The Role of Tissue Factor in Canine Hemangiosarcoma

A Thesis

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by

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ABSTRACT

Keywords: Hemangiosarcoma, Angiosarcoma, Tissue Factor

Hemangiosarcoma (HSA) and angiosarcoma (AS) are malignant endothelial neoplasms in dogs and human beings, respectively. Canine patients suffering from hemangiosarcoma often succumb to concurrent hemostatic disorders such as disseminated intravascular coagulopathy (DIC); human beings with AS are also prone to DIC. The cause of DIC in affected patients is unknown. Tissue factor (TF), a 47-kDa membrane-bound glycoprotein, is the main initiator of the coagulation cascade. Tissue factor has been found to be upregulated in some canine epithelial and mesenchymal cancer cells, but we currently do not know how TF is expressed in HSA and AS. Endothelial cells do not normally express TF or bind coagulation factor VII (FVII), the enzymatic binding partner for TF in plasma. Our global hypothesis is that TF contributes to the aggressive biological behavior of canine hemangiosarcoma, including the initiation of thrombin formation as seen in DIC. We hypothesize that hemangiosarcoma, a malignant endothelial neoplasm, is procoagulant due to the aberrant expression of TF. We found that HSA cell lines derived from primary tumors in dogs expressed TF mRNA and cell surface protein as determined by quantitative RT-PCR, and flow cytometry, respectively. In addition these cells produced thrombin as shown by calibrated automated thrombography (CAT) in canine plasma. Using human plasma deficient in specific factors and protein inhibitors, we found that thrombin generation by HSA cells is mediated by the extrinsic pathway of coagulation, requiring FVII and phosphatidylserine (PS), and is independent of contact activation. Conclusion: Our results show that HSA cells are procoagulant and this is most likely due to TF expression. Procoagulant TF expression could explain the pathogenesis of DIC in dogs with HSA. Future studies will examine if an AS-derived cell line has similar activity to establish canine HSA as a model for human AS. Tissue factor's interaction with protease-activated receptor 2 is involved several signaling pathways that promote hemostasis, repair, cell survival, and inflammation. These signaling

pathways can be subverted during cancer to promote tumor growth, angiogenesis, tumor survival and metastasis. We have developed a TF knockdown in a HSA cell line and plan to assess changes in cell growth, anchorage independence, and doxorubicin-sensitivity. This research could lead to future diagnostics for hypercoagulability associated with DIC and therapeutics for treating dogs with HSA.

BIOGRAPHICAL SKETCH

Lauren Witter grew up in Brooklyn, New York. She attended the Macaulay Honors College at the City University of New York's Hunter College where she received her Bachelor of Arts degree in biology with honors in 2012. In the fall of 2012 she joined the doctoral program in veterinary medicine at College of Veterinary Medicine at Cornell University. Lauren's work in Dr. Tracy Stokol's laboratory in the summer of 2013 during the Veterinary Investigator Program prompted her to persue a year of research continuing her project on canine hemangiosarcoma and tissue factor. She started her Master of Science in the field of comparative biomedical science at Cornell University in January of 2014. Funding for the year of research was provided by an NIH training grant for veterinary students interested in biomedical research.

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LIST OF ABBREVIATIONS

(Alphabetical order)

Active coagulation factor (F-a)

Angiosarcoma (AS)

Calibrated automated thrombography (CAT)

Coagulation factor (F-)

Disseminated intravascular coagulation (DIC)

Green fluorescent protein (GFP)

Epidermal growth factor receptor (EGFR)

Hemangiosarcoma (HSA)

Interleukin 1β (IL-1β)

Interleukin 8 (IL-8)

Lipopolysaccharide (LPS)

Median survival time (MST)

Median fluorescent intensity (MFI)

Microparticles (MPs)

Phosphatase and tensin homolog (PTEN)

Phosphatidylserine (PS)

Protease-activated receptor (PAR)

Quantitative real time PCR (qPCR)

RNA-induced silencing complex (RISC)

RNA interference (RNAi)

short hairpin loop RNA (shRNA)

small interfering RNA (siRNA)

Tissue factor pathway inhibitor (TFPI)

Transforming growth factor β (TGF β)

Tumor necrosis factor α (TNFα)

Urokinase Receptor (uPAR)

Vascular Endothelial Growth Factor (VEGF)

CHAPTER 1

LITERATURE REVIEW

Introduction

Hemangiosarcoma (HSA) is a common and aggressive malignant neoplasm in dogs with minimal treatment options and poor prognosis. Hemangiosarcoma is notoriously resistant to common chemotherapeutic drugs such as doxorubicin. In addition, canine patients with HSA have a higher incidence of thrombotic disorders including disseminated intravascular coagulation (DIC). Angiosarcoma (AS) is the human equivalent to HSA and while less common, AS is similarly aggressive with a poor response to chemotherapy and an increased risk of thrombosis. Tissue factor (TF) is known as the main initiator of thrombin generation during normal hemostasis. Tissue factor expression on tumors in human beings has been associated with an increased risk of thrombosis and metastasis. We have found that HSA upregulated TF compared to their non-neoplastic counterparts, which do not constitutively express TF under normal conditions. Previous research by others has confirmed upregulated TF expression in AS derived tumor cell lines. The role of TF in HSA is poorly understood. If TF on HSA and AS is functionally procoagulant then the pathogenesis of DIC in these cancers could be partially explained. Tissue factor expression on these cancers could be used as a marker of procoagulant propensity for diagnostic and therapeutic strategies to prevent death due to DIC. This thesis explores the role of TF in HSA procoagulant activity and tumor biology. In addition, this thesis demonstrates that canine HSA could serve as a model for human AS.

Tissue Factor and Physiological Hemostasis

Hemostasis is the vital process of keeping blood within a damaged blood vessel. A fine balance exists between coagulation, the process of clotting blood, and fibrinolysis, the process of breaking down blood clots. Abnormal hemostasis results from either too much or too little clotting, leading to thrombosis or hemorrhage, respectively. During clotting serine proteases are activated, which results in a cascade of activation of other serine proteases, which in turn forms a blood clot; this process is commonly referred to as the coagulation cascade. The coagulation cascade model is divided into two pathways; the extrinsic pathway and the intrinsic pathway. These two pathways converge on the common pathway to eventually generate a fibrin clot (Figure 1.1). The current cell-based model of coagulation expands the cascade model into a web of reactions that take place on cell surfaces, most of which are provided by activated platelets [1].

Tissue factor, also known as coagulation factor III and thromboplastin, functions as the initiator of coagulation. Tissue factor is a 47 kDa transmembrane glycoprotein [2]. Under non-pathological conditions TF is sequestered from coagulation factors circulating in the blood plasma. Upon injury to this hemostatic envelope, the TF constitutively expressed in smooth muscle and fibroblasts in the adventitia surrounding endothelial cells is exposed to coagulation factors in the blood stream, most importantly its binding partner coagulation factor VII (FVII) [2]. Blood clotting is maintained at the site of injury, because that is the only area where subendothelial TF is exposed. The interaction of TF with FVII initiates what is known as the extrinsic pathway of coagulation. Factor VII is serine protease that is secreted from the liver as an inactive zymogen. Upon binding to TF, FVII is converted to FVIIa (the lower case “a”

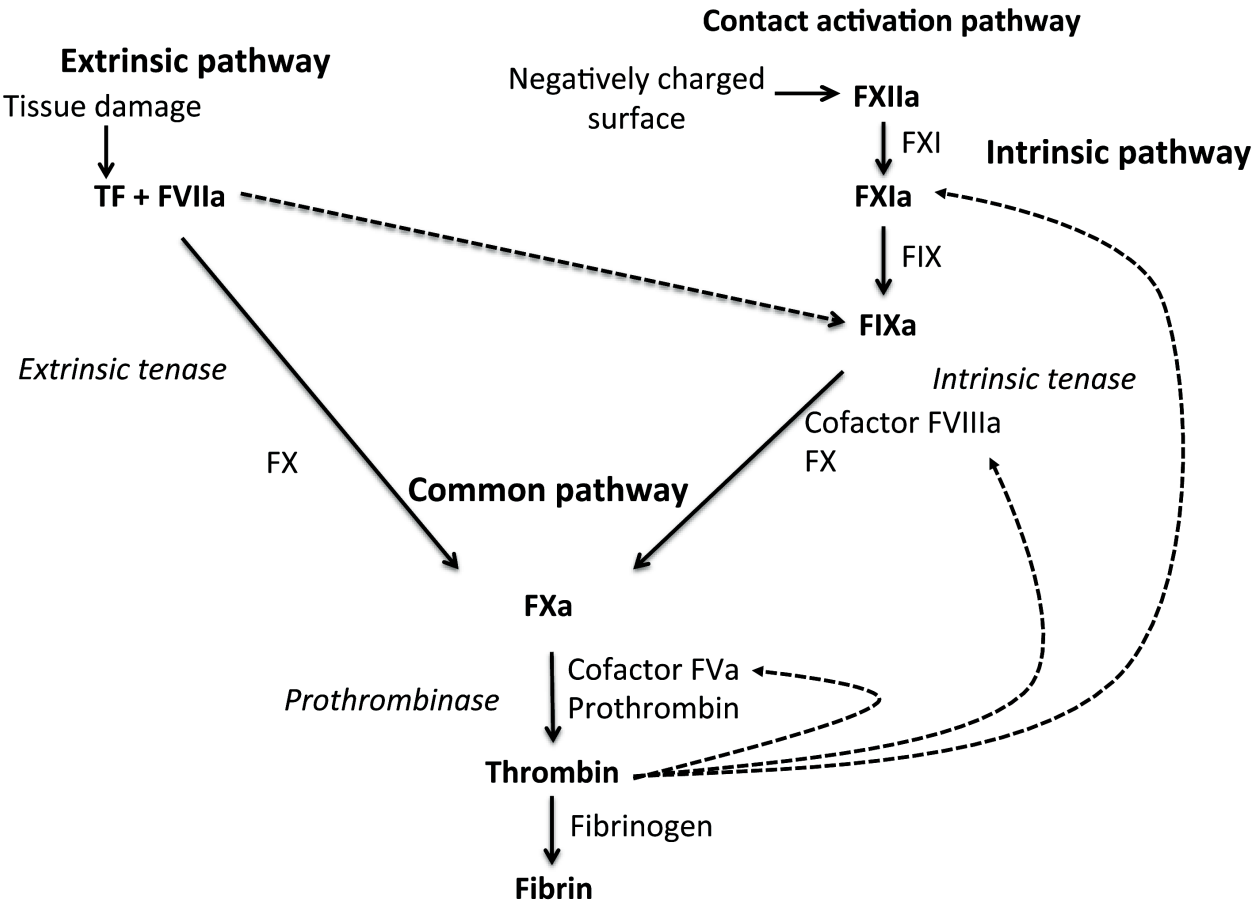

Figure 1.1- The coagulation cascade

The extrinsic and intrinsic pathways converge on the common pathway to create a fibrin clot. Physiologically during hemostasis the extrinsic pathway initiates thrombin generation once tissue damage exposes TF to plasma circulating FVIIa. The TF-FVIIa complex activates FX that in turn generates thrombin from prothrombin. The TF-VIIa complex also activates coagulation FIX secondarily (dashed line) which amplifies thrombin production by activating the intrinsic pathway. The small amounts of thrombin generated by the extrinsic pathway also works secondarily to activate coagulation factors and cofactors that amplify thrombin production. This burst of thrombin overcomes inhibitory anticoagulant proteins (not shown) and converts soluble fibrinogen into solid fibrin.

indicates the activated form of coagulation factors). Tissue factor binds to both FVII and FVIIa, but only creates an enzymatically active complex with FVIIa. Together TF and FVIIa with the help of a negatively charged phospholipid surface (see below) and calcium ions can initiate thrombin generation by the creation of active factor X (FXa) of the common pathway and factor IX (FIXa) of the intrinsic pathway. The TF-FVIIa complex is also known as the extrinsic tenase, i.e. activator of FX [1-3].

The initial production of FXa through the extrinsic pathway quickly begins the conversion of prothrombin to thrombin with the aid of cofactors factor Va and calcium. Thrombin has several important functions in coagulation. Most notably thrombin converts fibrinogen to fibrin creating a fibrin clot. Thrombin also activates platelets, which are integral to clot formation. Activated platelets provide a negatively charged phospholipid surface for the coagulation factor serine proteases to cleave their substrates and continue the coagulation [1]. Phosphatidylserine (PS) is the main negatively charged phospholipid that gets flipped to the outer leaflet of the plasma membrane upon platelet activation. Phosphatidylserine density on the cell surface greatly enhances thrombin generation as demonstrated in vitro with phospholipid vesicles of varying PS concentration. Thrombin generation in vitro can be inhibited by PS-binding proteins, such as annexin V [4]. Thrombin also starts the action of the intrinsic cascade by activating factor XI (FXI) leading to amplification of thrombin production. Thrombin-activated FXIa of the intrinsic cascade converts factor IX (FIX) to factor IXa. With activated cofactor factor VIII (FVIIIa) and calcium, FIXa converts FX to activated FXa. Factor IXa with the help of FVIIIa is known as the intrinsic tenase (Figure 1.1). The intrinsic tenase serine protease complex rapidly amplifies FXa production to create a localized burst of thrombin

necessary for fibrin clot formation. The extrinsic pathway begins thrombin generation physiologically in vivo, but the intrinsic pathway activation is necessary to reach the levels of thrombin needed to overcome clotting inhibitors. Activated platelets aggregate within the fibrin network and a clot is formed blocking blood loss from vessels [1, 3].

Coagulation during physiologic hemostasis is tightly regulated to keep coagulation at the site of injury and prevent systemic dissemination of activated coagulation proteins. Thrombin activates inhibitors of coagulation slowing down its own formation. Thrombin procoagulant activity is mitigated by its binding to thrombomodulin on endothelial cells. Thrombin that is complexed with thrombomodulin creates activated protein C. Activated protein C further dampens thrombin generation by inactivating the coagulation cofactors, FVa and FVIIIa by proteolytically cleaving them. Other inhibitors of thrombin generation include antithrombin and tissue factor pathway inhibitor (TFPI), both which are found in blood plasma. Antithrombin binds activated coagulation factors Xa, IXa, XIa, XIIa and thrombin, blocking their ability to function as serine proteases and propagate thrombin generation. Tissue factor pathway inhibitor reversibly binds to FXa and inhibits its activity. While bound to FXa, TFPI also inhibits TF-VIIa complexes and imposes an anticoagulant barrier that must be overcome by a sufficient amount of TF trigger to begin coagulation. These inhibitors counteract prothrombotic factors, sequestering hemostasis to the site of injury [3].

Thrombosis and Disseminated Intravascular Coagulation

Pathologic clotting that partially or totally occludes blood vessels is known as thrombosis. Thrombosis occurs because of increased clotting or a decrease in fibrinolysis, the break down of fibrin clots. Aberrant or excessive expression of TF pathologically initiate thrombosis, but the cellular source of TF can vary [5]. As stated earlier, the most common source of TF is from the subendothelium, specifically the cells surrounding blood vessels that become exposed upon vessel injury. Excessive vascular injury can expose large amounts of TF that can lead to thrombosis. Tissue factor can also be found on activated endothelial cells, monocytes and microparticles (MPs) derived from these cells [6, 7]. These cellular sources of TF can provide a mobile blood-borne trigger for thrombosis, which given sufficient concentration, can overcome the inhibition of TFPI and cause thrombus formation. Other cellular sources of TF also provide PS-rich surfaces to propagate coagulation reactions. Inappropriate activation of the coagulation cascade leads to pathologic hypercoagulability (defined as excessive formation of thrombin), which predisposes a patient to thrombosis.

