Tissue factor, also known as tissue thromboplastin or coagulation factor III, is best known as the main activator of the coagulation cascade. Tissue factor is a small transmembrane protein that is normally expressed in perivascular fibroblasts and smooth muscle cells. Extravascular tissue factor is exposed to its circulating plasma ligand, factor VII, when endothelial cells are injured. The resultant autolytic tissue factor–factor VII complex binds to and activates factor X in the extrinsic pathway of coagulation. This sets in motion additional enzymatic events that culminate in the generation of thrombin and fibrin clot formation. Expression of tissue factor was first identified in solid cancers by use of immunohistochemical techniques in 1992. Since that report, extensive studies have been performed on tissue factor in human cancers. Many cancer types, including epithelial and mesenchymal tumors, express high amounts of tissue factor, even if the normal tissue counterparts do not. Indeed, tissue factor is considered a marker of oncogenic transformation because expression coincides with loss of tumor suppressor genes or activation of oncogenes. Also, high tissue factor expression has been associated with increased mortality rates in patients with cancer. This is thought to be caused by the procoagulant effects of tissue factor, which result in paraneoplastic thrombosis.

Thrombosis frequently precedes or accompanies various forms of cancer in humans. Although there are several mechanisms by which thrombi can form in association with cancer, tissue factor is now accepted as one of the main causes. Cancer cells are thought to initiate thrombosis through their constitutive high tissue factor expression. Tissue factor expression was first identified in solid cancers by use of immunohistochemical techniques in 1992. Since that report, extensive studies have been performed on tissue factor in human cancers. Many cancer types, including epithelial and mesenchymal tumors, express high amounts of tissue factor, even if the normal tissue counterparts do not. Indeed, tissue factor is considered a marker of oncogenic transformation because expression coincides with loss of tumor suppressor genes or activation of oncogenes. Also, high tissue factor expression has been associated with increased mortality rates in patients with cancer. This is thought to be caused by the procoagulant effects of tissue factor, which result in paraneoplastic thrombosis.

Objective—To determine whether canine tumor cell lines express functional tissue factor and shed tissue factor–containing microparticles.

Sample—Cell lines derived from tumors of the canine mammary gland (CMT12 and CMT25), pancreas (P404), lung (BACA), prostate gland (Ace-1), bone (HMPOS, D-17, and OS2.4), and soft tissue (A72); from normal canine renal epithelium (MDCK); and from a malignant human mammary tumor (MDA-MB-231).

Procedures—Tissue factor mRNA and antigen expression were evaluated in cells by use of canine-specific primers in a reverse transcriptase PCR assay and a rabbit polyclonal anti-human tissue factor antibody in flow cytometric and immunofluorescent microscopic assays, respectively. Tissue factor procoagulant activity on cell surfaces, in whole cell lysates, and in microparticle pellets was measured by use of an activated factor X–dependent chromogenic assay.

Results—Canine tissue factor mRNA was identified in all canine tumor cells. All canine tumor cells expressed intracellular tissue factor; however, the HMPOS and D-17 osteosarcoma cells lacked surface tissue factor expression and activity. The highest tissue factor expression and activity were observed in canine mammary tumor cells and pulmonary carcinoma cells (BACA). These 3 tumors also shed tissue factor–bearing microparticles into tissue culture supernatants.

Conclusions and Clinical Relevance—Tissue factor was constitutively highly expressed in canine tumor cell lines, particularly those derived from epithelial tumors. Because tumor-associated tissue factor can promote tumor growth and metastasis in human patients, high tissue factor expression could affect the in vivo biological behavior of these tumors in dogs. (Am J Vet Res 2011;72:1097–1106)
suicidal factor expression (usually in a functionally active form) and release of procoagulant microparticles from their cell membranes. Microvesicles are small (<1-µm) membrane-derived vesicles that are enriched in procoagulant factors, including negatively charged phospholipids (e.g., phosphatidylserine) and tissue factor. Tissue factor–bearing microparticles are detected in human patients with carcinomas, particularly those with advanced or aggressive disease, and are associated with a prior history of thrombosis. Furthermore, microparticle-associated tissue factor activity may predict future thrombotic events and may be a prognostic indicator in such patients. Tissue factor is also known to affect cancer independently of its ability to induce thrombosis. Tissue factor can initiate signaling pathways within cells that promote tumor growth, angiogenesis, survival, and metastasis.

