. After washing in reaction buffer, a secondary fluorescent-conjugated goat anti-mouse IgG^r (1:200 dilution) was added, followed by incubation for 30 minutes on ice. Cells were washed twice in reaction buffer, then suspended in PBS and analyzed with a flow cytometer.^s For data acquisition, forward scatter settings were voltage of E-I, gain of 4.75 A, and linear mode; side scatter was set at voltage of 350, gain of 1.00 A, and linear mode. Fluorescence was set on log mode, and 10,000 events were counted in a gate set around the densest population of cells that was > 200 arbitrary units on the x-axis (to avoid inclusion of cellular debris) on a dot plot of forward versus side scatter. For analysis, dot plots of side scatter versus fluorescence were examined for positive events, which were defined as events with fluorescence greater than a threshold established with the isotype control for each cell line ($< 5\%$ of cells labeled with the isotype control fell within the gate established to identify positively labeled cells). The percentage and MFI of positively labeled cells identified in this manner were determined with commercial software^t and expressed as the mean $\pm$ SD of 3 independent experiments.

Preparation of plasma

Pooled canine plasma prepared from citrate-anticoagulated blood of healthy dogs (volunteered by their owners for the purpose of generating the pool),^u coagulation factor-replete human plasma,^v and human plasma deficient in specific coagulation factors of the extrinsic (FVII), intrinsic (FVIII, FIX, FXI, and FXII), or common (FX) pathways (obtained from individual patients with severe factor deficiencies via a commercial source)^v were used in the study. Commercially available human plasma was used because specific factor-deficient canine plasma and inhibitory antibodies against canine coagulation factors

were unavailable, and it has been shown that canine TF can efficiently bind to and act as a cofactor for canine and human FVII.³² Plasma was stored in single-use aliquots at -80°C and thawed to 37°C before use, and samples were depleted of microparticles before use in CAT by centrifugation at $13,523 \times g$ for 20 minutes, twice, at 4°C .

CAT

A CAT assay was used to measure thrombin generation in plasma over time.³³ The assay was performed with modifications as previously described.²⁵ Specifically, whereas human plasma is typically combined with an external clotting trigger (negatively charged phospholipids and recombinant human TF), no external trigger was added to test samples in the present study, and canine hemangiosarcoma cells were the source of these factors. Detached cells were washed free of culture medium with PBS and suspended at a concentration of 2×10^4 cells/20 μ L of HEPES-buffered saline solution (10mM HEPES,^c 137mM sodium chloride,^f 0.5% bovine serum albumin^d [wt/vol], 4mM potassium chloride,^f and 10 μ M glucose^f [pH, 7.05]) in a black 96-well plate.^w Next, 30 μ L of microparticle-depleted plasma (canine, factor-replete human, or specific factor-deficient human samples) was added, followed by 50 μ L (15mM) of calcium chloride-containing fluorogenic thrombin substrate.^x Fluorescence was then measured every 60 seconds at 360/460 nm for 2 hours at 37°C with a 96-well plate fluorescent spectrophotometer.^k A negative control microparticle-free plasma sample (canine and human plasma was used, depending on the experiment) containing no cells (only HEPES-buffered saline solution added) and a human microparticle-free plasma sample^y with a TF-containing trigger reagent that included a high concentration of phospholipid micelles^z (positive control) provided by the manufacturer were included in each run. All samples were run in duplicate, and the mean of results for each independent experiment was recorded. Raw fluorescence readings were converted to nanomoles of thrombin through use of the reagent manufacturer's software package^{aa} and human thrombin for calibration according to the manufacturer's instructions. Numerical results provided by the software included peak thrombin concentration, velocity of thrombin generation, and AUC of the thrombin concentration-versus-time curve (equivalent to the total amount of thrombin generated). The lag time, or time to initiation of thrombin generation, was measured manually in minutes, with time 0 designated as the time when the fluorescent thrombin substrate was added; lag time was defined as the time taken (in minutes) for the thrombin concentration to reach 4nM or greater (owing to a malfunction with the proprietary software that was discussed with the manufacturer, the automated lag time was inaccurate). In selected experiments, CTI^{bb} (a chemical inhibitor of FXIIa, used to assess whether activation of FXII through contact