Under normal circumstances in healthy endothelial cells there is no TF expression. The endothelium acts as an inhibitor of clotting by expressing high levels of thrombomodulin. Endothelial cells can be activated upon stimulation by inflammatory cytokines, such as interleukin 1β (IL-1β) and tumor necrosis factor-α (TNFα) during an immune or inflammatory stimulus. Endothelial cell activation is typified by a change in phenotype from antithrombotic to prothrombotic. The prothrombotic changes include loss of anticoagulant molecules like thrombomodulin and an increase in prothrombotic molecules like platelet activating factor, nitric

oxide and TF. New transcription of TF is mediated by nuclear factor κβ (NF- κβ), a protein complex that controls DNA transcription. NF- κβ is relieved from inhibition upon activation of several inflammatory signaling pathways and is able to move to the nucleus and initiate transcription of many proteins including TF [8, 9]. An activated endothelium facilitates leukocyte migration and extravasation during injury and infection. Endothelial activation has also been implicated in the pathogenesis of atherosclerosis and sepsis [10]. Monocytes are circulating leukocytes that patrol the blood for cytokines and eventually migrate into tissues to become macrophages. Monocytes also react to inflammatory cytokines TNFα and IL-1β by producing TF [11]. Monocytes and endothelial cells closely interact to generate TF. Both cells generate IL-1β and TNFα in response to inflammatory cytokines; in addition monocytes and endothelial cells respond to these cytokines by the induction of TF expression. Crosstalk between monocytes and endothelial cells by IL-1β and TNFα was demonstrated in vitro, demonstrating a positive feedback cycle of TF expression in response to inflammation [7].

Tissue factor exposure during sepsis commonly causes clotting in the blood stream. Sepsis arises from the release of bacteria into the bloodstream and the resulting host responses. A common sequela of sepsis is activation of coagulation. This is in part due to the expression of TF on monocytes triggering the initiation of coagulation within the bloodstream. Monocytes upregulate TF on their cell surface in response to lipopolysaccharide (LPS) and inflammatory cytokines during sepsis [11]. Activated monocytes and endothelial cells can shed MPs that carry active TF. Microparticles are small (0.1-1μm) membrane vesicles that are formed from plasma membrane blebs from apoptotic and activated cells [12]. Microparticles carry the same proteins as their parent cell's membrane. In addition to TF, MPs have PS exposed on their membranes.

This gives TF-bearing MPs the ability to form active extrinsic tenase complexes with circulating FVIIa in plasma. Microparticles have been implicated in pathological thrombus formation in DIC and have been shown to have procoagulant activity in vitro [13]. Expression of TF on monocytes and endothelial cells in response to inflammatory stimuli such as LPS and cytokines during sepsis is the main contributor to the pathogenesis of DIC in sepsis [14].

DIC is a pathological process whereby an underlying disease causes widespread clotting activation in small blood vessels throughout the body. It is characterized by excessive and uncontrolled thrombin generation that is disseminated on cell surfaces [15]. The two necessitating factors of DIC are: 1) excessive clotting activation and 2) cell surfaces that can circulate the resulting clotting throughout the body. Excess TF and phospholipid surfaces are required for induction of DIC. There are two recognized stages of DIC; nonovert DIC and overt DIC [16]. Because DIC is the result of an underlying disease, the body continually tries to compensate for the excess thrombosis. While the inhibitors can still compensate for the excessive incitement of coagulation, clinical signs are minimal. Screening blood tests might show increased breakdown products of blood clots including fibrin degradation products and D-dimers. Nonovert DIC usually remains unrecognized unless other clinical findings, like cancer, indicate a risk of DIC. Nonovert DIC is also known as chronic or low-grade DIC. The excessive clotting eventually exhausts the capability of inhibitory mechanisms and thrombus formation propagates. Circulating blood coagulation factors and platelets are depleted as excessive thrombin generation continues, leading to widespread bleeding and hemorrhage throughout the body. This decompensated stage is recognized as overt DIC. Blood tests of patients with decompensated DIC reflect their hypocoaguable state and can show prolonged blood clotting

times, low coagulation inhibitor levels, low platelet counts, and regenerative anemia [17]. Just as there is a spectrum of stages of DIC, DIC can manifest in patients as a range of bleeding disorders form small petechiae to fulminant life-threatening hemorrhages to multiple organ failure.

Thrombosis and DIC increase risk of morbidity and mortality in human and canine patients. The key to treatment of DIC is to address the underlying cause of hypercoagulability as soon as possible. This is complicated as current laboratory testing is not sensitive to compensated nonovert DIC [17]. Overt DIC is a life-threatening condition. Patients are at risk of excessive blood loss and ischemic damage to multiple organs. Treatment of patients in decompensated DIC consists of trying to regain hemostatic balance by replacing hemostatic factors including platelets and coagulation factors. Earlier indication of hemostatic unbalance could alter treatment and decrease risk of morbidity and mortality in patients.

Hypercoagulability and Cancer

For nearly two centuries the correlation between a hypercoagulable state and cancer has been recognized; this is known as Trousseau syndrome. This syndrome includes the following thrombotic conditions: DIC, deep vein thrombosis, and thromboembolism. Thrombosis occurs when there are abnormalities in three broad categories, known as Virchow's Triad. These three broad categories are: hypercoagulability, endothelial/vessel wall injury and hemodynamic changes [18].

Hypercoagulability is induced in cancer through various procoagulant mechanisms. Tumors can increase production of hemostatic factors like TF and shed TF-bearing MPs [19]. The aberrant expression of TF has been observed in several epithelial and mesenchymal cancers in human patients. Cancers more commonly associated with Trousseau syndrome are positive for TF expression by immunohistochemistry; these cancers include lung, pancreatic, breast, colon and gastric carcinomas [20]. TF overexpression in human pancreatic cancer in vitro increases thrombin generation, a marker for procoagulant activity [21]. In vivo, TF expression on pancreatic carcinoma is linked with a higher risk of venous thromboembolism [22]. Tissue factor expression and increased thrombotic risk is also seen in mammary carcinomas. A high TF-expressing human cell line, MDA-MB 231, has been shown to have increased thrombotic potential [23].

The link between cancer and inflammation was recognized as a hallmark of cancer in Hanahan and Weinberg's seminal writing. Chronic inflammation has been shown to increase the risk of genetic instability and as such increase the risk of developing cancer. In addition tumors activate surrounding inflammatory cells by manipulating their tumor microenvironment [24]. Inflammatory cells release cytokines and chemokines that aid in cancer progression by inducing angiogenesis and tissue invasion [25]. While many tumors actively avoid natural killer cells and other aspects of the immune system proinflammatory cytokines from tumor-associated macrophages produces cytokines that benefit many tumors such as myeloma, breast cancer and ovarian cancer among others. Tumor-promoting inflammation can also specifically induce local inflammatory cells to produce TNFα [26], which causes activation of the endothelium and monocytes causing TF translation and exposure. Proinflammatory cytokine expression enhances

tumor survival and triggers microenvironment remodeling and angiogenesis. This is one way cancer can lead to an increase in intravascular TF and hypercoagulability. In addition cancers are also now known to shed MPs. Tissue factor-bearing MPs from colorectal cancer have been shown to increase coagulation activation. Not only did patients with advanced colorectal cancer have increased TF-positive MPs over age-matched, and sex-matched controls, they also had increased evidence of coagulation activation and fibrinolysis [27]. Tissue factor-positive MPs have also been found in patients with pancreatic adenocarcinoma, metastatic breast cancer, non-small cell lung cancer and ovarian cancer and are increased after thrombotic incidents [28].

Hemodynamic changes, such as static or turbulent blood flow, can also cause thrombus formation. Metastatic tumors can cause alterations in blood flow by occluding vessels, promoting faulty vessel formation and rupturing vessels through metastatic invasion. Solid cancers promote angiogenesis, the creation of new blood vessels, which are often abnormal. Sometimes they do not fully barricade TF-rich subendothelium from flowing plasma; tumor endothelial cells do not form tight junctions with neighboring endothelial cells. Tumor-associated endothelial vessels are greatly enlarged, tortuous and thin-walled [29]. Altered blood vessel structures promote pooling of blood and increase the likelihood of coagulation.

Cancer-associated thrombosis resolves once the underlying prothrombotic insult is removed. In several human studies thrombotic symptoms manifested early in disease progression and improved with effective cancer therapy. Unfortunately symptoms of hypercoagulability returned after the cancer continued to progress [30]. Early recognition of cancer-associated hypercoagulability is important for patient prognosis and survival.

Hemangiosarcoma

Hemangiosarcoma is a malignant mesenchymal neoplasm of vascular endothelial cells in dogs. It is a very common tumor representing 21% of mesenchymal tumors in dogs; this is 2% of all canine cancer diagnoses [31]. Hemangiosarcoma affects mostly older dogs, greater than 8 years, but has been shown to occur in dogs as young as 4 years. Larger breed dogs, especially German Shepherds and Golden Retrievers, most often present with HSA. The tumor can be found anywhere as blood vessels with endothelial cells are everywhere in the body, but the most common sites include the spleen, right atrium of the heart, skin, subcutis and the liver [32]. The tumors of HSA are formed from many malformed blood vessels. Histological examination shows a range of various degrees of differentiated vascular endothelial cells. More differentiated cells form tortuous misshapen blood vessels creating soft red-brown masses with areas of hemorrhage and necrosis, making the histologic diagnosis readily apparent. Cells within HSAs can be less differentiated, forming firm, pale and grey foci similar in appearance to other sarcomas [33, 34]. Immunohistochemical probing for endothelial marker including von Willebrand factor and CD31 are commonly used to diagnose HSA [35].

Several studies have been done looking into the genetic events leading to HSA. A relatively common upregulation shown via genome wide expression profiles of HSA is an increased expression of epidermal growth factor receptor (EGFR) and urokinase receptor (uPAR) [36]. EGFR is a member of the receptor tyrosine kinase family that is commonly upregulated in the pathogenesis of human carcinoma [37]. uPAR functions physiologically during wound healing and tissue reorganization and is upregulated in several malignant tumors. The roles of EGFR and uPAR in the pathogenesis of canine hemangiosarcoma are still being

investigated but have been implicated in the high vascular endothelial growth factor (VEGF) production and chemoresistance [36]. Other genes that that are frequently altered in HSA are oncogenes CDKN2A, VEGF and SKI [38]. CDKN2A is a cyclin-dependent kinase inhibitor involved in promotion of cell cycle progression. Alterations in CDKN2A function could promote cell division and growth. VEGF promotes endothelial cells to divide and become resistant to apoptosis as well as other changes to vascular tissues. SKI is a part of the signaling transduction system of the transforming growth factor β (TGFβ) pathway. The TGFβ pathway regulates cell growth and division, differentiation, motility and apoptosis; all of these processes are altered in cancer. HSA tumors also show a global tendency to downregulate tumor suppressor phosphatase and tensin homolog (PTEN) potentially leading to the uncontrolled growth of HSA via deregulation of cell cycle progression [39]. The Golden Retriever breed has an even higher risk for developing HSA; one out of five Golden Retrievers will die from HSA in their lifetimes. In a study examining breed specific HSA genetic profiles HSA from Golden Retrievers clustered separately from HSAs of other breeds suggesting specific heritable genes are involved in HSA development. Proinflammatory and angiogenic genes were upregulated in Golden Retriever specific population. VEGF Receptor 1 (VEGFR1) was also overexpressed at the mRNA and protein level [40]. More research needs to be done exploring breed specific genetic events leading to HSA development.

Hemangiosarcoma is noted for its early and frequent metastasis. Clinical signs are uncommon until metastasis has occurred. Hemangiosarcoma commonly metastasizes to the liver and lung, but metastasis is also seen in the omentum, mesentery and brain. Seeding tumors are notoriously spread hematogenously and through frequent transabdominal bleeds that occur

through HSA rupture from malformed vessels. Dogs with HSA have a poor prognosis, with survival durations of less than 6 months after diagnosis. Stage I tumors, those defined as distinctly localized without any metastasis, have a median survival time (MST) of 151 days. Stage II tumors, where HSA tumors have ruptured but remained confined to the primary tumor site, have a MST of 107 days. Lastly, stage III tumors, those with noted distant metastasis, have a MST of 71 days [41]. Treatments include surgery and chemotherapy; most successful results use a combination of both. Unfortunately the benefits of surgery and chemotherapy only prolong survival times for dogs with HSA by less than a month. Aggressive surgery with doxorubicin, the chemotherapeutic drug of choice, only increases MST to 171 days 151 [42]. Doxorubicn is from the class of drugs called anthracyclines; it actively induces apoptosis in a wide range of tumors but is highly cardiotoxic [43]. Morbidity and mortality commonly occur from organ function disruption, internal hemorrhage from tumor rupture and hemostatic abnormalities in HSA.

The most common coagulopathy in dogs with HSA is DIC. Hammer et al, looked at the hemostatic profiles of 24 dogs with histologically confirmed HSA in a study in 1991. Overt DIC, as suggested by laboratory profiles, was documented in 50% of dogs with HSA and was directly responsible for the death in half of these dogs [44]. In a survey looking at coagulation abnormalities in dogs with solid cancers, only 16% of dogs had overt DIC [45]. A Japanese study looking at the incidence of DIC in dogs with solid malignancies in 2004, corroborated the study by Hammer et al. Incidence of DIC was highest for dogs with HSA and mammary gland carcinoma than for all of the other solid malignancies in the Japanese study [46]. While these studies are both small, this is strong evidence that dogs with HSA, versus dogs with other

cancers, have a higher risk for developing DIC. The excessive activation of coagulation seen in HSA, which culminates in DIC, is a major contributor to the high mortality rate.

As mentioned before, diagnosis of DIC is difficult. Progression of DIC from hypercoagulability to hypocoagulability throughout the course creates a wide range of diagnostic indicators of DIC. The progression of DIC is dynamic, depending on the rates of thrombosis and of consumption of clotting inhibitors. Diagnosing DIC in the early, hypercoagulable nonovert stage remains a challenge in the clinical setting. Early diagnosis and treatments targeted at removing the hypercoagulable insult could prevent death due to DIC in HSA. No studies have looked into the pathogenesis of DIC in canine HSA. The reason for HSA cancers being more clinically procoagulant than other solid tumors remains a mystery.

Angiosarcoma

In humans, the equivalent tumor to HSA in dogs is AS. Angiosarcoma is also a highly aggressive neoplasm of vascular endothelial origin. Sarcomas, cancers originating from mesenchymal tissue origin, account for 1% of tumor diagnosis in humans. Angiosarcoma accounts for 2-5% of sarcoma diagnosis [47]. These are rare tumors that have a poor prognosis and ineffective treatment options. Angiosarcoma has been associated with exposure to environmental carcinogenic factors, including dyes, and sites of radiation exposure. The face and scalp of elderly people are the most common sites for AS to arise however, AS is also known to arise on any anatomic site including the breast, bone, deep soft tissues and viscera. Tumors are often bruise-like lesions with the propensity for intratumoral bleeds. Large tumors often have

areas of necrosis and hemorrhage. Early in the course of the disease these lesions are often overlooked as benign. Symptoms of AS usually occur from disruption of normal organ function after metastasis or tumor rupture [47]. Just as in HSA in dogs, once symptoms from AS tumor progression are noticed, metastasis has likely already occurred. Metastasis in AS spreads hematogenously, with the lung being the most common site of metastasis. Visceral metastases are documented as well.