Presently, expression of tissue factor in canine tumors is unknown. This can largely be attributed to the lack of suitable reagents to detect tissue factor antigen or activity on canine cells. With the advent of the canine genome project, creation of canine tumor–derived cell lines, generation of polyclonal anti-human tissue factor antibodies, and development of chromogenic assays to measure TFPCA on cell surfaces, there are now suitable tools by which to assess the role of tissue factor in dogs with tumors. We hypothesized that tissue factor would be highly expressed and functionally active in tumor cell lines derived from various malignant canine epithelial and mesenchymal tumors. The objective of the study reported here was to determine whether specific canine tumor cell lines express tissue factor mRNA and protein, whether tissue factor is functionally active in these cells as detected by use of a 2-stage chromogenic assay for TFPCA on cell surfaces, and whether high tissue factor–expressing canine tumor cells shed tissue factor–bearing microparticles.

Materials and Methods

Cells—The canine tumor cell lines were of epithelial and mesenchymal origin and represented the following tumors: mammary gland tumors (CMT12 and CMT25), pancreatic carcinoma (P404), pulmonary adenocarcinoma (BACA), prostatic carcinoma (Ace-1), osteosarcoma (HMPOS, D-17, and OS2.4), and fibrosarcoma (A72). Most of the cell lines, with the exception of P404 and BACA, have been used previously. The P404 and BACA cell lines were established from histologically confirmed primary tumors in dogs and are positive for cytokeratin via immunostaining, confirming the derived lines are of epithelial origin. The present study also evaluated a renal epithelial cell line that represented normal canine epithelium (MDCK) as a canine-derived positive control. A human mammary carcinoma cell line (MDA-MB-231) that highly expresses tissue factor has been used as a positive control. The cells were cultured at 37°C in a humidified chamber with 5% carbon dioxide. They were maintained in specific growth media (Dulbecco modified Eagle medium for MDA-MB-231, MDCK, and A72; RPMI 1640 for osteosarcoma, P404, BACA, and Ace-1; and L-15 for the canine mammary tumor cells), supplemented with 10% fetal bovine serum (20% for BACA), sodium pyruvate (1mM), L-glutamine (2mM), penicillin (100 U), and streptomycin (100 µg). When required, cells were detached with 0.25% trypsin–EDTA, and trypsin was neutralized with growth medium. All experiments were performed on cells that had been passaged <30 times.

mRNA analysis for tissue factor expression in canine cells—Total RNA was isolated from harvested cells, and cDNA was reverse transcribed by use of oligo(dT) primers. Gene-specific sequences were amplified from cDNA by use of 35 cycles with the following temperatures: 94°C for denaturing, 47°C for annealing, and 72°C for extension. The gene-specific primer sequences were designed on the canine cDNA sequence in GenBank and spanned an intron: GCCAGGAGAAGGTAGTG (forward primer) and GGTGAGACGACCATATCTTG (reverse primer). The PCR product (expected product size, 214 bp) was separated on a 0.9% agarose gel, stained with ethidium bromide, and examined under UV light.

Flow cytometry—Detached cells (5 x 10⁵ cells/reaction) or yeast (2 x 10⁶ yeast/reaction) in PBS solution with 1% BSA and 0.05% azide were labeled with the polyclonal rabbit anti-human tissue factor antibody or rabbit IgG (both at 20 µg/mL) for 15 minutes on ice, followed by a secondary Alexa488-conjugated goat anti-rabbit IgG (1:200) for 15 minutes on ice. Induced and uninduced (as a negative control) yeast were similarly labeled with the rabbit anti-human tissue factor antibody as well as a murine monoclonal anti-myc antibody (1:50; clone 9E 10) or murine IgG, followed by an Alexa488-conjugated goat anti-mouse antibody (1:200). After washing, cells or yeast were suspended in PBS and analyzed with a flow cytometer. For data acquisition on cells, forward scatter was set at a voltage of E-1, amperage gain of ±7.4, and linear mode. Side scatter was set at a voltage of 350 V, amperage gain of 1.00, and linear mode. For yeast, forward and side scatter were on log mode. For cells and yeast, fluorescence was set on log mode and 10,000 events were counted. For analysis, histogram plots were examined for positive cells (those with fluorescence ≥10² arbitrary units [i.e., the IgG negative controls]), and the percentage and MFI of positive cells were determined.