with negatively charged surfaces such as plastic contributed to thrombin formation; 50 $\mu\text{g}/\text{mL}$) or human placenta-derived annexin V^f (a protein that binds and blocks exposed phospholipids, particularly phosphatidylserine) was added to plasma before addition of cells. For experiments with annexin V, the cells were diluted in annexin-binding buffer^{cc} (10mM HEPES, 150mM sodium chloride, 5mM potassium chloride, 1mM magnesium chloride, and 1.8mM calcium chloride); annexin V (0, 5, or 10 μM) was added to the cell suspensions, and the samples were incubated for 20 minutes at 20° to 23°C. Cells were then washed once with PBS and once with HEPES-buffered saline solution (to remove the calcium prior to measuring thrombin generation by CAT) before addition of plasma. In other experiments, various numbers of SB-HSA cells (0.5 $\times 10^4$ cells/reaction, 1 $\times 10^4$ cells/reaction, and 2 $\times 10^4$ cells/reaction) were tested by CAT to assess the influence of decreasing cell numbers and, accordingly, decreasing amounts of surface TF, on thrombin production. Numerical results were recorded as the mean $\pm$ SD of ≥ 3 independent experiments, and composite thrombin generation curves from all experiments were created.

Statistical analysis

Results of Shapiro-Wilk tests indicated that all numerical data had Gaussian distributions; these were expressed as mean $\pm$ SD. Comparisons of mean results for different treatments (or cell numbers) of the same cell line or for different cell lines that underwent same treatment were performed with paired and 2-tailed student *t* tests, respectively. Comparison of 3 or more means (eg, percentage of cells that tested positive for various factors by flow cytometry and MFI data) was completed by 2-way ANOVA followed by a Tukey multiple comparisons posttest. Comparison of means for test samples to control sample data was performed by 2-way ANOVA with a Dunnett posttest. Statistical analysis was performed with commercially available software.^{dd} Values of $P < 0.05$ were considered significant.

Results

Expression of TF mRNA and surface expression of TF protein by canine hemangiosarcoma cell lines

All cell lines (CnAoEC [control endothelial cells], Emma, Frog, Dal-4, and SB-HSA) expressed TF mRNA. Relative to CnAoEC (assigned a unitless value of 1), mean $\pm$ SD TF mRNA expression in hemangiosarcoma cells ranged from 11.5 $\pm$ 4.7 (Dal-4) to 45.6 $\pm$ 9.7 (SB-HSA; **Figure 1**). Mean TF mRNA expression was significantly ($P < 0.05$) higher in all hemangiosarcoma cell lines than in CnAoEC and significantly ($P < 0.005$ for all comparisons) higher in the SB-HSA and Emma cell lines than in the Frog and Dal-4 cell lines. A significantly ($P < 0.05$ for all comparisons) higher percentage of SB-HSA cells expressed TF antigen, compared with

Figure 1—Mean $\pm$ SD relative TF mRNA expression for the canine hemangiosarcoma cell lines SB-HSA, Emma, Frog, and Dal-4 in vitro. Expression is shown relative to that in non-neoplastic CnAoEC (assigned a value of 1) and normalized to expression of the ribosomal protein subunit 5 as a reference gene by use of the $2^{-\Delta\Delta C_t}$ method. *Value is significantly ($P < 0.005$) different from that for SB-HSA and Emma cells.

other hemangiosarcoma cell lines, and both SB-HSA and Emma cells had significantly higher MFI (reflecting TF antigen expression) than did other hemangiosarcoma cell lines (**Figure 2**; **Table 1**).

CAT evaluation of thrombin generation

All cell lines generated thrombin in canine plasma with all hemangiosarcoma cell lines, generating significantly ($P < 0.05$) more thrombin than CnAoEC (which had minimal thrombin generation; **Figure 3**). The SB-HSA cells had a significantly ($P < 0.01$) shorter lag time, but generated a similar total amount of thrombin (as measured by the AUC), compared with the other primary hemangiosarcoma cell lines in canine plasma. All canine cell lines generated more thrombin, with a longer lag time, in factor-replete human plasma than in canine plasma ($P < 0.001$ for all comparisons); similar to results for canine plasma, all hemangiosarcoma cell lines generated significantly ($P < 0.05$) more thrombin with a significantly ($P < 0.05$) shorter lag time, compared with results for CnAoEC. In contrast to results for canine plasma, there was no significant difference in lag time between SB-HSA cells and Emma cells in human plasma. Thrombin generation in canine and factor-replete human plasma was cell dependent; no thrombin was generated in the absence of cells (HEPES buffer alone in canine or human plasma; data not shown). The positive control human plasma sample that included a trigger reagent generated large amounts of thrombin in all experiments (data not shown).