There is no defining sequence of genetic events leading to AS but rather a spectrum of genetic abnormalities. Primary AS describes those tumors that arise spontaneously. These are mostly seen in older patients but have been documented in young patients as well. Secondary AS develops following exposure to carcinogenic dyes and radiation therapy. Genetic upregulation of vascular-specific receptor tyrosine kinases, TIE1, KDR, TEK and FLT1 occur in both types of AS. KDR encodes for VEGFR2, VEGF-Receptor-2, which is upregulated in AS leading to increased sensitivity to VEGF [48]. Like these mutations 40% of AS analyzed had a driver mutation for angiogenesis signaling. Other enhancements of angiogenic signaling included truncations in PTPRB, an endothelial phosphatase that negatively regulates vascular growth factor tyrosine kinases [49]. Secondary AS amplifications of MYC andFLT4 genes are consistently reported. FLT4 encodes VEGF receptor 3, which is also involved in the transduction of VEGF signaling. MYC is a classic proto-oncogene that is subverted in cancer and has a crucial role in regulating cell growth, differentiation and apoptosis. MYC specifically causes upregulation of regulatory microRNAs that cleave angiogenesis inhibitors thrombospondin and connective tissue growth factor; this has been seen in AS tumors with high MYC expression. No alterations in PTEN have been observed in secondary AS in contrast to other sarcomas [48]. The

dependence of AS tumors on KDR mutations is currently being investigated as a route for VEGF receptor-directed therapy [50]. Since there are so few cases of AS and even fewer of primary AS specific genetic alterations in primary AS are still being explored.

While AS mostly affects elderly people over the age of 65, it has been documented in children as young as 6 months [51]. The median survival time for all patients with AS is 7 months after diagnosis. The prognosis for AS in children is extremely poor, often less than 4 months [51]. Overall, 5 year survival rate for AS is 25-35% of patients compared to 60% for other primary soft-tissue sarcomas[30, 47]. Prediction of tumor behavior is dependent on the size of the primary lesion and the presence of distant metastases. Patients with distant metastasis treated with doxorubicin-based chemotherapy have 3-5 months of halted progression of AS but this does not carry over to increased survival time. This is the same treatment most commonly used in dogs. Most successful treatment for AS occurs from radical surgical removal with wide surgical margins. If the primary tumor is completely removed before metastasis, the prognosis is good, however, complete removal is difficult to achieve due to diffuse tissue infiltration by AS [52].

The occurrence of consumptive coagulopathies, such as DIC, has been regularly identified in AS patients [53, 54]. The prevalence of DIC in AS was analyzed in a study by Mount Sinai Medical Center. This study reviewed 42 cases retrospectively that were seen between 2000 and 2013 [30]. Eleven of the 42 patients were in overt DIC during the course of their illness and 7 patients (17% of all cases) had no known reason for the pathological development of DIC. The International Society of Thrombosis and Hemostasis (ISTH) scores

DIC based on changes in four hemostasis assays: platelet count, fibrinogen degradation products, prothrombin time and fibrinogen levels, with overt DIC being classified as a score over 5 [55]. These 7 patients had scores over 5, based on their clinical blood work. Symptomatic worsening of patient coagulopathy increased as AS disease progressed. In this small cohort of patients there was a very short MST of only 6 months. The incidence of DIC in human sarcomas was only 4%; in solid tumors DIC only complicated 7% of human cases [56, 57]. Based on this single study, patients with AS appear to have a substantially higher rate of DIC than other patients.

Angiosarcoma in humans is a devastatingly aggressive disease with a poor prognosis. Reviews of case reports and current management protocols show that today's standards of treatment are not efficacious in preventing metastasis and death in AS. There are many similarities between canine HSA and human AS. Both cancers have the same cellular origin, localization and disease progression. There are some notable differences as well. Dogs develop HSA spontaneously while the majority of AS cases develop secondary to radiation or exposure to carcinogenic chemicals. Genetically, HSA tumors have high incidence of tumor suppressor PTEN dysfunction while this is not seen in secondary AS. Both AS and HSA have enhancement of VEGF signaling and through overproduction of VEGF and alteration of VEGF receptors. Additionally, surgical and chemotherapeutic measures are similar with poor results for both cancers. Current research on AS uses a mouse model. Mice do not spontaneously develop HSA and can only develop it when they have no tumor suppressor p53. In addition mice with HSA do not develop HSA-associated coagulopathies. Notably mice with cancer in general are hypocoaguable [58]. This is a stark contrast to humans who are continually observed to be hypercoagulable with cancer. Dogs on the other hand are hypercoagulable compared with

humans, especially in the case of endothelial cell tumors. Dogs overall have increased risk of DIC due to cancer compared to humans [59]. Canine HSA is a good model for understanding procoagulant risk in human AS. Understanding how the clotting system is affected in HSA progression in dogs could lead to new ways to treat complications of DIC during HSA or AS.

TF and Biologic Behavior of Cancer

The role of TF in biological signaling beyond initiating coagulation has been heavily investigated. While it is generally accepted that excess TF expression can lead to DIC, TF expression has been linked to increased levels of metastasis, cancer progression and chemoresistance. One of the first clues to a signaling role came from sequencing TF. Researchers expected TF to have homology to other coagulation factors, but instead it showed more similarity to the cytokine receptor subfamily [2, 60]. This receptor subfamily, which includes endothelial growth factor receptor, combines into homodimers or heterodimers and signals via the cytoplasmic domain. Currently TF has not been observed to act like other cytokine receptors however the TF cytoplasmic domain does increase rates of metastasis in immune-deficient murine models of metastasis [61]. In addition the cytoplasmic domain is required for vascular endothelial growth factor (VEGF) production in malignant human melanoma cell lines [62]. Another clue that TF might function in other pathways came from TF knockout mice. Tissue factor null mice do not survive past embryonic day 8.5 due to abnormalities in yolk sac vasculature. Yolk sac blood vessels showed abnormal vessel formation with poorly developed vessel walls [63]. While other coagulation factors have known deficiencies and subsequent

hemophilia there is no naturally occurring TF deficiency. From these studies and others TF signaling has been linked to angiogenesis, metastasis and cell survival [64].

Coagulation factor VII is required for most observed TF signaling [64]. Besides via the TF cytoplasmic domain, signaling is also mediated by the TF-FVIIa complex via protease-activated receptor 2 (PAR2) [60]. Specific serine proteases act on PARs to proteolytically process extracellular residues that leave an active autocatalytic domain. Protease-activated receptors are 7 transmembrane-spanning cell surface receptors that mediate cell signaling through various G-protein cascades. There are 4 known PARs that are activated by serine proteases, such as thrombin, FXa and FVIIa [65]. PAR2 and to a lesser extent PAR1 signaling preferentially respond to TF-FVIIa complexes. Under normal conditions PAR signaling is activated by proteases released during tissue injury and result in cellular responses that promote hemostasis, wound healing, cell survival, and inflammation [65, 66]. These signaling pathways are commonly subverted during cancer to promote tumor growth, angiogenesis, tumor survival and metastasis. At physiologic concentrations TF-FVIIa complexes activated PAR2 in human breast cancer cells. This signaling prevented apoptosis and promoted cellular migration in human breast cancer cells [67, 68]. A TF knockdown in human melanoma reduced pulmonary metastasis in a nude mouse [69]. These studies show that TF-FVIIa signaling can alter tumor behavior and promote aggressive cancer phenotypes.

One way TF-FVIIa signaling mediates these benefits is through the upregulation of VEGF and interleukin 8 (IL-8). Normally increased VEGF and IL-8 following injury lead to new blood vessel formation during wound healing [70]. Angiogenesis, the process of creating new

blood vessels, involves endothelial cell proliferation and migration via extracellular matrix remodeling. For tumor growth and metastasis, neovascularization is very important. Chemokine IL-8 normally acts as chemoattractant for neutrophils and lymphocytes but has also been shown to stimulate invasion and metastasis in cancer cells. PAR2 signaling induced by the TF-FVIIa complex upregulates IL-8 production in human breast carcinoma cells in vitro. This upregulation of IL-8 was specific for TF-FVII versus PAR2 activation by other proteases notably thrombin and FXa [71]. In canine HSA cells IL-8 is highly upregulated. Genome wide gene expression profiling of high and low IL-8-expressing HSA cells revealed enhancement of genes associated with influencing tumor microenvironment. The tumor microenvironment is a niche in which a tumor's growth is fostered. Chemokine IL-8 also increases vascular density in tumors and is implicated in supporting angiogenesis. In addition IL-8 has been shown to attract tumor-infiltrating macrophages, which also enhance the microenvironment for tumor growth and cancer progression. Follow up studies with mouse xenografts supported the role of IL-8 in HSA tumor development. When IL-8 signaling was blocked the transplanted HSA tumors were reduced in growth by 50% compared to controls [72]. The connection between TF and IL-8 expression in HSA has not been explored. PAR2 by TF-FVII also induces expression and release of VEGF. Glioblastomas, which are highly vascularized aggressive cancers, release VEGF after PAR2 signal transduction [73]. Compared to hemangiomas, benign endothelial growths, HSA has higher VEGF expression as seen by immunohistochemistry [74]. Interestingly in p53-deficient mice inhibition of VEGF signaling in endothelial cells reduced the incidence of spontaneous murine HSA development. This indicates that VEGF signaling is involved in progression of endothelial cell transformation into HSA [75]. Interestingly the use of low molecular weight heparin in patients with cancer-associated coagulopathies has decreased rates of metastasis.

Many studies have observed a connection between the presence of TF and aggressive metastatic cancer phenotypes and the mechanisms of TF signaling involvement in cancer progression, metastasis and tumor microenvironment is still actively being researched. Further characterization of the role of TF could lead to developments in cancer-associated coagulopathy treatments.

Conclusion

The studies presented in this thesis examine the role of TF expression in the procoagulant activity seen in HSA using an in vitro system. No previous studies have reported TF expression on HSA. One study has reported TF expression in an AS derived cell line, but no further studies into the role of TF in AS were performed [76]. No studies have examined the thrombotic potential of cells derived from AS and HSA tumors. TF-dependent procoagulant activity associated with these cancers could drive future target therapy to reduce coagulopathies in patients with HSA and AS. In addition coagulation profiling could lead to earlier diagnosis of TF-expressing HSA in dogs. Chapter 2 demonstrates the expression of procoagulant TF on HSA cell lines. Hemangiosarcoma cells are shown to produce TF mRNA and express TF on their surface. In addition these cells generate thrombin in plasma. Thrombin generation was shown to be dependent on the presence of FVII and PS, supporting a role for TF in procoagulant activity of cells derived from HSA. Chapter 3 summarizes conclusions based on the presented data and explores future directions. A functional TF knockdown was created that could be useful for future experiments examining the role of TF in the cancer progression and metastasis. Further

characterization of the role of TF in HSA and AS is crucial for the development of targeted therapies and diagnostics.

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CHAPTER 2

PROCOAGULANT TISSUE FACTOR EXPRESSION IN HEMANGIOSARCOMA CELL LINES*

Abstract

Background: Hemangiosarcoma (HSA) is a common, highly aggressive and metastatic endothelial cell tumor that is frequently associated with disseminated intravascular coagulation (DIC) in dogs.

Objective- To determine whether canine hemangiosarcoma cells express tissue factor (TF) and are procoagulant.

Samples- Cell lines derived from a mouse passaged canine HSA (SB), metastatic (Emma brain) and primary splenic canine HSA (Frog, Dal-4) and non-neoplastic canine aortic endothelium (CnAoEC).

Procedures- TF mRNA and antigen expression were evaluated by quantitative reverse transcriptase PCR and flow cytometric analysis with a murine monoclonal anti-canine TF antibody, respectively. Procoagulant activity was measured as lag time and area under the curve (AUC) generated from calibrated automated thrombography using canine plasma and human replete or specific coagulation factor (F)-deficient plasma.

Results-All HSA cells expressed more TF mRNA and surface antigen than CnAoEC. TF mRNA and antigen was differentially expressed in HSA cells, with highest expression in SB cells. Canine HSA cells generated significantly more thrombin than CnAoEC in canine and human replete plasma. Thrombin generation in SB cells was cell number dependent and was significantly reduced and abolished in human FVII- and FX-deficient plasma, respectively.

Residual thrombin generation in FVII-deficient plasma was abolished with the addition of Annexin V, a phosphatidylserine-binding protein. Thrombin generation in SB cells was unaltered in human plasma deficient in factors XII, IX, XI, and VIII or by addition of corn trypsin inhibitor, a FXII inhibitor. The non-mouse passaged HSA lines also generated thrombin in a FVII- and FX-dependent manner, but to a lesser degree than SB cells.

Conclusions and Clinical Relevance- HSA cell lines express procoagulant functional TF. TF expression on tumor cells could explain the high incidence of hemostatic dysfunction in dogs with HSA. TF on cancer cells could serve as a biomarker for potential DIC and potential therapeutic target in dogs with HSA.

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Author Contributions: LW designed and performed calibrated automated thrombography, flow cytometry, real time PCR experiments with SB cells and primary HSA cell lines, performed statistical analysis and drafted the manuscript. EG helped conceive of and design the study providing preliminary data for the experiments. FLZX gathered primary cell calibrated automated thrombography data. JD and CD validated the mouse monoclonal canine tissue factor antibody (133-2). BW provided the hybridomas and helped develop the mouse monoclonal canine tissue factor antibody (133-2). TS developed the mouse monoclonal tissue factor antibody (133-2), conceived of the study, participated in its design and coordination, helped with statistical analysis, and helped draft the manuscript.

Introduction

Hemangiosarcoma (HSA) is a malignant tumor of endothelial origin in dogs. Dogs with HSA often present with concurrent hemostatic abnormalities particularly disseminated intravascular coagulation (DIC) [1]. It has been estimated that 50% of dogs suffer from concurrent DIC, which contributes to the immediate death in up to 25% of affected dogs. DIC is defined as uncontrolled thrombin generation as a consequence of activation of coagulation. Tissue factor (TF), also know as thromboplastin and coagulation factor III, is the main initiator of coagulation. During normal hemostasis, TF on fibroblasts in the perivascular space is exposed to coagulation factors in the blood upon endothelial cell injury. Once exposed to plasma, TF binds to and acts as a cofactor for coagulation factor VII (FVII). The TF-FVIIa complex subsequently binds and activates factor X (FX), leading to thrombin generation and eventual fibrin clot formation [2]. TF is not normally expressed on endothelial cells but can be upregulated by inflammatory cytokines in disease states, such as sepsis [3, 4]. In the latter diseases, TF is considered the main trigger for initiation of DIC [5].