Cloning of canine tissue factor—To provide a positive control for the rabbit polyclonal antibody used to detect tissue factor protein in this study, the ectodomain of canine tissue factor was cloned from MDCK cells. Only the ectodomain was cloned because most tissue factor protein (with accessible antigenic sites) is extracellular. Messenger ribonucleic acid was purified from total RNA isolated from MDCK cells by use of commercial kits. Canine tissue factor cDNA was reverse transcribed and amplified from the mRNA by use of gene-specific primers. The gene-specific primers were designed from the canine tissue factor gene sequence in GenBank (AB200288.1) and spanned the ectodomain of the transcribed protein. These primer sequences were as follows: CCTCAGGTCAGCAGATGT (forward primer) and ACCACACGGCCACAAATCTCCAATG (reverse primer). The 685-bp product was cloned into
a bacterial vector1 and sequenced for correctness. The insert was then subcloned into an inducible yeast expression vector (pCTCON), which expresses the protein under a galactose promoter with a myc-tag fused at the C-terminal. Expression of canine tissue factor on the surface of yeast cells (Saccharomyces cerevisiae) was induced with medium containing 2% galactose for 24 hours at 30°C with shaking.30

**Immunofluorescent microscopy**—For immunofluorescent detection of tissue factor, tumor cells (0.7 × 10^5 to 5 × 10^5) cultured on fibronectin (10 µg/mL)—coated glass coverslips were fixed in 2% paraformaldehyde, then labeled with a rabbit polyclonal anti—tissue factor antibody (20 µg/mL) or rabbit IgG (20 µg/mL; as a negative control), followed by a secondary Alexa488-conjugated donkey anti-rabbit antibody (1:200). For detection of tissue factor on cell surfaces, cells were incubated with the primary antibody without permeabilization. For detection of intracellular tissue factor, cells were permeabilized with 0.1% Triton X-100 in PBS solution with 1% BSA and 0.05% azide before application of the primary antibody. The primary antibody (diluted in PBS solution with 1% BSA and 0.05% azide) was added for 40 minutes at 4°C. The cells were washed, and the detection antibody (diluted in PBS solution with 1% BSA and 0.05% azide) was applied at room temperature (24°C) for 40 minutes. The cells were washed and the coverslips were mounted on glass slides with mounting medium containing a DNA-binding dye (4',6-diamidino-2-phenylindole).6

**Surface TFPCA**—In this 2-stage chromogenic assay, the tissue factor—activated factor VII complex on cell surfaces activated exogenous factor X. The generation of activated factor X was measured spectrophotometrically by use of an activated factor X—dependent chromogenic substrate.4 To perform the assay, cells (3 × 10^4/well) were plated onto 96-well plates in culture medium. After 24 hours, adherent cells were washed twice with HEPES buffer (10mM HEPES, 137mM sodium chloride, 5mM calcium chloride, 4mM potassium chloride, 10mM glucose, and 0.5% BSA; pH, 7.4). Activated recombinant human activated factor VII (1nM) diluted in HEPES buffer was added for 10 minutes at 37°C, followed by a 15-minute incubation with recombinant human factor X (75nM) at 37°C. The chromogenic substrate (final concentration, 250µM) was added to the reaction mixture, and the ensuing color change (via OD) was measured kinetically every 30 seconds for 10 minutes at 405 nm by use of an ELISA plate reader.6 For comparison among cell lines, surface TFPCA was expressed as the change in OD per minute (equating to the slope of the OD vs time curve) by use of the first 3 data points. Activity was also quantified as the amount of activated factor X (µg/mL) generated per minute by comparison of the rate of change in OD over time for each cell line to that of a standard curve generated from serially diluted human recombinant activated factor X (starting concentration, 0.5 µg/mL). The standard curve was linear between 0.008 and 0.025 µg/mL (equating to a change in OD from 5 ± 1 U/min to 108 ± 24 U/min, respectively). Only the first 3 data points were included in the calculation for all samples because...
negative controls) was tested in duplicate and mean results were calculated for each experiment. Data are represented as mean ± SD values. Comparison among multiple means was performed with a 1-way ANOVA followed by a Tukey multiple comparison test, as indicated. The mean surface and whole cell TFPCas of activated factor VII–negative versus other negative controls combined (no factor X, no chromogenic substrate, and no components) were compared for each cell line by use of a paired t test. Means of different samples (TFPCA of human mammary carcinoma cells vs A72 canine fibrosarcoma cells) were compared by use of an unpaired t test. A P value of < 0.05 was considered significant.