Assessment of thrombin generation by canine hemangiosarcoma cells in specific factor-deficient human plasma

Thrombin generation induced by SB-HSA cells was abolished in FX-deficient plasma, whereas lag

Figure 2—Representative flow cytometry output (dot plots) used to assess surface expression of TF on CnAoEC (A) and canine hemangiosarcoma cell lines SB-HSA (B), Emma (C), Frog (D), and Dal-4 (E) in vitro. Cells were incubated with mouse anti-dog monoclonal IgG antibody or a mouse IgG isotype control. The TF-positive cells (R2 gate; gray dots) were quantified within a gate created on the basis of fluorescence units (intensity) versus side scatter (SSC) characteristics (not shown). Black dots (95% excluded from the R2 gate) represent cells labeled with the mouse IgG isotype control.

time and the total amount of thrombin generated (on the basis of the AUC) were largely unaffected for SB-HSA cells in plasma deficient in intrinsic pathway factors (FVIII, FIX, FXI, and FXII; **Figure 4**). However, the mean rate of thrombin generation (as determined by the slope of the thrombin generation curve) was significantly ($P < 0.001$) higher in FXII-deficient than in factor-replete plasma (**Table 2**). In contrast, SB-HSA cells incubated in (extrinsic pathway-specific) FVII-deficient plasma generated significantly ($P < 0.001$ for all comparisons) less thrombin at a slower rate and with a longer lag time than did those in factor-replete plasma. Changes in lag time and thrombin generation for other canine hemangiosarcoma cell lines and for CnAoEC in FVII-deficient plasma (**Supplemental Figure S1**, available at avmajournals.avma.org/doi/suppl/xxx) and in other intrinsic pathway factor-deficient plasma samples (data not shown) were similar to those for SB-HSA cells.

Table 1—Results of flow cytometric evaluation for TF expression on the surface of nonneoplastic (CnAoEC) and hemangiosarcoma (SB-HSA, Emma, Frog, and Dal-4) cells of canine origin in vitro as detected by use of a mouse monoclonal anti-canine TF antibody.

Cell line	TF-positive cells (%)	MFI of TF-positive cells (arbitrary units)
CnAoEC	0.9 ± 0.3	5.5 ± 0.2
SB-HSA	83.3 ± 0.5*	20.3 ± 4.4*
Emma	16.8 ± 7.9*†	20.6 ± 9.1*
Frog	6.8 ± 4.4†	7.4 ± 1.9††
Dal-4	13.9 ± 3.8*†	7.2 ± 1.8††

Data are mean ± SD.

*Value is significantly ($P < 0.05$) different from that for CnAoEC.

†Value is significantly ($P < 0.05$) different from that for SB-HSA cells.

‡Value is significantly ($P < 0.05$) different from that for Emma cells.

Addition of the FXIIa inhibitor CTI did not affect thrombin generation induced by SB-HSA cells in factor-replete or FVII-deficient plasma (**Figure 4**). This confirmed that contact activation of FXII did not contribute to thrombin generation in either plasma type.

Effects of cell number and phospholipid-expressing membranes on residual thrombin generation in FVII-deficient and factor-replete human plasma

To investigate whether reducing the amount of available TF would eliminate residual thrombin generation induced by SB-HSA cells in FVII-deficient plasma, CAT was performed with 2×10^4 SB-HSA cells/reaction, 1×10^4 SB-HSA cells/reaction, and 0.5×10^4 SB-HSA cells/reaction. The amount of thrombin generated was smaller (mean ± SD AUC, 165 ± 107 nM X minutes vs $1,735 \pm 1,052$ nM X minutes, respectively; $P = 0.001$), and lag time was longer (mean ± SD, 24 ± 4 minutes vs 14 ± 4 minutes, respectively; $P = 0.005$) for 0.5×10^4 cells than for 2×10^4 cells (**Figure 5**). Lag time was not significantly affected by cell numbers in factor-replete plasma, although the amount of thrombin generated was significantly ($P = 0.001$) less for 0.5×10^4 cells (mean ± SD AUC, $3,345 \pm 250$ nM X minutes) than for 2×10^4 cells ($4,444 \pm 396$ nM X minutes).