Thrombosis is a known sequela of malignant tumors in humans [6], with higher rates of thrombosis in patients with metastatic cancer [7]. Prothrombotic conditions, such as thromboembolism and DIC, increase the morbidity and mortality of cancer patients [8, 9]. Angiosarcoma, the human equivalent of hemangiosarcoma, is a highly metastatic tumor with a poor prognosis. In one retrospective study, human patients with angiosarcoma had a higher risk of DIC (17%) compared to those with other types of solid tumors (7%) [10]. Aberrant or over-expression of TF is now thought to be one of the main mechanisms by which tumors activate

hemostasis, including DIC [11]. Various types of cancer, including those of epithelial and mesenchymal origin, express high concentrations of TF, with expression being recognized as a marker of thrombotic risk [12], histologic grade and prognosis [13, 14].

Hypercoagulability (excessive activation of coagulation), thrombosis and hemostatic dysfunction are clinical complications in dogs with various cancers [15]. The pathogenesis of hypercoagulability in cancer in dogs is unknown. Functional TF expression was recently demonstrated in canine epithelial and fibrosarcoma tumor cell lines in vitro [16]. A recent study examined TF expression in canine mammary tumors in relation to markers of hemostasis and inflammation and found evidence of hemostatic dysfunction, including DIC, in dogs bearing tumors with higher TF expression [17]. These results suggest TF expression on cancer cells may be the cause of hypercoagulability and DIC in dogs with HSA. It is not currently known if TF is expressed and functionally procoagulant (i.e. can generate thrombin) on canine HSA. We hypothesized that canine HSA cells would express TF and demonstrate TF-dependent procoagulant activity, specifically the ability to activate FX and generate thrombin. The purpose of this study was to test this hypothesis in vitro using a combination of mRNA and flow cytometry for protein expression and calibrated automated thrombography (CAT) for thrombin generation on HSA cell lines derived from primary tumors in dogs and normal canine aortic endothelial cells.

Materials and Methods

Cells— The following canine HSA cell lines were used in the study and were a gift from Dr. J. Modiano (University of Minnesota): Mouse-passaged SB cells derived from a subcutaneous HSA, a metastatic brain HSA (Emma Brain), and primary splenic tumors from two dogs (Frog, Dal-4). HSA cell lines were cultured in FK-12 media supplemented with 10% fetal bovine serum, 0.05mg/mL endothelial cell growth supplement (ECGS) (BD Bioscience, San Jose, CA, USA), 10mM HEPES (Gibco, Grand Island, NY, USA), 0.01mg/mL heparin (Sigma-Aldrich, St. Louis, MO, USA), and 100ug/mL primocin (InvivoGen, San Diego, CA, USA). Normal canine endothelial cells (CnAoEC, Cell Applications, San Diego, CA, USA) were cultured in canine endothelial cell growth media (Cell Applications). All cells were maintained in 5% CO_2 humidified atmosphere at 37°C. Cells were detached with 0.25% trypsin–EDTA, and trypsin was neutralized with growth medium. Cells were used in the log phase of growth between 60-80% confluency and were 90% viable based on trypan blue exclusion.

Quantitative real time PCR—Total RNA was isolated from harvested cells using a commercial kit (Qiagen RNeasy, Valencia, CA, USA) with concentration and purity of total RNA determined by UV measurements at 280nm/260nm (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA). cDNA was synthesized from 1ug total RNA using a commercial kit with oligo$(dT)_{20}$ primers (Superscript® III RT system, Life Technologies, Grand Island, NY, USA). Quantitative real time PCR (qRT-PCRT) was performed using the SYBER® Green Master Mix (Applied Biosystems, Austin, TX, USA), with primers specific for canine TF that were designed to span the exon 2 and 3 junction of canine TF DNA (Forward: 5' AGTGGGAACCCAAACCCATC 3';

Reverse: 5' ATGGAGGCTCCCCAGAGTAG 3') (IDT, Coralville, IA, USA). The canine ribosomal protein subunit 5 (RPS5) (Forward: 5' TCACTGGTGAGAACCCCCT 3'; Reverse: 5'GTTCTCATCGTAGGGAGCAAG 3', IDT) was used as a house-keeping gene, using published primer sequences [18]. The following PCR conditions were used: 2 min at 50°C ,10 min at 95°C, followed by 40 cycles of 15 s at 95°C, then 1 min at 57°C (PTC 200 PCR Thermal Cycler, MJ Research, Ramsey, MN, USA). Amplification curves and cycle threshold (Ct) values were collected and analyzed by specific software (StepOne Software v2.3, Life Technologies). Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with canine TF mRNA expression being normalized to the endogenous control RPS5.

Flow cytometry—Detached cells (1 X 10^5 cells/ reaction) were resuspended in phosphate-buffered saline (PBS) with 1% bovine serum albumin and 0.05% sodium azide (PBSA) and were incubated with a murine IgG_1 monoclonal anti-canine TF antibody (clone 133-2) that was generated in the laboratory or murine IgG_1 (both at a final concentration of 20 μg/mL) for 30 minutes on ice. The mouse monoclonal anti-canine TF antibody (clone 133-2) was developed with the help of Dr. Bettina Wagner and validated previously in our lab (data not shown). After washing this was followed by a secondary Alexafluor® 488-conjugated goat anti-mouse IgG (1:200, Life Technologies, Eugene, OR, USA) for 30 minutes on ice. Cells were washed twice in PBSA and then suspended in PBS and analyzed with a flow cytometer (FACSCalibur, BD Bioscience, San Jose, CA, USA). For data acquisition, forward scatter (FSC-H) was set at a voltage of E-1, ampere gain of 4.75, and linear mode. Side scatter (SSC-H) was set at a voltage of 350 V, ampere gain of 1.00, and linear mode. Fluorescence was set on log mode and 10,000 events were counted in a gate set around the majority of cells on a FSC versus SSC dot plot (R1,

Figure 2.1). For analysis, dot plots of SSC by fluorescence were examined for positive reactions, being defined as events falling within a gate established with the isotype control for each cell line (R2, Figure 2.2). Less than 5% of cells labeled with the isotype control fell within this R2 positive fluorescence gate. The percentage and median fluorescent intensity of positive cells in the R2 gate was determined using commercial software (FlowJo V.10, Tree Star Inc., Ashland, OR, USA).

Preparation of plasma—Pooled canine plasma (a gift from Dr. M. Brooks in the Comparative Coagulation Laboratory, Cornell University), human factor-replete plasma (Full) containing all coagulation factors and human plasma deficient in specific coagulation factors (obtained from patients with severe factor deficiencies, George King Biomedical, Overland Park, KS, USA) were depleted of microparticles before use in thrombin generation assays. This was accomplished by 2x 20 min centrifugation at 13,523*g* at 4°C. Plasma was aliquoted and stored at -80°C until use and was discarded after thawing at 37°C.

Calibrated automated thrombography— An *in vitro* global coagulation assay, which measures thrombin generation over time in plasma, reflecting initiation, amplification and termination phases, was used [19]. In clinical settings, patient plasma is usually combined with an external clotting trigger, consisting of negatively charged phospholipids and recombinant human TF. In our in vitro manipulation of this assay, canine HSA cells were the source of negatively charged phospholipid and TF. No external trigger was added. To perform this assay, detached cells were washed free of culture media with PBS and suspended at various concentrations (0.5 to 2 x 10^4) in 20uL HEPES-saline buffer (10mM HEPES, 137uM sodium

chloride, 4mM potassium chloride, 10uM glucose, pH 7.05) in a black 96-well plate (FluoroNunc with MaxiSorp, Fisher Scientific, Pittsburgh, PA, USA). Then, 30ul of microparticle-depleted plasma was added, followed by 50ul of fluorescent substrate (Technothrombin TGA substrate, Technoclone GmbH, Vienna, Austria). Fluorescence was then measured every minute at 360nm/460nm for 2 hours at 37°C on a 96-well plate fluorescent spectrophotometer (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA). A plasma control containing no cells (only HEPES-saline buffer added) and a positive human control from the substrate manufacturer (TGA CH and RCH, Technothrombin) were included in each run. All samples were run in duplicate and experiments performed a minimum of three times. Thrombograms were generated by comparing raw fluorescence output to a standard curve of thrombin calibrator (Technothrombin Calibrator), using specific software (Technothrombin software). Numerical results provided from the software were peak thrombin concentration, velocity of thrombin generation and area under the curve (AUC) or total amount of thrombin generation (Figure 2.1). Due to a malfunction with the proprietary software that was discussed with the manufacturer the lag time or time to initiation of thrombin generation was measured manually as the first minute when thrombin concentration increased above 4nM. In select experiments, corn trypsin inhibitor (CTI, 50ug/mL, Haematologic Technologies, Essex Junction, VT, USA) and phosphatidylserine-binding protein, Annexin V (human placenta-derived, Sigma Aldrich, St. Louis, MO, USA), were added to plasma before addition of cells. For Annexin V studies, the cells were diluted in Annexin binding buffer (10 mM HEPES, 150 mM sodium chloride, 5mM potassium chloride, 1mM magnesium chloride, and 1.8 mM calcium chloride, Trevigen, Gaithersburg, MD, USA).

Statistical analysis—Data was Gaussian and expressed as means ± standard deviations. Comparison of mean results of the same cell line with different treatments or different cell lines with the same treatment was done with a paired or student T test, respectively. Comparison of 3 or more means was performed using a 2-way Analysis of Variance with a Tukey's multiple comparison post-test. Comparison of means to a control was done using a 2-way Analysis of Variance with a Dunnet's post-test as well. Statistical analysis was performed with commercially available software (Prism 5, GraphPad Software, INC, La Jolla, CA). *A P* value set at <0.05.

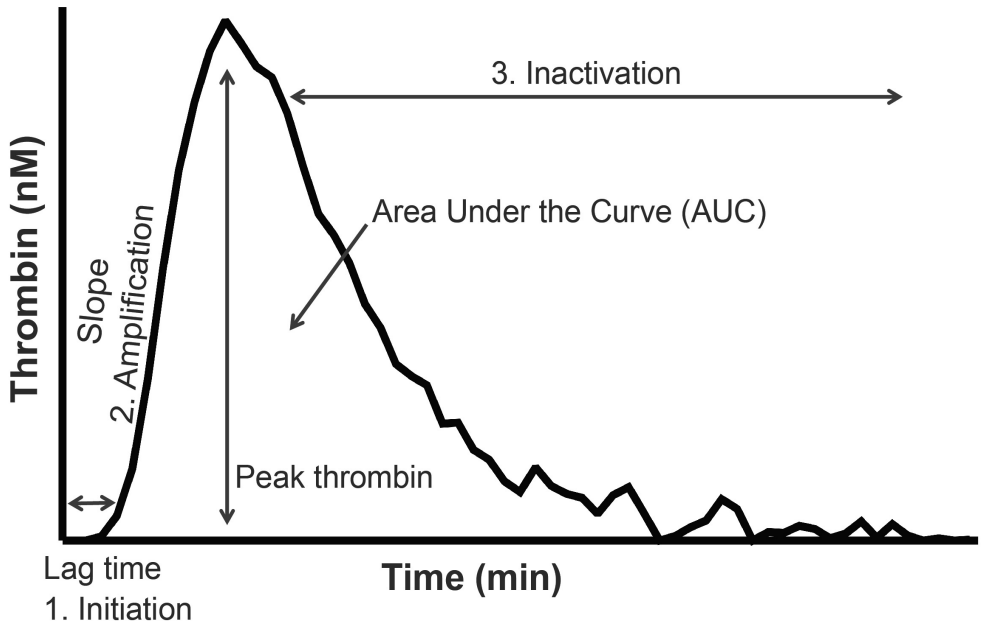

Figure 2.1- Thrombin generation curves generated by calibrated automated thrombography. This technique creates a thrombogram of thrombin generation in nM over time in minutes, which reflects initiation, amplification and inactivation of thrombin generation. 1. Initiation is the time it takes for a small amount of thrombin to be produced (4 nm) and is quantified as the lag time. The lag time is dependent on the trigger (in this study, the cancer cells). 2. Amplification reflects the thrombin burst created by thrombin activation of the intrinsic pathway of coagulation on phospholipid-rich surfaces and is quantified by the slope of the thrombogram, peak thrombin and time to peak (not shown). This phase is dependent on coagulation factors in the added plasma, their assembly into complexes on phosphatidylserine-rich surfaces as well as inhibitors, which limit the amount of thrombin generated. 3. Inactivation of thrombin generation occurs once thrombin inhibitors in the plasma stop thrombin generation. The area under the curve (AUC) reflects the total amount of thrombin generation or the combination of all phases.

Results

Canine HSA TF mRNA and protein expression

To confirm the presence of TF mRNA and cell surface expression in HSA cells we used a combination of qRT-PCR and flow cytometry. All cell lines expressed TF mRNA, with higher TF mRNA amplification in all HSA cell lines compared to CnAoEC. mRNA expression was highest in the mouse-passaged SB and metastatic-HSA Emma Brain cells compared to the primary splenic HSA cell lines, Frog and Dal-4 (Figure 2.3). Canine HSA cells also expressed more TF antigen on their cell surfaces than CnAoEC, as measured by flow cytometry, with expression matching mRNA results (Figure 2.1, Table 2.1). A significantly higher percentage of SB cells expressed TF antigen compared to the other HSA cell lines and both SB and Emma brain cells had significantly higher median fluorescent intensity of TF antigen expression than the other HSA cell lines.