Results

All evaluated canine tumor cells and the normal canine renal epithelial cells (MDCK) expressed mRNA for tissue factor. Samples of genomic DNA, water, and RNA controls yielded negative results for the predicted PCR product (Figure 1).

Flow cytometric analysis of yeast induced to express myc-tagged canine tissue factor—Analysis revealed that the myc-tag product was expressed in induced but not in uninduced yeast (Figure 2) and that use of the rabbit polyclonal antibody detected the expressed canine tissue factor in a similar percentage of yeast. Similarly, use of the rabbit polyclonal antibody also detected a protein on the surface of MDCK cells and human mammary carcinoma cells (MDA-MB-231). In contrast, canine tissue factor was variably expressed on the surface of the canine tumor cells, with mammary tumor cells (CMT12 and CMT25) and bronchoalveolar carcinoma cells (BACA) having the highest protein expression with little expression in the 2 osteosarcoma cell lines, D-17 and HMPOS (Table 1).

Immunofluorescent microscopy was performed on fixed cells to determine the cellular location of tissue factor in canine tumor cells. Positive results of staining for tissue factor were observed on membrane surfaces of unpermeabilized cells. The intensity of membrane staining matched the degree of expression detected via flow cytometry in that the canine mammary tumor cells (CMT12 and CMT25) had strong tissue factor staining, whereas the osteosarcoma cells (D-17 and HMPOS) did not stain for tissue factor on their membranes. The human mammary carcinoma cells strongly stained for membrane surface tissue factor, as expected (Figure 3). In permeabilized canine (CMT25) and human mammary carcinoma cells, high concentrations of tissue factor were located in a perinuclear area corresponding to the Golgi body, as described for fibroblasts, with some cell membrane staining. In contrast, little intracellular tissue factor was observed in HMPOS or D-17 osteosarcoma cells, indicating these cells expressed little tissue factor protein intracellularly or on their membrane surfaces (Figure 4).

Surface-expressed tissue factor, as measured by use of the TFPCA assay, on the canine tumor cells was

![Figure 2](image-url)

**Table 1**—Results (mean ± SD of the percentage of positive cells and MFI of positive cells) of flow cytometric analysis of tissue factor antigen expression on the surface of canine tumor cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Percentage (arbitrary units)</th>
<th>MFI (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreatic carcinoma (P404)</td>
<td>96 ± 3</td>
<td>68 ± 9a</td>
</tr>
<tr>
<td>Prostatic carcinoma (Ace-1)</td>
<td>83 ± 5</td>
<td>37 ± 11f</td>
</tr>
<tr>
<td>Bronchial carcinoma (BACA)</td>
<td>95 ± 4</td>
<td>105 ± 26a</td>
</tr>
<tr>
<td>Mammary gland carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMT12</td>
<td>92 ± 6</td>
<td>242 ± 199a</td>
</tr>
<tr>
<td>CMT25</td>
<td>97 ± 3</td>
<td>255 ± 96a</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMPOS</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>D-17</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>OS2.4</td>
<td>95 ± 2</td>
<td>48 ± 13a</td>
</tr>
<tr>
<td>Fibrosarcoma (A72)</td>
<td>96 ± 1</td>
<td>67 ± 24a</td>
</tr>
<tr>
<td>Canine renal epithelium (MDCK)</td>
<td>96 ± 2</td>
<td>255 ± 93</td>
</tr>
<tr>
<td>Human mammary carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MDA-MB-231)</td>
<td>98 ± 1</td>
<td>348 ± 55</td>
</tr>
</tbody>
</table>

ND = Not determined.