Incubation of 2×10^4 SB-HSA cells with 0, 5, and $10 \mu\text{M}$ annexin V (to assess the contribution of cell membrane-expressed phosphatidylserine to thrombin generation) had no significant influence on lag time in factor-replete plasma, although the amounts of thrombin generated with $5 \mu\text{M}$ and $10 \mu\text{M}$ annexin V (mean ± SD AUC, $1,714 \pm 673$ nM X minutes and $1,646 \pm 839$ nM X minutes, respectively) were significantly ($P < 0.001$ for both comparisons) less than that for the same number of cells without annexin V exposure ($3,289 \pm 766$ nM X minutes; **Figure 6**). In FVII-deficient plasma, addition of annexin V abolished residual thrombin generation in reactions that included 2×10^4 cells ($P < 0.001$ in 2 of 3 experiments, and lag time was accordingly increased ($P < 0.001$ at both annexin V concentrations); only small

Figure 3—Representative thrombograms (A and B), mean $\pm$ SD lag time (ie, time from addition of thrombin substrate [time 0] to initiation of thrombin production; C), and mean $\pm$ SD total thrombin production (as measured by AUC for thrombin concentration vs time; D) for CnAoEC, SB-HSA, Emma, Frog, or Dal-1 cells in microparticle-depleted canine or human plasma (2×10^4 cells/reaction). Data in panels A and B represent thrombin generation in canine and human plasma, respectively; notice that the scale of the y-axis differs between the panels. In panels C and D, reactions in canine and human plasma are represented by white bars and gray bars, respectively. *Value differs significantly ($P < 0.05$) from that for CnAoEC in the same type of plasma. †Value differs significantly ($P < 0.01$) from that of SB-HSA cells in the same type of plasma. ‡Value differs significantly ($P < 0.001$) from that of the same cell line in canine plasma.

Figure 4—Representative thrombograms (A and B), mean $\pm$ SD lag time (C), and mean $\pm$ SD total thrombin production (D) for SB-HSA cells (2×10^4 cells/reaction) in factor-replete human plasma (FRHP) or human plasma deficient (–) in specific coagulation factors, with or without addition of the FXIIa inhibitor CTI (50 μ g/mL). In panel A, notice the substantially lower thrombin generation curve for cells in FVII-deficient plasma, compared with that in factor-replete plasma, and lack of detectable thrombin generation in FX-deficient plasma. Conversely, the thrombograms were only slightly altered for the same cell line in FVIII-, FIX-, FXI-, or FXII-deficient plasma, indicating that thrombin generation was dependent on the extrinsic, but not the intrinsic, coagulation pathway. In panel B, addition of CTI had no detectable effect on the thrombograms for cells in factor-replete or FVII-deficient plasma. *Value is significantly ($P < 0.001$) different, compared with that for factor-replete plasma with or without CTI added. †Value differs significantly ($P < 0.05$) from those for other factor-deficient plasma types.

amounts of thrombin were generated in 1 of 3 experiments.

Discussion

Results of the present study showed that hemangiosarcoma cell lines derived from primary tumors in dogs constitutively produced TF mRNA and expressed TF antigen on their membrane surfaces. Tissue factor primarily functions as the initiator of coagulation in physiologic and pathological states.⁵ Hemangiosarcoma cell lines generated much more thrombin than nonneoplastic CnAoEC in canine plasma in vitro, as assessed by means of CAT. Thrombin generation in this assay was triggered by the extrinsic coagulation pathway and was dependent on the presence of FVII, cell number (which would influence the available amount of surface TF), and cell membrane-expressed phosphatidylserine. Although the cause of DIC is likely multifactorial in dogs with hemangiosarcoma, our data supported the hypothesis that aberrant procoagulant TF expression on hemangiosarcoma tumor cells is 1 mechanism involved in this paraneoplastic syndrome.