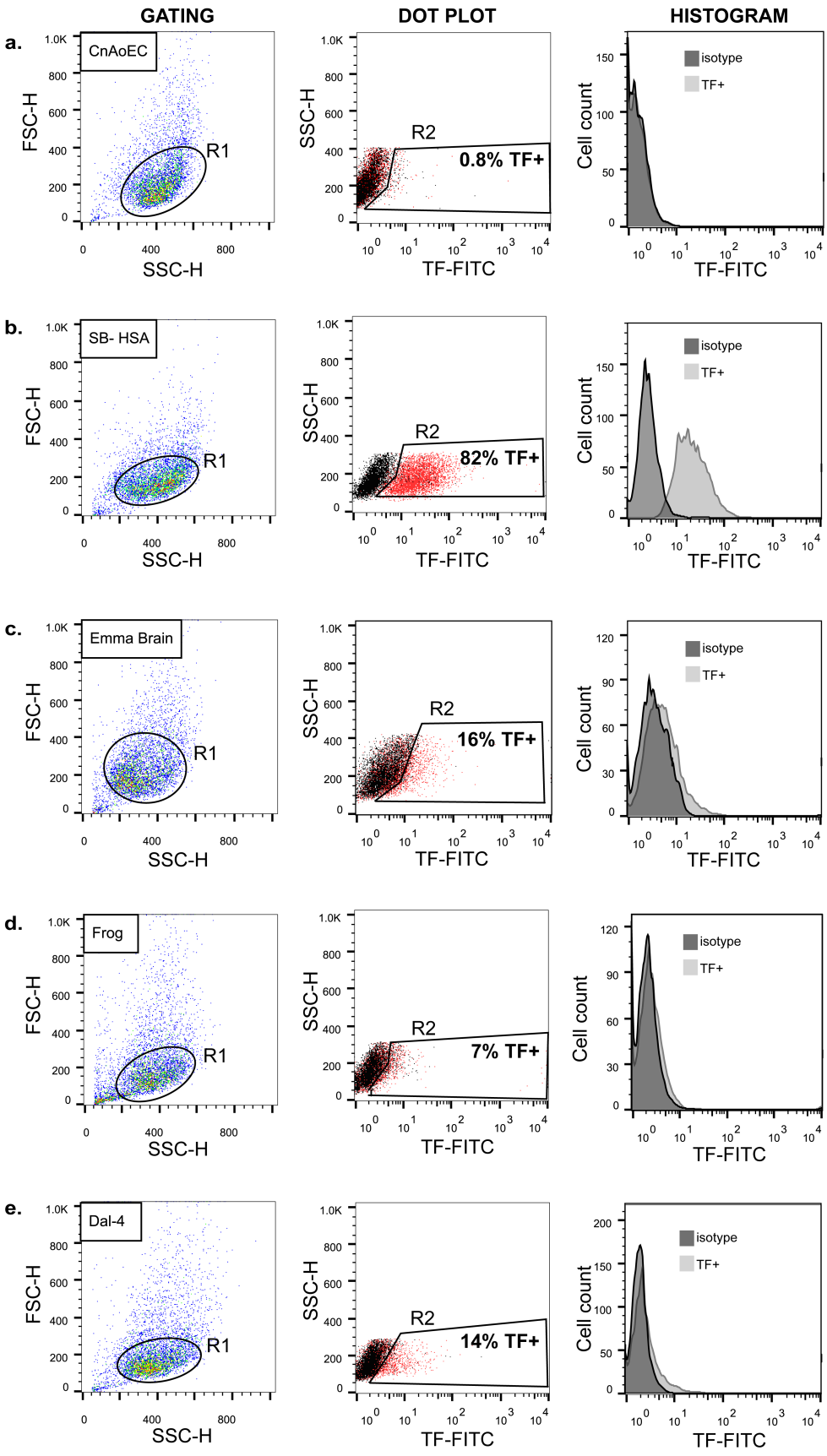
GATING
DOT PLOT
HISTOGRAM
a.
CnAoEC
R1
R2
0.8% TF+
b.
SB- HSA
R1
R2
82% TF+
c.
Emma Brain
R1
R2
16% TF+
d.
Frog
R1
R2
7% TF+
e.
Dal-4
R1
R2
14% TF+
FSC-H
SSC-H
TF-FITC
Cell count
isotype
TF+

Figure 2.2- HSA cell express surface TF compared to canine endothelial cells.

Gating strategies for flow cytometric detection of tissue factor (TF) surface expression on canine normal and neoplastic cell lines: a. Normal canine aortic endothelial cells (CnAoEC), b. mouse xenograft canine HSA (SB), c. Metastatic brain HSA (Emma Brain), d. Primary splenic HSA (Frog), and e. Primary splenic HSA (Dal-4).

All cell lines express TF antigen to various degrees, with the least and highest expression on the CnAoEC and SB cells, respectively.

Left panel (gating): Cell events were gated (R1) on a forward scatter (FSC-H, measure of cell size) and side scatter (SSC-H, measure of cell complexity) dot plot. The R1 gate was drawn around the densest population of cells that was above 200 arbitrary units on the X-axis to avoid cell debris.

Middle panel (dot plot): Within the R1 gate, TF-positive cells were quantified within a gate (R2) created on fluorescence (TF-FITC) versus SSC-H dot plot of cells labeled with a FITC-conjugated murine monoclonal antibody against canine TF (red events). The R2 was based on isotype control (black events).

Right panel (histogram): Frequency distribution histograms of cells labeled with the isotype control (dark grey) or anti-canine TF antibody (light grey).

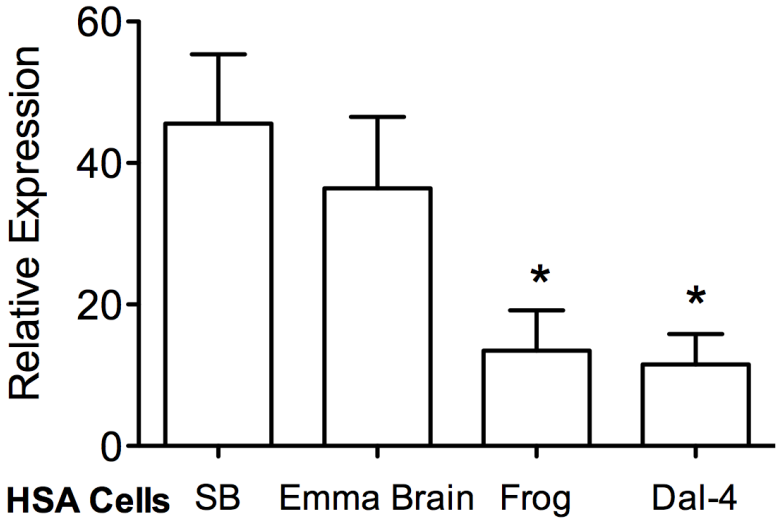

Figure 2.3: Tissue factor (TF) mRNA expression in canine HSA cells.

TF mRNA is expressed in higher concentrations in canine neoplastic compared to normal endothelial cells. Mean ± SD of relative mRNA expression is shown for mouse xenograft hemangiosarcoma (HSA) (SB), metastatic brain HSA (Emma Brain), and primary splenic HSA (Frog and Dal-4) cell lines. TF mRNA expression is relative to canine endothelial cells (given a value of 1) and normalized to expression of the ribosomal protein subunit 5 as a reference gene, using the $2^{-\Delta\Delta Ct}$ method (n=3). All HSA cell lines expressed more TF than normal canine aortic endothelial cells. The highest mRNA expression was seen in SB and Emma Brain cells, both of which were significantly greater than the expression in Frog and Dal-4 (*P <0.005).

Canine cell line	Percentage	MFI (arbitrary units)
CnAoEC	0.89 ± 0.27	5.5 ± 0.17
Mouse xenograft HSA (SB)	83.3 ± 0.5*	20.3 ± 4.4*
Brain metastatic HSA (Emma Brain)	16.8 ± 7.9*†	20.6 ± 9.1*
Primary splenic HSA (Frog)	6.82 ± 4.4†	7.4 ± 1.9
Primary splenic HSA (Dal-4)	13.9 ± 3.8*†	7.2 ± 1.8

* $P < 0.05$ versus CnAoEC
† $P < 0.05$ versus SB cells

Table 2.1- Flow cytometric evaluation of surface TF expression.

Mean ± SD percentage and median fluorescent intensity (MFI) of normal canine aortic endothelial cells (CnAoEC) and hemangiosarcoma (HSA) cell lines positive for surface tissue factor (TF) as detected by a murine monoclonal anti-canine TF antibody with flow cytometry (n=3). The TF -positive cells were located in a gate based on the autofluorescence of an isotype control for each cell line (R2, see Figure 1).

HSA cells generate thrombin

We first determined if HSA cells generated more thrombin than the non-neoplastic CnAoECs. We found that all HSA cells, but not CnAoECs, generated thrombin in canine plasma (Figure 2.4a). The mouse xenograft SB cell line generated significantly more thrombin with a shorter lag time than the other primary HSA cell lines (except for Emma Brain AUC) in canine plasma (Figure 2.4c-d). Similarly in human factor-replete plasma, all HSA cell lines generated significantly more thrombin with a shorter lag than CnAoECs (Figure 2.4b-d). However, in contrast to canine plasma, CnAoEC did generate thrombin in human factor-replete plasma and differences in lag time and total thrombin generation between HSA cell lines were no longer apparent (Figures 2.4c-d). In addition, the lag times were significantly slower with higher total thrombin generation in human factor-replete versus canine plasma. Thrombin generation in both canine and human factor-replete plasma was cell-dependent, since no thrombin generation occurred in the absence of cells (HEPES buffer alone in canine or human plasma; data not shown). The positive human control generated large amounts of thrombin in all experiments (data not shown).

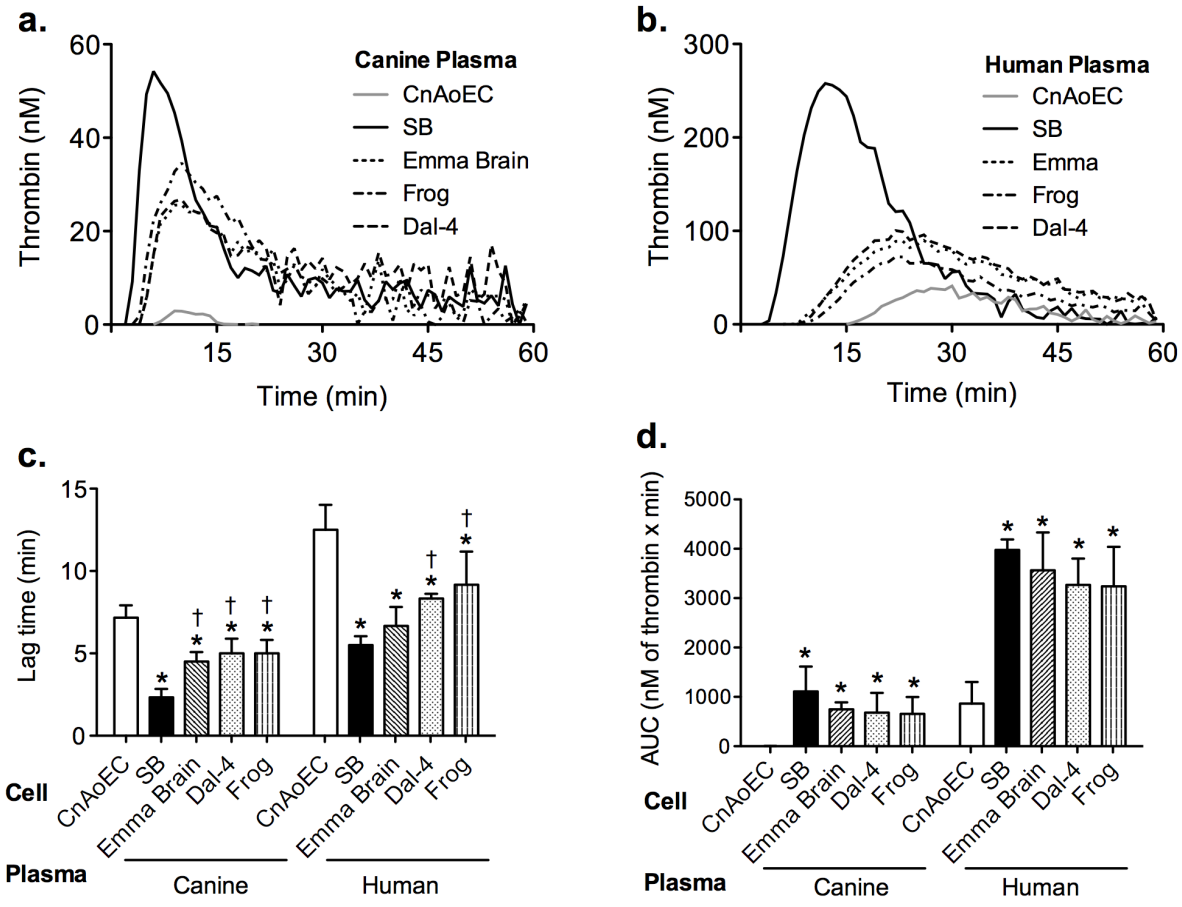

Figure 2.4: Thrombin generation in canine and human plasma.

Canine hemangiosarcoma cell lines (HSA) generate more thrombin in canine and human plasma than canine aortic endothelial cells (CnAoEC). Compilation thrombograms of CnAoEC, mouse xenograft HSA (SB), brain metastatic HSA (Emma Brain), and splenic HSA (Frog and Dal-4) in canine plasma (**a**) and human plasma (**b**). Lag times were significantly shorter (**c**) and more total thrombin (area under the curve, AUC) was generated (**d**) in HSA cells compared to CnAoEC in both canine and human plasma, using $2x10^4$ cells in the reaction (n=3). The lag times were longer and more thrombin was generated in human versus canine plasma for all cell lines (P <0.001).

* P <0.05 versus CnAoEC in canine or human plasma.

† P <0.05 versus SB cells in canine or human plasma.

Thrombin generation is triggered by the extrinsic pathway of coagulation and is independent of the contact pathway of coagulation

Thrombin generation can be initiated through the extrinsic or intrinsic pathways of coagulation. In the extrinsic pathway, the TF/FVII complex initiates thrombin generation via activating FX of the common pathway. The intrinsic pathway is activated by factor XII (FXII), which initiates a cascade of enzymatic reactions, culminating in FX activation. Activated FX complexes with cofactors to form a prothrombinase, which generates thrombin from prothrombin [20]. Our next objective was to parse out which of these pathways was initiating thrombin generation in HSA cells. Because specific-factor deficient canine plasma and inhibitory antibodies against canine coagulation factors are not available, we used commercially available specific factor-deficient human plasma to elucidate the role of the different coagulation factors in thrombin generation by HSA cells. Rationale for the use of human plasma comes from a previous study showing that canine TF can efficiently bind to and act as a cofactor for both canine and human FVII [21]. We first measured thrombin generation by SB cells (as our model HSA line) in FX-deficient plasma to assess if thrombin was generated by activation of FX and not a cancer-associated prothrombinase. Thrombin generation by SB cells was abolished in the FX-deficient plasma, indicating that activated FX in the human plasma is responsible for thrombin generation (Figure 2.5a, c, d). We then tested intrinsic pathway factors and found that the total amount of thrombin generated and lag time was unaffected in SB cells suspended in plasma deficient in intrinsic pathway factors (Figure 2.5a, c, d). Although AUC was not significantly different in human factor-replete and intrinsic coagulation factor-deficient plasma, we did find a trend towards a decrease in the rate (slope) of thrombin generation in plasma deficient in FIX and FVIII (Figure 2.5a, Table 2.2). This highlights the role of FIX and its

cofactor FVIII in amplifying thrombin generation [20]. We found that there was a faster rate of thrombin generation in human plasma deficient in factors XI and XII, which we attributed to individual variation in the donors that provided the plasma. In contrast to intrinsic coagulation factor-deficient plasma, SB cells generated significantly less thrombin at a slower rate with a slower lag time in FVII-deficient human plasma ($p<0.001$ for AUC and lag time, Figure 2.5a, c, d, Table 2.2). Similar results were seen for the other cell lines (Supplemental Figure 1).

In vitro studies of coagulation can be complicated by artificial activation of FXII through contact with plastic surfaces. While our results with FXII-deficient plasma suggest that the contact pathway is not triggering thrombin generation with SB cells, we confirmed these results by adding a chemical inhibitor of FXII, CTI, to human-replete plasma. We also added CTI to FVII-deficient plasma to determine if contact activation was responsible for the residual thrombin generation in this plasma. Addition of CTI did not affect thrombin generation of SB cells in either plasma (Figure 2.4b), indicating that contact activation of FXII is not occurring in the system or responsible for residual thrombin generation in FVII-deficient plasma. Taken together, our data with factor-deficient plasma and CTI show that activated FX mediates thrombin generation in HSA cells and FX is activated through the extrinsic pathway of coagulation, i.e. the TF-FVIIa complex on tumor cell surfaces.