**Among canine tissue factor–expressing tumor cell lines, mean MFI values with different superscript letters are significantly (P < 0.05) different.**
functionally active, with amounts of TFPCA matching surface expression as detected by use of flow cytometry (ie, the high tissue factor–expressing canine mammary tumor cells had the highest surface TFPCA). For the high tissue factor–expressing cells (canine mammary tumors, pancreatic and bronchoalveolar carcinoma, and fibrosarcoma), the amount of tissue factor was consistently higher than the upper limit of the standard curve of diluted human recombinant activated factor X (> 0.25 µg of activated factor X/mL/min). In contrast, no procoagulant activity (< 0.008 µg of activated factor X/mL/min) was observed with both canine osteosarcoma cells (D-17 and HMPOS), which lacked surface tissue factor expression. Tissue factor was also constitutively active in high concentrations (> 0.25 µg of activated factor X/mL/min) on the surfaces of normal canine renal epithelial cells and the human mammary carcinoma cells (Figure 5). The TFPCA was exclusively dependent on the generation of activated factor X because the various negative controls lacking factor X (cells without factor X, cells with substrate only, or cells with no added assay component) did not cleave the substrate to a greater degree than background values in any cell line. However, small amounts of activated
factor X (representing 2.0% to 4.7% of total activity) were generated in the absence of exogenous activated factor VII (significantly greater than the other negative controls) in the highest-expressing canine epithelial tumor cells (CMT12, CMT25, and BACA). This activated factor VII–negative procoagulant activity was not observed in the other canine tumor cells or the positive controls (normal canine renal epithelial cells or human mammary carcinoma cells).

Tissue factor procoagulant activity was identified in whole cell lysates (standardized to 10 µg of protein) from all cell lines, with the exception of canine HMPOS osteosarcoma cells (the change in OD/min/10 µg of protein was not significantly higher than that of negative controls; Figure 3). However, TFPCA was observed in HMPOS whole cell lysates when > 10-fold protein concentrations (> 100 µg) were tested, indicating that this tumor cell line expressed small amounts of tissue factor intracellularly. The TFPCA was high in the canine mammary and bronchoalveolar tumor cells and the human mammary carcinoma cells, reaching a plateau within the first minute after substrate addition. This
prevented accurate quantification of the rate of substrate cleavage with time, so the protein concentration of those whole cell lysates was decreased to 2.5 µg. At this protein concentration, the rate of substrate cleavage was consistently linear; this rate was multiplied by a factor of 4 to achieve the final result of change in OD/min/10 µg of protein. Activated factor VII–negative controls of the whole cell lysates of both canine mammary tumor cells had significantly more procoagulant activity than the factor X–negative or other negative controls. This activated factor VII–independent activated factor X generation in whole cell lysates represented little of the total procoagulant activity (0.5% to 1.6%) in these whole cell lysates.

The TFPCA was measured in centrifuged microparticle preparations from the highest tissue factor–expressing canine epithelial (CMT12, CMT25, and BACA) and mesenchymal (A72) tumor cells as well as the human mammary carcinoma cells. The TFPCA activity was detectable in microparticle pellets in the 3 evaluated canine epithelial tumor lines. The TFPCA in the microparticle pellets rapidly reached a plateau in both canine mammary tumor cells (CMT12 and CMT25), and these samples had to be diluted to obtain accurate results. Tissue factor procoagulant activity was also detected in microparticle pellets from the human mammary carcinoma cells but not from the canine fibrosarcoma cells (A72, Figure 5). The TFPCA of activated factor VII–negative controls of the microparticle pellets was not significantly higher than background in any of the cell lines.

**Discussion**

In this study, we found that most cell lines derived from epithelial and mesenchymal tumors in dogs constitutively expressed tissue factor on their surfaces. The highest tissue factor expression was in cells from epithelial tumors of the mammary gland or lung, with less expression in cells from pancreatic or prostatic tumors or fibrosarcomas. Cells derived from canine osteosarcomas varied considerably in their constitutive tissue factor expression, with 2 cell lines (HMPOS and D-17) having no or minimal surface expression, whereas the third evaluated cell line (OS2.4) had amounts of surface tissue factor comparable with that of the lower-expressing epithelial and fibroblast-derived tumor cells. These results are similar to that reported in human cancers, in which epithelial tumors constitutively express more tissue factor than mesenchymal tumors, typically on their membrane surfaces. We and others have found that expression of tissue factor also varies between cell lines derived from human breast carcinoma and osteosarcoma. We speculate that these differences in tissue factor expression may correlate to in vivo tumor behavior in dogs; however, this remains to be tested in future studies. In human patients with various types of cancer, tissue factor expression correlates to histologic grade and is associated with increased risk of death.