Expression of TF mRNA production did not correspond to the percentage of cells with TF surface antigen expression. Although the expression of TF mRNA (relative to that of CnAoEC) was similar between the SB-HSA and Emma cell lines, a significantly greater percentage of SB-HSA cells expressed surface TF antigen. The discrepancy between TF mRNA and protein expression in these cell lines could be explained by differences in intracellular stores, rates of recycling, and differential translation. The factors controlling translation and surface expression of TF are poorly understood, but most of the translated protein is stored intracellularly in the Golgi apparatus.³⁴ These stores could be mobilized in response to inflammatory cytokines or following surface TF-FVIIa complex activation of protease-activated receptors 1 and 2, as described for human fibroblasts.³⁵ Further studies are needed to characterize the intracellular pools and protein dynamics of TF in CnAoEC and canine hemangiosarcoma cell lines. The discrepancy between mRNA expression and surface protein expression could also be attributable to changes in transcriptional reg-

ulation. Several alternatively spliced TF mRNAs have been found in normal and malignant human cells, with greater amounts in tumor cells.³⁶ The function of these untranslated alternatively spliced TF mRNAs is still unknown, although current hypotheses favor a regulatory function. Only one of these alternatively spliced mRNAs is known to have a translation product, a truncated TF protein called alternatively spliced TF,³⁷ and its function, including its ability to trigger thrombin generation, is still under debate. Considering that surface TF expression determines the procoagulant potential of cells to some extent, understanding and controlling the factors that dictate expression of the protein may be important for reducing the incidence of DIC in patients with hemangiosarcoma.

Several differences in TF protein expression were observed among the tumor cell lines in the present study. The SB-HSA line had the highest proportion of cells (mean, 83%) with surface TF expression. This cell line was generated through xenografting and serial passage of resultant hemangiosarcoma tumors in immune-deficient mice. When the cell line was established, it was found to express markers of mitotically activated endothelial cells, including vascular endothelial growth factor receptors 1 and 2 and $\alpha_v\beta_3$ integrin, and to produce endothelial growth factors and cytokines such as vascular endothelial growth factor, basic fibroblast growth factor, and interleukin-8.²⁷ Enhanced TF expression in these cells could be an artifact of xenografting or activation; however, we also found that primary hemangiosarcoma cell lines that were not mouse-passaged also expressed TF, albeit in fewer cells, suggesting that TF expression is a feature of these cells and not an artifact of xenografting. Differences in TF mRNA and antigen expression were also identified among the primary non-mouse-passaged hemangiosarcoma cell lines. Emma cells, derived from a metastatic hemangiosarcoma in the brain of a dog, had the highest TF mRNA and MFI (indicative of antigen expression) of the primary cell lines. It is possible that these differences could be related to metastatic and procoagulant potential, with higher TF expression in more aggressive tumors. In a study²⁶ assessing TF expression in canine mammary tumors, metastatic tumor cells in a lymph node from a dog were classified among those with the highest percentage of TF-positive cells (as determined by immunohistochemistry), and the same dog had labora-

tory evidence of DIC. Similarly, a greater amount of TF protein was found in canine gliomas of higher histologic grades than in those of lower grades.²⁵ Tissue factor expression is often upregulated in metastatic tumors, compared with nonmetastatic tumors, in human patients^{38,39} and is thought to be associated with metastatic potential. This could also be true for canine hemangiosarcoma, but further studies, which particularly examine TF expression in primary and metastatic hemangiosarcoma in situ, are needed.

Figure 5—Representative thrombograms (A), mean $\pm$ SD lag time (B), and mean $\pm$ SD total thrombin production (C) for 2.0×10^4 SB-HSA cells, 1.0×10^4 SB-HSA cells, or 0.5×10^4 SB-HSA cells in factor-replete or FVII-deficient human plasma. In panel A, notice the lower peaks with decreasing cell number (denoted in abbreviated form above each curve) in both plasma types. In panels B and C, cells in factor-replete plasma and FVII-deficient plasma are represented by white and gray bars, respectively. *Within a plasma type, value is significantly ($P \leq 0.005$) different from that for 2×10^4 cells. †Value is significantly ($P < 0.001$) different from that for the same number of cells in factor-replete plasma. See Figure 4 for remainder of key.