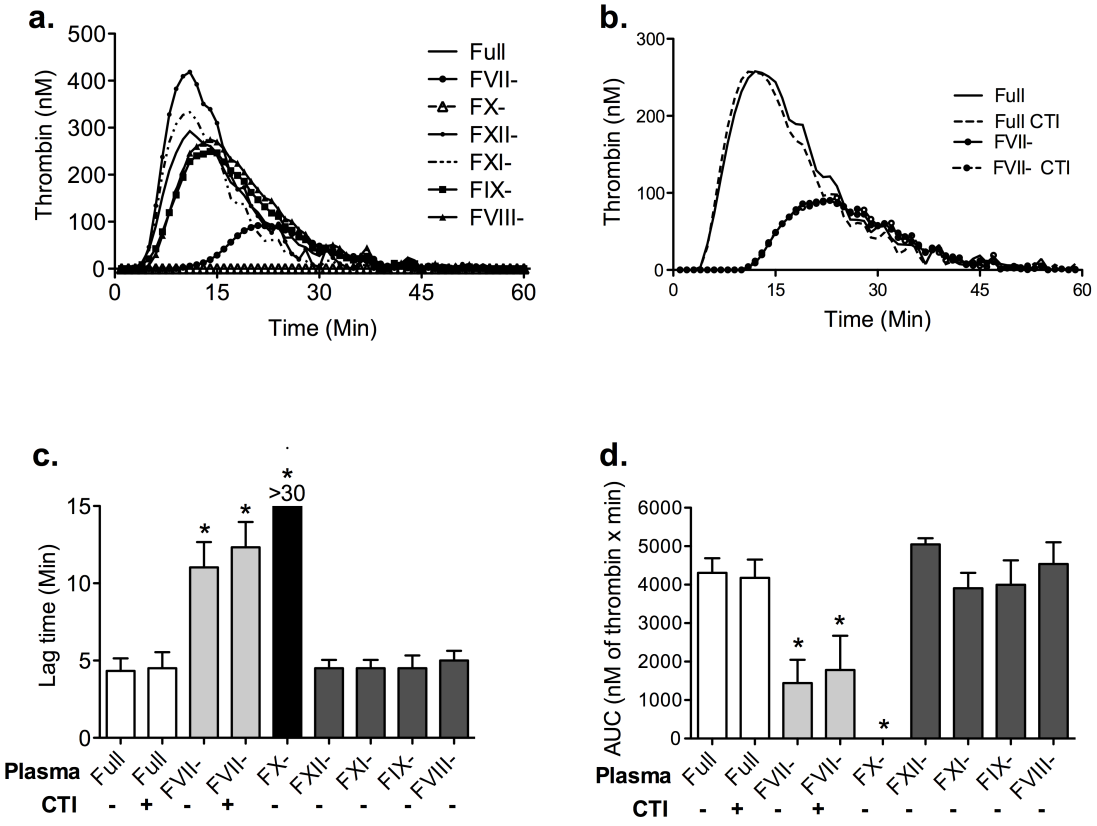

Figure 2.5- Thrombin generation is dependent on the extrinsic and not the intrinsic pathway of coagulation.

Compilation thrombograms for mouse xenograft hemangiosarcoma (SB) cells ($2x10^4$/reaction) in human plasma replete in all factors (Full) or deficient in specific factors (F-) showed no and reduced thrombin generation in FX- and FVII-deficient plasma, respectively. Thrombin generation was largely unaltered in plasma deficient in intrinsic coagulation factors XII, XI, IX and VIII (**a**). Representative thrombograms of SB cells in Full or FVII-deficient plasma, showing that addition of corn trypsin inhibitor (CTI, 50ug/mL) had no effect on the thrombin curves (**b**). The lag time was significantly longer (**c**) with significantly lower total thrombin generation (area under the curve, AUC, **d**) in FX- and FVII-deficient plasma than in full plasma in the absence or presence of CTI (n=3).

* *P* <0.001 versus Full plasma for lag time and AUC.

Human plasma type	Slope (min/nM thrombin)
Factor-replete (Full)	45.4 ± 12.5
Factor VII-deficient (FVII-)	13.1 ± 4.8*
Factor XII-deficient (FXII-)	83.9 ± 18.2*
Factor XI-deficient (FXI-)	62.9 ± 19.6
Factor IX-deficient (FIX-)	29.6 ± 5.8
Factor VIII-deficient (FVIII-)	30.2 ± 7.4

* $P < 0.001$ versus factor-replete plasma

Table 2.2: Amplification of thrombin generation as represented by slope of thrombogram curves in factor-deficient plasmas.

Mean ± SD of the slope of thrombin generation of mouse xenograft hemangiosarcoma cells (SB) in human plasma replete in all coagulation factors or deficient in specific coagulation factors (n=3). The slope of the thrombogram represents the rate of thrombin generation, specifically amplification and propagation, and is dependent on coagulation factors and inhibitors in the plasma. The rate of thrombin generation was slower in plasma deficient in the intrinsic tenase coagulation factors (FIX, FVIII) and extrinsic coagulation factor VII, whereas it was faster in FXII-deficient plasma.

Residual thrombin generation in FVII-deficient plasma is dependent on cell number and phosphatidylserine

Since thrombin generation was dependent on FVII-mediated activation of FX, we were surprised that it was not abolished in FVII-deficient plasma in SB cells. Although the commercial plasma is identified as FVII-deficient, it is not totally lacking in FVII (<1% per supplier product information sheet). Very small amounts of TF (100 pM) are capable of triggering coagulation in normal plasma [22]. Thus, we reasoned that the residual thrombin generation in FVII-deficient plasma was due to the strong surface TF expression in the mouse xenograft HSA (SB) cells, which was sufficient to bind to and form a complex with the small amount of FVII remaining in the plasma. Accordingly, we found that thrombin generation in the lower-TF antigen expressing other primary HSA cell lines (Emma Brain, Frog, Dal-4) was nearly abolished in FVII-deficient human plasma at $2x10^4$ cells pre reaction (Figure 2.6). Thus, we reasoned that reducing the amount of available TF would eliminate the residual thrombin generation in SB cells in FVII-deficient plasma. We accomplished this by reducing the cell numbers added to the reaction (from 2×10^4 to 1 and 0.5×10^4) and found that thrombin generation was almost completely eliminated with 0.5×10^4 cells, with significantly longer lag times and lower AUC compared to 2×10^4 cells. Reduction in cell number had less of an effect in coagulation factor-replete human plasma (Figure 2.7). This supported our theory that residual thrombin generation by SB cells in FVII-deficient plasma is due to the high TF expression on the cells and reducing surface TF availability by decreasing cell numbers in the reaction below a critical threshold prevents thrombin generation in this system.

We also tested the role of phosphatidylserine in thrombin generation by the SB cells. Phosphatidylserine, a negatively charged phospholipid, is crucial for the full activity of the TF-FVIIa and other coagulation factor enzymatic complexes [23]. Annexin V is a protein that binds to and blocks exposed phosphatidylserine, preventing the subsequent binding of coagulation factors to the TF-bearing phosphatidylserine-containing cell membranes. To test whether Annexin V would inhibit thrombin generation by the SB cells, we added 5 and 10uM Annexin V from human placenta to $2x10^4$ SB cells for 20 min at room temperature in Annexin binding buffer before adding coagulation factor-replete and FVII-deficient human plasma. Cells incubated in Annexin binding buffer alone (0uM Annexin V) served as a negative control. The 20 min incubation at room temperature slightly, but not significantly, lengthened lag time and reduced the AUC (data not shown). There was no significant difference in lag time with the addition of Annexin V in factor-replete plasma (Figure 2.8a-b), whereas Annexin V at both concentrations significantly reduced AUC (P <0.0001) in this plasma (Figure 2.8a,c). This data indicates that initiation (by the extrinsic pathway) was unimpaired by inhibition of phosphatidylserine but amplification or the activity of assembled intrinsic factor complexes on the cancer cell membranes was affected, decreasing amplification. In FVII-deficient plasma, addition of Annexin V almost completely abolished residual thrombin generation at the highest SB cell number (P <0.0001 for lag time and AUC); only small amounts of thrombin were generated in 1 of 3 experiments. Both concentrations of Annexin V were equally efficacious in both plasma types. The data indicate that the residual thrombin generation in FVII-deficient plasma is dependent on cell number (and available TF) and phosphatidylserine for initiation of thrombin generation and maximal thrombin generation by the membrane-assembled coagulation factor complexes.

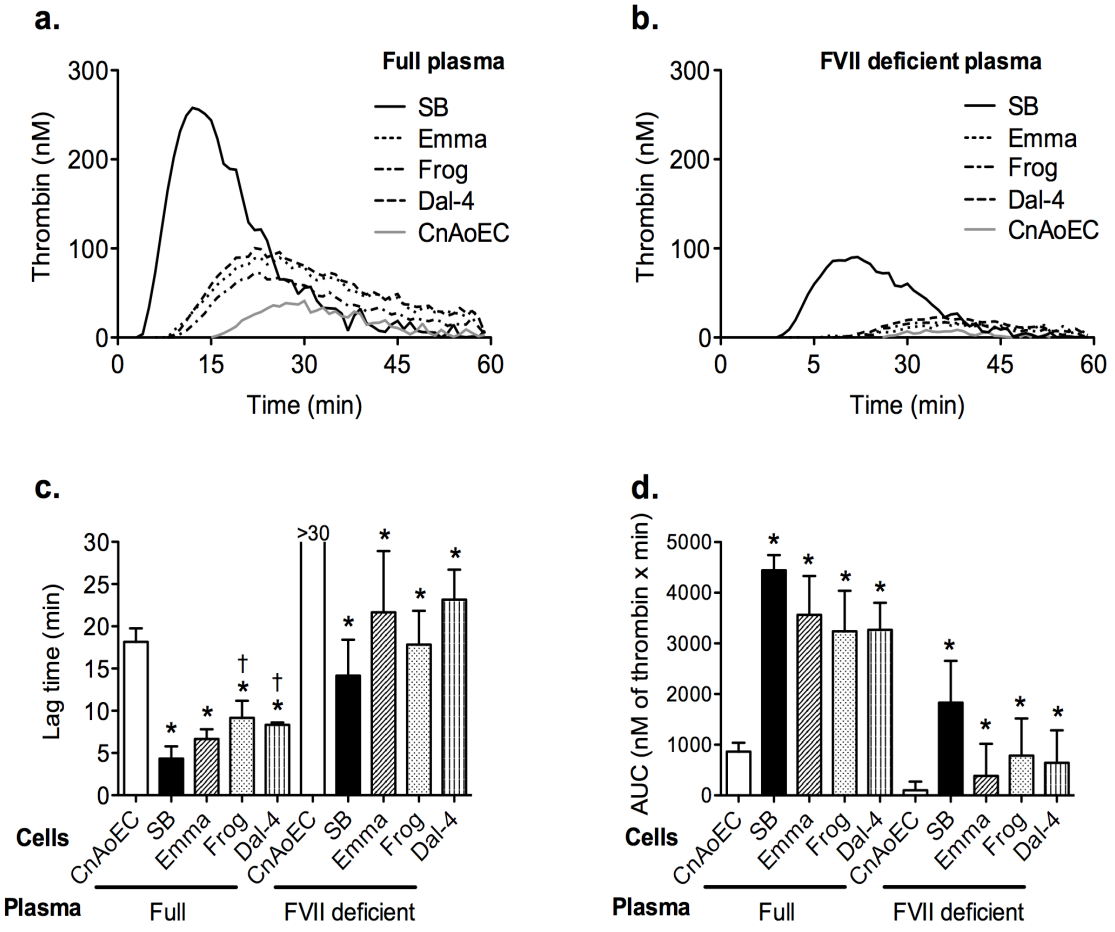

Figure 2.6: Canine primary hemangiosarcoma (HSA) cells produce thrombin in a Factor X (FX)- and FVII-dependent manner.

Compilation thrombin generation curves of canine HSA cell lines (SB, Emma Brain, Frog, Dal-4) and the normal canine aortic endothelial cells (CnAoEC) factor-replete human plasma (**a**) and FVII-deficient human plasma (**b**) at 2 x 10^4 cells/reaction. The lag time (**c**) and total amount of thrombin generated (area under the curve, AUC, **d**) was significantly greater for HSA cells than CnAoEC in factor-replete and FVII-deficient plasma. All canine HSA cells had significantly delayed and reduced thrombin generation in FVII-deficient versus factor-replete human plasma (P <0.005). In contrast to SB cells which had stronger thrombin generation compared to the other HSA cell lines, thrombin generation was almost eliminated in the lowering TF antigen-

expressing cell lines from the metastatic brain HSA (Emma Brain) and primary splenic HSA (Frog and Dal-4) in FVII-deficient plasma (n=3).

* $P < 0.05$ versus CnAoEC

† $P < 0.05$ for SB cells versus the other HSA cell lines.

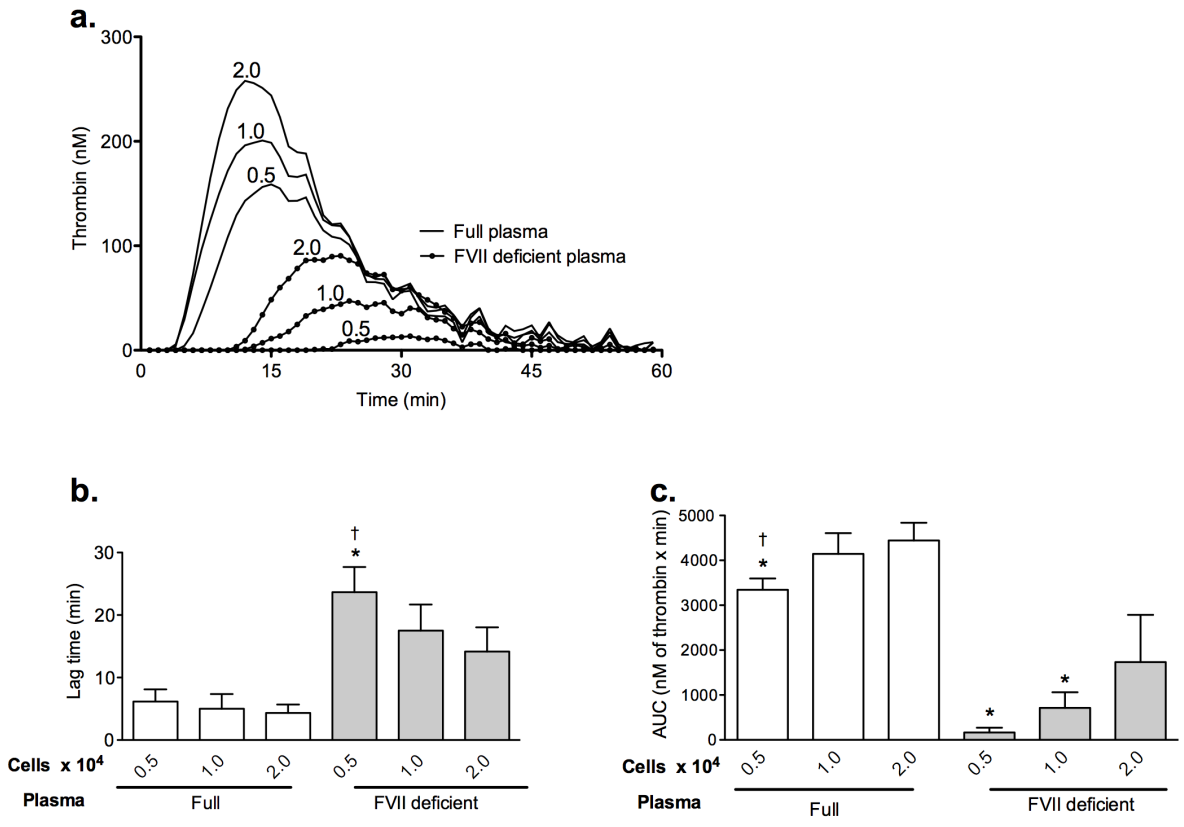

Figure 2.7: Thrombin generation in factor VII (FVII)-deficient plasma is cell number dependent in mouse xenograft hemangiosarcoma (SB) cells.