Intracellular and membrane-expressed tissue factor is mostly present in an encrypted form and must be activated before tissue factor can function as a procoagulant protein. The surface-associated tissue factor on the canine tumor cells in the present study was
functionally active because the cells could cleave factor X in the presence of exogenous activated factor VI without prior activation, suggesting that tissue factor was in a decrypted state in these cells. The mechanism of tissue factor decryption is unknown but is presently under intense investigation. Activation may be associated with cleavage of a key disulfide bond in the extracellular domain (which is conserved in canine and human tissue factor), dedimerization of tissue factor, or calcium-induced movement of tissue factor between cholesterol-rich lipid rafts and phosphatidylserine-rich microdomains in cell membranes. The surface TFPCA of the tumor cells mimicked the protein expression as detected by means of flow cytometry (expressed as MFI) in that cells with the highest TFPCA also had the highest protein expression. The surface TFPCA also matched the TFPCA of whole cell lysates, with the exception of the canine HMPOS and D-17 osteosarcoma cells. These cells expressed tissue factor mRNA, but tissue factor was not detected on cell surfaces via flow cytometry, immunofluorescent microscopy, or the TFPCA chromogenic assay. Osteosarcoma cells (HMPOS and D-17) did express tissue factor intracellularly, as indicated by the small amounts of TFPCA in whole cell lysates (although none was detected on the permeabilized cells via immunofluorescent microscopy, which can be attributed to different sensitivity of these techniques for the detection of canine tissue factor). However, this intracellular expression may not be biologically relevant because only surface-expressed tissue factor is functional and able to initiate coagulation or downstream signaling. It should also be noted that results of the whole cell TFPCA assay do not indicate that the intracellular tissue factor is already in an active state because the sonication procedure decrypts tissue factor.

Tissue factor protein expression was weaker on both canine mammary tumor cells, compared with the human mammary carcinoma cells (this was particularly evident when evaluated via immunofluorescent microscopy), even though the canine cells had significantly higher surface TFPCA. This can be attributed to lower sensitivity of the rabbit polyclonal anti-human tissue factor antibody against the canine protein, compared with the human protein. This was indicated by the lower MFI obtained with this antibody in yeast transfected with the canine tissue factor ectodomain, compared with the human mammary carcinoma cells. This result is not surprising because the ectodomains of human and canine tissue factor only have 72% homology at the amino acid level. We and others have found that few monoclonal antibodies directed against human tissue factor cross-react with canine tissue factor.

Microparticles are membrane-bound vesicles that are shed from the surfaces of many cells, including platelets, RBCs, endothelial cells, and leukocytes. They are distinguished by their small size (< 1 μm), phosphatidylserine content, and cell-specific surface proteins. Low concentrations of tissue factor–bearing microparticles (also termed intravascular, circulating, or blood-borne tissue factor) are present in the blood of healthy humans and can be detected by use of sensitive ELISAs. Although the cellular source of these microparticles is unclear, monocytes are thought to be a major contributor. High concentrations of monocyte-derived microparticles have been implicated in the pathogenesis of disseminated intravascular coagulation in sepsis. Microparticle release from most cells requires prior activation or apoptosis; however, cancer cells constitutively shed these vesicles, which have been associated with thrombotic events and a negative disease outcome in patients with cancer. Microparticle-associated tissue factor can be detected by use of immunologic-based methods, such as flow cytometry or ELISA, or procoagulant activity–based methods, including the 2-stage chromogenic assay used in the present study. We found that the high tissue factor–expressing canine epithelial tumor cells (mammary and pulmonary tumors) released tissue factor–bearing microparticles into the culture supernatants. In contrast, these microparticles were not detected in supernatants from A72 fibrosarcoma cells. This could be caused by the degree of surface tissue factor expression on the tumor cells because the canine mammary and pulmonary tumor cells had higher surface TFPCA than did the fibrosarcoma cells. However, there may also be inherent differences in the ability of tumors to shed these microparticles, with mesenchymal tumors shedding fewer microparticles than do epithelial tumors. This was supported by the detection of tissue factor–bearing microparticles from the human mammary carcinoma cells, which expressed similar surface TFPCA to the canine fibrosarcoma cells (Figure 5) in the present study. Furthermore, circulating microparticles are frequently detected in carcinomas in human patients, are associated with thromboembolic events, and are poor prognostic indicators.