Table 2—Mean $\pm$ SD rate of thrombin generation by SB-HSA cells in factor-replete and specific factor-deficient human plasma, as represented by the slope of the thrombography curve.

Plasma type	Slope (min/nM thrombin)
Factor-replete	45.4 $\pm$ 12.5
Factor VII-deficient	13.1 $\pm$ 4.8*
Factor VIII-deficient	30.2 $\pm$ 7.4
Factor IX-deficient	29.6 $\pm$ 5.8
Factor XI-deficient	62.9 $\pm$ 19.6
Factor XII-deficient	83.9 $\pm$ 18.2*

*Value is significantly ($P < 0.001$) different from that for factor-replete plasma.

Figure 6—Representative thrombograms (A), mean $\pm$ SD lag time (B), and mean $\pm$ SD total thrombin production (C) for 2.0×10^4 SB-HSA cells in factor-replete or FVII-deficient human plasma following incubation with the phosphatidylserine blocker annexin V added to final concentrations of 5 μ M or 10 μ M, or with no annexin V treatment (0 μ M). In panel A, peaks of the thrombin generation curves were substantially lower in both plasma types after addition of annexin V at either concentration. In panels B and C, cells in factor-replete plasma and FVII-deficient plasma are represented by white and gray bars, respectively. *Within a plasma type, value is significantly ($P < 0.001$) different from that for cells without annexin V. †Value is significantly ($P < 0.001$) different from that for cells in factor-replete plasma that received the same annexin V treatment. See Figure 4 for remainder of key.

Fairly small populations ($< 20\%$) of Emma, Frog, and Dal-4 cells expressed surface TF protein in the present study; however, all of the hemangiosarcoma

cell lines generated thrombin in canine plasma. Similarly, only a subset of canine mammary tumors express TF in situ.²⁶ Initiation of coagulation only occurs with surface-expressed TF,⁵ and it is therefore likely that these few cells were responsible for the observed procoagulant activity in the primary cell lines. The variability of TF expression within a tumor cell line is expected and is thought to be a consequence of tumor heterogeneity.⁴⁰ Functional variations arise in cancer cells as a result of different sets of acquired mutations, microenvironmental cues, and other factors. One model used to explain cancer cell heterogeneity is the cancer stem cell model. In this model, a hierarchy of subpopulations arise from stem cell-like tumor cells that are capable of dividing and promoting tumor progression.⁴¹ Tissue factor is currently under investigation for its role in the formation of the cancer stem cell niche⁴² and may be a marker of cancer stem cells, as has been shown in a human carcinoma cell line,⁴³ but further investigation is needed.

To test the roles of the different pathways in thrombin generation by canine hemangiosarcoma cells, we used human plasma deficient in specific coagulation factors. Human FVII can form a functional extrinsic tenase with canine TF, with canine and human TF working equally well to initiate coagulation in human plasma.³² In the present study, canine cells triggered thrombin generation in canine and human plasma; however, there were notable differences. There was a longer lag time and larger amounts of thrombin were generated in factor-replete human versus canine plasma. Results of a cross-species study³⁰ revealed that canine FVIIa is a more active serine protease than human FVIIa. This could account for the shorter lag time for thrombin generation by hemangiosarcoma cells in canine plasma, which is primarily dependent on the trigger for thrombin generation, in this case the TF-FVIIa complex or extrinsic tenase. The greater amount of total thrombin generated in human plasma by the canine cells was likely attributable to differences in coagulation factor complex assembly, amplification, and inhibition. The thrombography curves showed that AUCs were greater in human plasma because of higher peaks and slower rates of termination of thrombin generation, resulting in broader curves than were generated for canine cells in canine plasma. This suggested that inactivation of coagulation was less efficient in human than in canine plasma. Alternatively, slower enzyme kinetics could have been associated with conformational or structural differences in assembly of canine versus human enzymatic coagulation complexes on canine cell membranes. Human coagulation factor inhibitors, including TF pathway inhibitor and antithrombin, may be less efficient at inhibiting enzymatic complexes bound to canine cells. To our knowledge, no studies to date have examined cross-species enzymatic activity of natural anticoagulants. We also observed that CnAoEC generated substantial amounts of thrombin in factor-replete human, but not canine, plasma. Normal endothelial cells are not expected

to express TF (which was supported by our flow cytometry results for surface TF antigen expression in CnAoEC) unless stimulated by bacterial endotoxin or proinflammatory cytokines.⁴⁴ Because the thrombin generation by CnAoEC in human plasma was FVII-dependent (thrombin generation was significantly reduced in FVII-deficient plasma), we attributed this to stimulation of TF expression by the heterologous plasma. Regardless of the underlying mechanisms, the enhancement of thrombin generation by canine cells (whether or not they were neoplastic) in human plasma did not alter interpretation of the results of our study. This phenomenon was also not unique to these cell lines because we previously found similar results with other canine tumor cells in human plasma.²⁵