Decreasing cell numbers (2.0, 1.0 and 0.5x10^4) were added to factor-replete plasma (Full) and FVII-deficient human plasma and thrombin generation was measured. Compilation thrombograms showing changes in thrombin generation with decreasing cell number (denoted above each curve) in both plasma types (**a**). Lag time was only significantly decreased with the lowest cell number in FVII-deficient plasma (**b**) whereas total thrombin generation (area under the curve, AUC) was significantly decreased in both factor-replete and FVII-deficient plasma at the lower cell numbers (**c**) (n=3). Both lag time and AUC were significantly longer and smaller in FVII-deficient versus factor-replete human plasma at each cell concentration (P <0.0005).

* P <0.005 versus 2 x 10^4 cells.
† P <0.005 versus 1 x 10^4 cells.

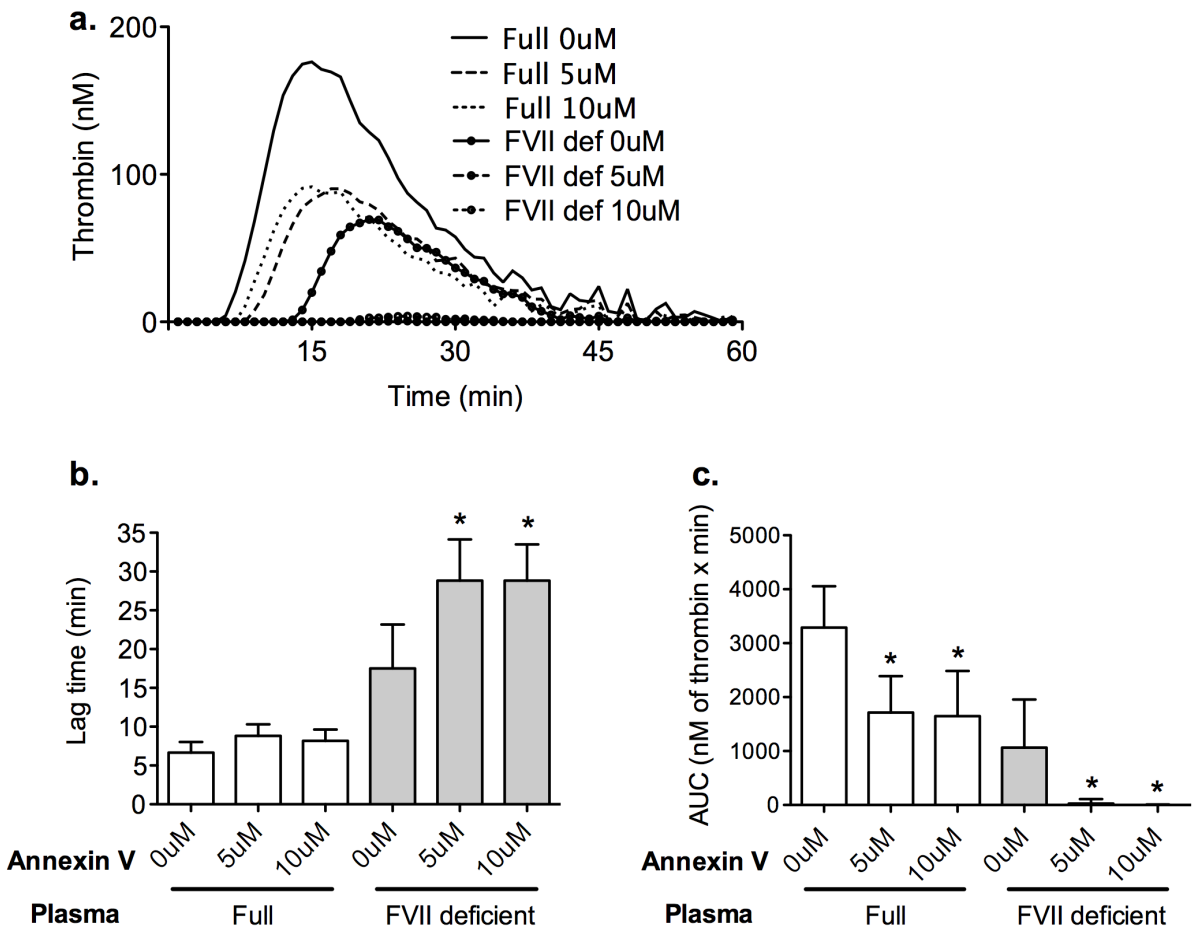

Figure 2.8: Thrombin generation is boosted by phosphatidylserine.

Annexin V, a phosphatidylserine inhibitor, was added (5 and 10uM) to $2.0x10^4$ mouse xenograft hemangiosarcoma (SB) cells in factor-replete and factor VII (FVII)-deficient human plasma. Compliation thrombograms with or without Annexin V in both plasma types (**a**). Lag time was significantly lengthened by addition of Annexin V in FVII-deficient human plasma only (**b**), whereas total thrombin generation (area undet the curve or AUC) was significantly decreased by Annexin V in factor-replete and FVII-deficient plasma (**c**) (n=3). Lag time and AUC were significantly lower in FVII-deficient versus factor-replete plasma with or without Annexin V (P <0.0005).

* P <0.0001 versus 0uM Annexin V control (0uM).

Discussion

Hemangiosarcoma is a highly malignant endothelial neoplasm in dogs. Many affected dogs succumb to thrombosis, including disseminated intravascular coagulation (DIC), even before the tumor is definitively diagnosed [1]. The cause of DIC in dogs with HSA has not been previously explored. In this study, we found for the first time that canine HSA cell lines derived from primary tumors constitutively produced TF mRNA and expressed TF antigen on their membrane surfaces. Tissue factor primarily functions as the initiator of coagulation in physiologic and pathologic states [2]. We concurrently found that HSA cell lines generate thrombin in a canine plasma-based in vitro CAT assay. The thrombin generation in this in vitro assay was triggered by the extrinsic pathway of coagulation and was dependent on FVII, cell numbers (associated with surface TF antigen expression) and cell membrane phosphatidylserine. Our data supports our theory that aberrant procoagulant TF expression on HSA is one of the mechanisms for initiation of DIC in dogs with this neoplasm.

We found that TF mRNA production did not predict the percentage of cells with surface antigen expression. The SB and Emma Brain HSA cells had similar levels of mRNA but significantly different percentages of cells with surface TF expression; with more SB cells expressing TF antigen than Emma Brain cells. The discrepancy in TF mRNA and protein expression in the 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 within the Golgi apparatus [24]. These stores could be mobilized in response to inflammatory cytokines, as

described for human fibroblast cells. These cells also mobilize TF to the cell surface following surface TF/FVIIa complex activation of protease-activated receptors 1 and 2 (PAR1, 2) [25]. Further studies are needed to characterize the intracellular pools and protein dynamics of TF in the canine aortic endothelial and HSA cell lines. Intracellular TF could be examined in several ways, such as performing flow cytometry, immunofluorescent staining on permeabilized cells or western blotting. Surface dynamics could be explored through biotinylation studies and the cells could be stimulated with pro-inflammatory cytokines to determine if they mobilize intracellular TF stores, as reported for human endothelial cells. Since inflammatory cytokines may be increased in dogs with HSA, higher levels of TF expression on tumor cells could be seen in vivo and may help trigger hypercoagulability in affected dogs. The difference between mRNA and protein levels could also be caused by changes in transcriptional regulation. Several alternatively spliced TF mRNAs have been found in normal and malignant cells, with higher levels in tumor cells [26]. The function of these untranslated alternatively spliced TF mRNAs is still unknown; current hypotheses suggest a regulatory function. Only one of these alternatively spliced mRNA has been shown to translated into a truncated TF protein, called alternatively spliced TF or asTF [27]. The function of this truncated TF, including its ability to trigger thrombin generation, is still under debate. Since TF surface expression will determine, to some extent, the procoagulant potential of the cells, understanding and controlling the factors that dictate surface expression may be important for reducing the incidence of DIC with this tumor.

Several differences were observed among the tumor cells in TF expression. SB cells had the highest cell surface TF expression with nearly all cells expressing the protein. The SB-HSA cell line was selected after causing tumor formation in immune-deficient mice. When this cell

line was established, it was shown to express markers of mitotically activated endothelial cells including vascular endothelial growth factor receptors 1 and 2, and $\alpha_v\beta_3$ integrin; and produce growth factors and cytokines, such as vascular endothelial growth factor, basic fibroblast growth factor and interleukin-8 [28]. Enhancement of TF expression could be an artifact of xenografting or activation, however we still found that non-mouse passaged primary HSA cell lines also expressed TF, albeit in fewer cells, suggesting that TF expression is a feature of these cells and not an artifact of xenografting. It is still possible that TF expression is an artifact of the cell culture system and it would be important to confirm in situ TF in canine tumors as described recently for canine mammary tumors [17].

Differences in TF mRNA and antigen expression were seen between the non-mouse passaged cell lines. Emma Brain, a cell line derived from a HSA metastasis to the brain, had the highest TF mRNA and antigen MFI of the primary cell lines. It is possible that these differences could relate to metastatic and procoagulant potential, with higher TF expression in more aggressive tumors. In a recent study assessing TF expression in canine mammary tumors, tumor cells from one dog with lymph node metastasis had the highest TF expression and was associated with DIC [17]. Tissue factor is often upregulated in metastatic compared to non-metastatic tumors in human patients [29, 30] and is thought to be associated with metastatic potential. This could also be true for canine HSA, but further studies, particularly those examining TF expression in primary and metastatic tumors in situ, are needed.

Fairly small populations (less than 20%) of Emma Brain, Frog and Dal-4, expressed TF on their surface and yet all HSA cell lines generated thrombin in canine plasma. Similar results

were reported for in situ expression of TF in canine mammary tumors [17]. Only surface-expressed TF is able to initiate coagulation [2], thus it is likely that these few cells are responsible for the observed procoagulant activity in the primary cell lines. The variability of TF expression within a tumor cell line is not unanticipated and thought to be a consequence of tumor heterogeneity, a well-established tumor characteristic. Functional variations arise in cancer cells as a result of different sets of acquired mutations, different microenvironmental cues and other factors. One model 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 [31]. Tissue factor is currently under investigation for its role in the formation of the cancer stem cell niche [32] and may be a marker of cancer stem cells, as shown for a human carcinoma cell line [33]. We have observed that all HSA cell lines express mRNA of the stem cell marker, SOX2 (unpublished data), but have not yet associated this with TF expression.

In order to test the role of the different activating pathways for thrombin generation, we used human plasma deficient in specific coagulation factors for thrombin generation. Human FVII can interact with canine TF and produce a functional extrinsic tenase; canine TF and human TF function equally well to initiate coagulation in human plasma [21]. In our study, canine cells triggered thrombin generation in canine and human plasma, however there were notable differences. Overall there was a longer lag time but larger amounts of thrombin generated in human compared to canine plasma. Canine FVIIa is more active than human FVIIa in a cross-species human-canine compatibility study [21]. This could account for the shorter lag time of HSA cells in canine plasma. The higher total thrombin generation is attributed to differences in

amplification and inhibition. The thrombogram curves showed that the AUC was increased in human plasma because of a higher peak and slower rate of termination, resulting in a broader curve. This suggests that inactivation is less efficient in human than canine plasma; potentially due to conformational or structural differences in how canine and human enzymatic coagulation complexes assemble on canine cell membranes. Human coagulation factor inhibitors, including tissue factor pathway inhibitor and antithrombin, may be less efficient at inhibiting enzymatic complexes bound to canine cells. No studies to date have looked into cross-species enzymatic activity of natural anticoagulants. We also noted that human but not canine plasma generated thrombin in CnAoEC. Since normal endothelial cells are not expected to express TF (as shown with our flow cytometric results for surface TF antigen expression) unless stimulated by bacterial endotoxin or proinflammatory cytokines [34], this result was unexpected. As the thrombin generation by CnAoEC in human plasma was FVII-dependent, we attributed this to stimulation of TF expression by the heterologous plasma. Regardless of the underlying mechanisms, the enhancement of thrombin generation of canine cells (normal or neoplastic) in human plasma did not alter interpretation of the results of our study.

We used FVII depletion as a surrogate for the role of surface TF in the procoagulant activity on HSA cells. Ideally, to directly test the role of TF in thrombin generation, we would need to inhibit TF with specific antibodies [35]. We tested several inhibitory anti-human TF antibodies including a murine monoclonal (hTF1) and a rabbit polyclonal antibody against human TF (Knudsen T, Novo Nordisk, personal communication), as well as our own murine anti-canine TF monoclonal antibodies, but unfortunately, none of these inhibited canine TF procoagulant activity in the CAT assay. The activity of activated FVII (FVIIa) is enhanced 5,000

fold by binding of TF and membrane surfaces, with the subsequent allosteric change in FVIIa structure [36]. At supraphysiologic concentrations (administration of recombinant FVIIa) FVIIa can activate FX and initiate thrombin generation without TF, but this is unlikely to occur at the levels found in normal canine or human plasma. In addition to TF, FVII can bind to the endothelial protein C receptor on endothelial cells. However, this interaction does not initiate coagulation and only occurs at supraphysiologic conditions of FVIIa [37]. Furthermore, it is unknown if FVIIa binds to the endothelial protein C receptor in dogs. Although not ideal, the only method currently available to evaluate the role of TF in procoagulant activity is indirectly by using FVII-deficient plasma as we did in this study.

In accordance with our observation that the TF-FVIIa complex is likely triggering thrombin activation in the canine HSA cells, we expected to see higher procoagulant activity in the metastatic HSA cell line correlating with Emma Brain's higher level of protein expression. Instead we saw that all primary cell lines had similar thrombin generation profiles and generated analogous amounts of thrombin with comparative lag times in canine and human plasma. A current theory is that TF is found in two states, an inactive/encrypted state and an active/decrypted state. Encryption is thought to act as an additional level of control to prevent excessive coagulation. The mechanism of decryption is still unknown but could involve TF dimerization, cleavage of a disulfide bond on TF, or phospholipid characteristics of the cell membrane [38]. Recently encrypted TF was shown to induce signaling activity without initiating coagulation [39]. It is possible that TF is more "encrypted" on the brain metastatic (Emma Brain) cells, thus it is similarly procoagulant, despite being expressed on more cells and with higher intensity than the primary splenic HSA cell lines, Frog and Dal-4. Alternatively, tumor-specific

alterations in TF conformation or ability to bind to FVII may also be responsible for the observed differences in procoagulant activity.