In human patients with cancer, tissue factor has been ascribed the major role for induction of paraneoplastic thrombosis. Patients with cancer, particularly those with metastatic pancreatic, ovarian, mammary, and pulmonary carcinomas and osteosarcomas, are at risk of developing thrombosis or suffering from recurrent thrombotic episodes, which contributes to an increased risk of death. Furthermore, adults have a higher risk of having cancer diagnosed within 1 year after an initial idiopathic thromboembolic event. In contrast, thrombosis is infrequently recognized or diagnosed in dogs with malignancies. This could be attributable to the lack of suitable laboratory or imaging tests for identifying microvascular thrombosis in animals. Thromboelastography is a global test of hemostasis that is being increasingly used in veterinary medicine to diagnose hypercoagulable (thrombotic) disorders in animals. A recent study that used thromboelastography found that hypercoagulability (on the basis of a high clot shear elastic modulus or G value, which is a mathematical derivation of clot strength) was present in 50% of dogs with various malignant tumors. Furthermore, 31% of dogs with benign tumors, all of which were epithelial in origin, had hypercoagulability. Overt thrombosis was not described for any of the dogs in the study. These data suggest that, similar to human patients, dogs with neoplasia are at risk of thrombosis, particularly if the tumor is malignant and of epithelial origin. Furthermore, this documented hypercoagulability in canine patients with malignancies could be attributable to consti-
tively high tissue factor expression by the tumor cells. However, additional studies are required to directly test this hypothesis and to evaluate the role of tissue factor in canine tumors known to induce thrombosis, such as hemangiosarcoma.46

In recent years, it has become increasingly apparent that tissue factor can affect tumor cell behavior, independently of thrombosis. Various in vitro and in vivo studies have revealed that tissue factor can promote tumor cell growth, invasion into extracellular matrix, angiogenesis, survival, and metastasis. The nonthrombotic effects of tissue factor on tumor cells are thought to be mediated through G-protein–mediated intracellular signal transduction pathways, initiated directly via tissue factor through its cytoplasmic tail or indirectly via the formation of tissue factor–activated factor VII or tissue factor–activated factor VII–activated factor X complexes or the generation of thrombin, which activates PARs on the surface of tumor cells.8,19,47 Four PARs have been identified to date,48 of which PAR-1 and -2 are thought to be involved in mediating tissue factor–induced signals in tumor cells.8,19,47 The expression of PARs and their role in tissue factor–induced signaling in tumor cells is not known.

In the present study, the 2 canine mammary tumor cell lines and the bronchoalveolar carcinoma cells could activate factor X in the absence of exogenous activated factor VII in the TFPCA chromogenic assay. This was most prominent on cell surfaces and was not found in the microparticle preparations. This tissue factor–independent generation of activated factor X activity could be associated with ectopic production by the tumor cells of canine factor VII, which then binds to tissue factor constitutively expressed on the cell surface. This has been described for various types of human cancer cells.49 We attempted to address this by incubating cells with an activated factor VII–dependent chromogenic substrate,5 but in preliminary trials, we found that activated factor X was more effective at cleaving the substrate than was activated factor VII because the substrate is not specific for activated factor VII. Another potential explanation for the tissue factor–independent activated factor X activity is the presence of another protease that is capable of cleaving factor X. There is a report49 of a cancer-associated protease, which is a cysteine protease found on human cancer cells of epithelial origin. Because this tissue factor–independent activity was surface expressed, it is possible that a similar tumor-associated protease exists on canine epithelial tumor cells. However, the contribution of this protein to the procoagulant activity of these cells is quite small, indicating that most of this activity can be attributed to tissue factor, as in human cancer cells.

In the present study, canine tumor cells, particularly malignant epithelial tumors of mammary and lung origin, constitutively expressed high levels of active tissue factor on their surfaces and spontaneously released tissue factor–bearing membrane-derived microparticles. Further studies are warranted to assess the functional relevance of these findings for canine tumor cell behavior in vivo and to determine whether tissue factor is a potential prognostic indicator for metastasis in dogs with various forms of neoplasia.

References