We used FVII depletion as a surrogate means to assess the role of surface TF in thrombin generation by hemangiosarcoma cells. Ideally, this test would have been performed directly by inhibition of canine TF with specific antibodies.⁴⁵ We previously tested several inhibitory anti-human TF antibodies including a monoclonal murine antibody (anti-human TF1) and a murine anti-canine TF monoclonal antibody generated in our own laboratory,³¹ but none inhibited canine TF procoagulant activity in the CAT assay. The activity of FVIIa is enhanced 5,000-fold by binding of TF and membrane surfaces, which results in an allosteric change in FVIIa structure.⁴⁶ At supraphysiologic concentrations of FVIIa, such as those achieved by pharmacological administration of the recombinant protein, FVIIa can activate FX and initiate thrombin generation without TF. This is unlikely to occur at the concentrations found in normal canine or human plasma and thus was not believed to have affected results of the present study. In addition to TF, FVII can bind to endothelial protein C receptor on human endothelial cells. However, this interaction does not initiate coagulation and only occurs at supraphysiologic conditions of FVIIa.⁴⁷ Also, it is not known whether FVIIa binds to the endothelial protein C receptor in dogs.

Considering that thrombin generation was dependent on FVII-mediated activation of FX, we were surprised that thrombin production induced by SB-HSA cells was not abolished in FVII-deficient plasma. However, the commercial plasma is not totally lacking FVII but contains < 1% of FVII activity as determined by 1-stage clotting assay.⁴⁸ Also, as previously mentioned, very small amounts of TF are capable of triggering coagulation in normal plasma.⁴⁹ We considered it possible that the strong surface expression of TF by SB-HSA cells was sufficient to bind to and form a complex with the residual FVII present, and indeed, results of experiments evaluating the effect of reduced numbers of SB-HSA cells (as well as data for hemangiosarcoma cell lines that had weaker expression of TF) on thrombin generation in FVII-deficient plasma in this study supported the possibility that thrombin production was lower when less surface TF was present.

Preincubation of SB-HSA cells with annexin V diminished thrombin generation in factor-replete human plasma and nearly abolished its production in FVII-deficient human plasma. These data indicated that cell membrane-expressed phosphatidylserine also contributed to thrombin generation by these cells. The hemangiosarcoma cells were considered the most likely source of phosphatidylserine, and we have previously shown that other neoplastic cells (canine mammary tumor and bone cancer cell lines) express this product.²⁵ However, the canine and human plasma samples used in this study were depleted but not free of microparticles, and phosphatidylserine on these endogenous microparticles might have contributed to the observed thrombin generation. In factor-replete plasma, annexin V had a larger inhibitory effect on amplification than initiation of thrombin generation (as evidenced by the lack of a significant change on lag time) by SB-HSA cells. This has been previously reported with other cell types, particularly those with high TF expression (cancer cells and lung fibroblasts), which provides a strong trigger for coagulation.⁵⁰ Our group previously showed that annexin V treatment can increase the lag time for initiation of thrombin generation and decrease the amount of thrombin produced by canine mammary tumor cells.²⁵ It is possible that the lack of a significant effect of annexin V on lag time for SB-HSA generation of thrombin in factor-replete human plasma in the present study was attributable to detachment of some annexin V during the washing steps (because binding to phosphatidylserine is reversible in the absence of calcium). Preincubation with higher concentrations of annexin-V than were used in the present study and inclusion of annexin V throughout the thrombin generation assay (ie, adding the protein to the plasma) would be expected to inhibit all stages of thrombin generation in this model, but this was not investigated in the present study.

In accordance with the observation that the TF-FVIIa complex was likely acting as a trigger for thrombin production by canine hemangiosarcoma cells, we expected to find greater thrombin production by the Emma (metastatic brain hemangiosarcoma) cell line, corresponding to its greater TF protein expression (as assessed by MFI with flow cytometry), compared with results for the primary splenic hemangiosarcoma cell lines Frog and Dal-4. Instead, the 3 primary hemangiosarcoma cell lines had similar thrombin generation profiles and generated analogous amounts of thrombin with comparable lag times in canine or factor-replete human plasma. It has been demonstrated that TF exists in 2 states: an inactive or encrypted state and an active or decrypted state *in vitro*.⁵¹ Encryption is thought to provide an additional level of control to prevent excessive coagulation.⁵² The mechanism of decryption is not known, but it could involve TF dimerization, cleavage of a disulfide bond on TF, or phospholipid characteristics (particularly the proportion

of phosphatidylserine to other phospholipids) of the cell membrane.⁶ Encrypted TF was shown to induce intracellular signaling without initiating coagulation.⁵³ It is possible that TF expressed on Emma cells was more frequently encrypted than that on the Frog and Dal-4 cells, resulting in thrombin generation characteristics similar across all 3 cell lines. Alternatively, tumor-specific alterations in TF conformation, the ability of TF to bind to FVII and its binding affinity, or the degree of cell surface expression of phosphatidylserine could have contributed to the observed differences in procoagulant activity.

Our results supported that hemangiosarcoma cells express high amounts of TF, compared with nonneoplastic CnAoEC, and suggested that aberrant expression of TF on canine neoplastic endothelial cells likely contributes to DIC, a devastating complication in dogs with hemangiosarcoma. Further research should investigate whether TF is similarly expressed in high amounts on tumor cells in vivo and whether expression of this factor on cancer cells or their released microparticles in plasma could serve as a biomarker for canine patients at risk of developing DIC and thus be useful for earlier diagnosis and anticoagulant treatment for such patients.

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Footnotes

- a. Provided by Dr. Erin Dickerson, Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, Minn, under a materials transfer agreement with Dr. Stuart Helfand, Department of Clinical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Ore.
- b. Gift of Dr. Jaime Modiano, Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, Minn.
- c. Gibco, Life Technologies, Grand Island, NY.
- d. Atlanta Biologicals, Flowery Branch, Ga.
- e. BD Biosciences, San Jose, Calif.
- f. Sigma-Aldrich, St Louis, Mo.
- g. Invitrogen, Carlsbad, Calif.
- h. Primocin, InvivoGen, San Diego, Calif.
- i. Cell Applications, San Diego, Calif.
- j. Qiagen RNeasy, Valencia, Calif.
- k. Spectramax M3, Molecular Devices, Sunnyvale, Calif.
- l. Superscript III RT system, Life Technologies, Grand Island, NY.
- m. SYBR Green Master Mix, Applied Biosystems, Austin, Tex.
- n. Integrated DNA Technologies, Coralville, Iowa.
- o. PTC 200 PCR Thermal Cycler, MJ Research, Ramsey, Minn.
- p. StepOne Software, version 2.3, Life Technologies, Grand Island, NY.
- q. Life Technologies, Eugene, Ore.

- r. Alexafluor -488 secondary antibody, Life Technologies, Eugene, Ore.
- s. FACS caliber, BD Biosciences, San Jose, Calif.
- t. FlowJo, version 10, Tree Star Inc, Ashland, Ore.
- u. Gift of Dr. Marjory Brooks, Comparative Coagulation Laboratory, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY.
- v. George King Biomedical Inc, Overland Park, Ks.
- w. Nunc Thermo Fisher Scientific, Waltham, Mass.
- x. 1 mM Z-Gly-Gly-Arg-AMC (7-amino-4-methylcoumarin hydrochloride), Technoclone GmbH, Vienna, Austria.
- y. TGA control 2, Technoclone GmbH, Vienna, Austria.
- z. RC High, Technoclone GmbH, Vienna, Austria.
- aa. Technothrombin TGA Excel Software, Technoclone GmbH, Vienna, Austria.
- bb. Haematologic Technologies, Essex Junction, Vt.
- cc. Trevigen, Gaithersburg, Md.
- dd. Prism, version 5.0, GraphPad Software Inc, La Jolla, Calif.

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