The next step for elucidating the role of TF in HSA is to evaluate TF expression in vivo. We plan to probe for TF expression in situ in HSA tumors using immunohistochemical methods, as recently described for canine mammary tumors [17]. To further explore the pathogenesis of DIC in relation to HAS, we are currently assessing thrombin generation by microparticles released by HSA cells in culture. Microparticles are small (0.1-1um) membrane-derived vesicles that are shed from activated cells, including platelets, apoptotic cells and tumor cells [40]. Tissue factor-bearing tumor microparticles have been implicated in increased thrombotic risk in human patients with pancreatic, breast, and colorectal cancer [41, 42]. We also currently lack reliable diagnostic tests for hypercoagulability, which leads to under-diagnosis of thrombosis in dogs with cancer [43]. Tissue factor expression on cells or their released microparticles in plasma could serve as biomarker for canine patients at risk of developing DIC and may lead to earlier diagnosis and anticoagulant treatment for dogs with HSA.

In this study, we demonstrate for the first time that cell lines derived from primary hemangiosarcomas in dogs express TF mRNA and surface TF antigen and are procoagulant in vitro. In contrast, normal CnAoEC lack surface TF and do not generate thrombin in canine plasma, supporting abnormal expression of TF in endothelial cells as a consequence of neoplastic transformation. Our results suggest that aberrant expression of TF on canine neoplastic endothelial cells likely contributes to DIC that is a devastating complication and clinical feature

of this neoplasm. Tissue factor may prove to be a useful therapeutic target or tumor biomarker. Future studies are needed to corroborate these findings in vivo.

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CHAPTER 3

SUMMARY, PRELIMINARY EXPERIMENTS AND FUTURE DIRECTIONS

Summary

Procoagulant activity of cancer cells has been shown to be a poor prognostic indicator for human patients [1]. In vitro studies have shown aberrant TF expression and coagulation activation in several cancers [2, 3]. Hemangiosarcoma is an aggressive cancer in dogs with a high rate of cancer-associated thrombosis [4, 5]. This is the first study to examine the underlying mechanism of the activation of coagulation in HSA. The work presented in chapter two shows that thrombin generation in HSA is likely mediated by TF in our in vitro system. This could partially explain the pathogenesis of DIC in dogs with HSA.

Preliminary Experiments and Future Directions

Angiosarcoma is the human equivalent cancer of HSA in dogs. It shares many characteristics of HSA. Both cancers are derived from endothelial cells, are prone to rapid metastasis, and have high rates of cancer-associated thrombosis like DIC [6-8]. We have recently performed pilot studies examining the expression of TF and thrombin generation of an AS cell line, (a kind gift from Dr. Cristina R. Antonescu at Memorial Sloan-Kettering). We have found these AS cells do aberrantly express TF where non-transformed human umbilical vein endothelial cells do not, confirming results of an older study which showed TF expression in AS

[9]. Interestingly, the AS cells had a similar proportion of TF antigen surface-expressing cells as did the primary canine HSA cells (data not shown, n=1). The AS cells also generated thrombin in our CAT assay (data not shown, n=1). While these are still preliminary results, it is promising that canine HSA is a useful model system for human AS.

The clinical picture of HSA in dogs is what prompted our examination of TF expression in vitro. A necessary continuation for this project would be to look for TF expression in tumors in vivo. We need to ensure that our results in cell culture are applicable to dogs with HSA. Furthermore it would be prudent to examine clinical HSA samples for in situ TF expression using immunohistochemistry. Since we saw variable surface TF expression between the HSA cell lines we tested, we expect to see a similar variation in TF expression in clinical samples. Tumor origin and the presence of metastasis could play a large role in TF expression. Emma brain, the cell line from a brain metastasis, had a higher median fluorescence intensity of TF expression by flow cytometry compared to primary splenic cell lines, Frog and Dal-4. We hypothesize that there will be variable TF expression, with stronger expression in metastatic HSA tumors.

Microparticles have also been implicated in the pathogenesis of thrombotic disorders such as DIC in people. Microparticles are small membrane-derived vesicles (0.1-1.0 uM) released from cells. Microparticles were first observed around activated platelets and were described as platelet dust [10]. Since then, MPs have been found to aid hemostasis under normal conditions by adding additional PS-rich surfaces for coagulation activation [11]. In addition to platelets, cancer cells spontaneously release MPs by blebbing off pieces of their plasma

membrane. Cancer-associated TF-bearing MPs have been associated with an increased risk of thrombosis in people with pancreatic, breast and colorectal cancer [12]. For instance, human patients with colorectal cancer have been shown to have an increased activation of coagulation when there are circulating TF-bearing cancer MPs in their plasma [13]. Microparticles derived from several cancers, including human breast and pancreatic carcinoma, have been shown to be procoagulant in vitro [14]. Previous work in the Stokol laboratory found that TF-bearing MPs were released from canine mammary tumor and pulmonary carcinoma cell lines into the tissue culture supernatant [15]. Hemangiosarcoma has yet to be evaluated for release of TF-bearing MPs, however preliminary studies have been conducted in our lab that shows thrombin generation from cell-conditioned media from HSA cells in the CAT assay (data not shown). These preliminary results suggest that HSA cells are able to produce procoagulant MPs which could explain the pathogenesis of DIC (figure 3.1). We plan to further investigate HSA cell lines for MPs and procoagulant activity from these MPs. We can isolate MPs from cell-free media from culture cells to test for procoagulant activity via FX activation and calibrated automated thrombography. Tissue factor-bearing MPs released into the blood stream by cancer cells could act as a vehicle for dissemination of procoagulant particles necessary for initiation of DIC. If we find that HSA cells shed TF-bearing MPs, this would expand our knowledge regarding the pathogenesis of DIC in HSA. Furthermore, plasma samples from canine patients with HSA could also be analyzed for TF-bearing MPs, which could prove to be a useful diagnostic or prognostic marker in vivo as has been shown for human pancreatic cancer [12].

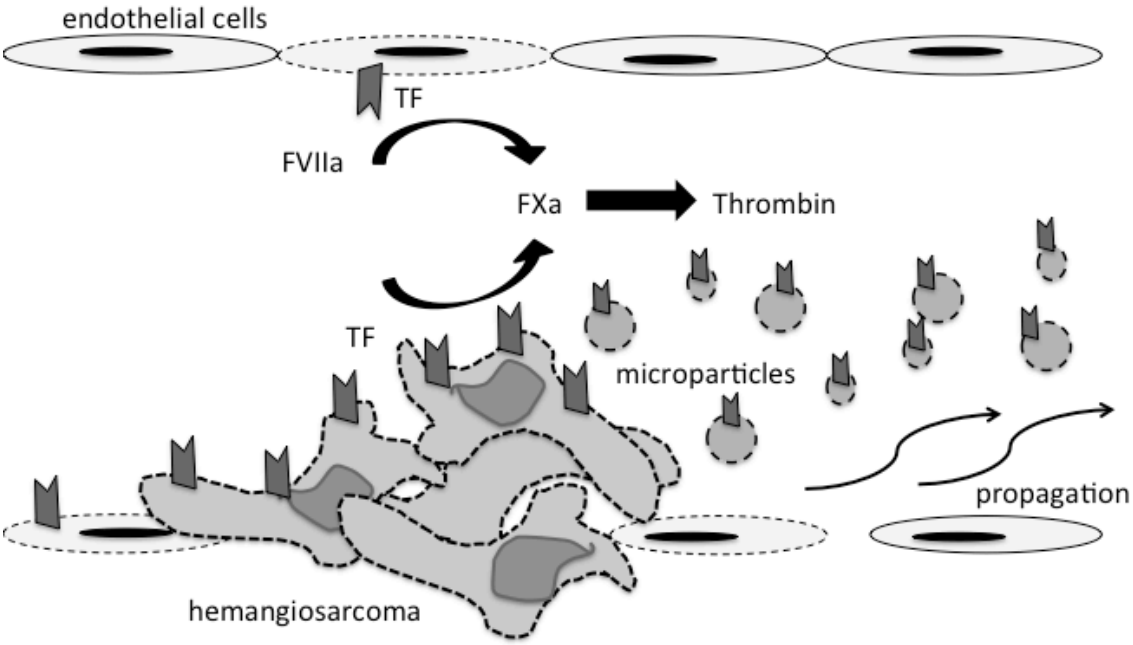

Figure 3.1- The dissemination and propagation of thrombin generation in canine HSA. HSA cells (irregularly shaped grey shapes) are derived from endothelial cells lining blood vessels (round white shapes). HSA display characteristics of an activated endothelium (shown as dashed line) which also results in TF upregulation(dark grey receptor shape). In addition to producing their own TF, HSA activates surrounding endothelial cells causing upregulation of TF and leaky junctions. FVIIa found in the blood plasma upon meeting TF rapidly activate FX, which generates thrombin from prothrombin. Microparticles (MP) with the same activated membrane characteristics are suspected to be shed from HSA (shown as circles that carrying TF). MP production from HSA could lead to dissemination and propagation of thrombin as MPs travel away from the tumor site into the blood stream. These significant changes caused by HSA lead to the observed hypercoagulable state and high incidence of DIC in dogs with HSA.

The driving force behind this thesis is to understand the role of TF in HSA biology (growth and metastasis). HSA is frequently called the occult killer because it is difficult to diagnose and has often metastasized by the time of diagnosis. We hope to potentially manipulate TF to create new treatments or diagnostic tests for patients with this cancer in hopes of detecting HSA earlier and treating it. Functional TF expression on HSA and AS could partially explain the propensity for metastasis, angiogenesis and poor prognosis in these cancers. Beyond its known procoagulant function, the TF-FVIIa complex can induce intracellular signaling via the TF cytoplasmic domain and through interaction with PARs [16]. TF-FVIIa-mediated PAR 2 signaling has been shown to be important for the invasion, metastasis and angiogenesis of tumors [17-19]. Silencing TF expression with short hairpin loop RNA (shRNA) in the high TF-expressing human breast cancer, MDA-MB-231, resulted in decreased tumorigenicity and vascularity of tumors in nude mice [20].

In order to address the role of TF in HSA tumor biology, we produced a TF knock down in the SB canine HSA cell line. This was done using lentivirally-transduced short hairpin RNA-mediated gene silencing. The RNA interference (RNAi) pathway in eukaryotes regulates post-transcriptional cell gene expression [21]. To manipulate gene expression, small interfering RNA (siRNA) is designed to degrade targeted mRNA. Short hairpin RNAs (shRNAs) are a form of siRNA. The RNAi pathway effectively recognizes and processes shRNA into siRNA that leads to gene silencing. The siRNA, created from the shRNA, is loaded into the RNA-induced silencing complex (RISC). Activation of the RISC can cause mRNA degradation through binding of the siRNA guide to mRNA. Once loaded, the passenger strand of RNA is degraded while the remaining guide strand is used by the RISC to target and cleave mRNA. The lentiviral

transduction system takes advantage of the ability of lentivirus to insert its genome into the host creating a stable, long-lasting knockdown. The insertion of the shRNA sequence into the host sequence can induce constant production of the shRNA. Injection of siRNA only results in knockdown of host mRNA for 2-3 days, whereas with lentiviral transduction stable knockdown can be immortalized in a cell. It is important to note that the insertion of the lentivirus genome is random and can occur anywhere within the host genome causing an increased risk of transcriptional silencing and insertional oncogenesis [21, 22]. We designed the shRNA to the canine TF gene taking into account positional effects of siRNA noted in human TF [23, 24]. Materials and methods are presented in Appendix 1.

We were able to successfully decrease TF expression in the canine SB cells. Flow cytometric evaluation confirmed a mild decrease in the percentage of TF-surface expressing cells. The intensity of TF fluorescence was decreased by 50% with the TF specific knockdown, whereas the scramble control had similar surface expression and intensity of fluorescence of TF to the un-transduced control (Figure 3.2). For both scramble and TF-specific transduced cell populations there was a high green fluorescent protein (GFP) expressing group and a low GFP-expressing group. Heterogeneously GFP-expressing cells could also be seen by fluorescent microscopy (data not shown). Cells with stronger GFP expression are suspected to have received multiple copies of the shRNA. There is differential TF expression in the TF shRNA transduced cell population. Cells with higher GFP fluorescence had a lower percentage of surface-TF expressing cells (Figures 3.2, C). This shows that transduction efficiency and multiplicity of infection between individual cells was different. Perhaps concentrating the lentiviral stocks would increase the multiplicity of infection and create a uniformly infected population of cells.

We attempted to isolate highly GFP-expressing cells by fluorescence activated cell sorting. Unfortunately too few cells were isolated during our attempt due to a small starting sample, but the populations were quite (99%) pure (data not shown). Cell sorting is a promising avenue for isolating pure populations of highly GFP-expressing knocked down cells for future experiments.

Quantitative PCR expression was also performed and showed that both scrambled and TF-specific knockdown cells had significantly decreased TF mRNA expression compared to non-transduced SB cells (data not shown). TF mRNA reduction in the TF-specific shRNA was significantly decreased even further from the scrambled control. The surface expression of TF for scramble sequence transduced SB cells did not change significantly but total TF mRNA was decreased compared to non-transduced cells (data not shown). Future replicates might help to quantify the TF mRNA in these cells by using purer populations of cells. It is common to see some changes in mRNA production in scramble control as the process of infection and the scrambled sequences themselves can have unknown effects on the cell. It is interesting to note that the cells transduced with the scrambled sequence reached the cycle threshold more quickly compared to non-transduced and TF-specific shRNA transduced cells. This suggests some off target changes in global transcription are caused by the scrambled sequence. In addition it is possible that the lowered TF mRNA in the scrambled transduction may be due to testing a mixed population of cells, which resulted in high variability in the replicate data for qPCR experiments as seen by the standard deviation. Samples of RNA for quantitative PCR analysis were taken before the selection protocol was optimized. During the first transduction we witnessed cell populations becoming less selected over time, losing GFP fluorescence after 10 days. This escape from selection is most likely explained by using a concentration of puromycin that was

too low. During the second transduction we used a higher concentration of puromycin (7ug/ml) and found that the cells maintained GFP fluorescence after two weeks. With the selection protocol optimized, it is now possible to isolate purer populations of cells, which should result in a clearer mRNA quantification. In future studies, given more time, we could also isolate single colonies of highly expressing cells to assess a clonal population.

Preliminary work with this SB HSA knockdown cell line shows decreased procoagulant activity. There is decreased FXa generation in a one-step amidolytic assay compared to normal SB cells and scramble knockdown cells (Figure 3.3b). We further analyzed the effect of silencing TF in the CAT in vitro system we used in chapter 2. This system is more reflective of coagulation in vivo using all factors found in plasma. In factor-replete human plasma, the lag time, (the measure of time to initiation of thrombin generation) was significantly increased in the TF knockdown SB cells compared to scramble-transduced and non-transduced SB cells (Figure 3.3 C-D). It was exciting to see a decrease in coagulation initiation in the TF knockdown SB cells. Since only small amounts of TF, on the picomolar level, are needed to initiate coagulation we were not expecting to see a decrease in procoagulant activity in our TF knockdown [25]. There are several experiments that can be done in the future to further characterize the procoagulant activity. Additional replicates are needed for all of the above experiments. SB cells have increased surface expression of TF compared to primary HSA cell lines as shown in chapter 2. It would be interesting to see if the TF shRNA similarly affects procoagulant behavior in lower TF-expressing primary HSA cell lines. The TF silencing could potentially reduce procoagulant activity of the primary HSA cells to non-neoplastic endothelial cell levels.

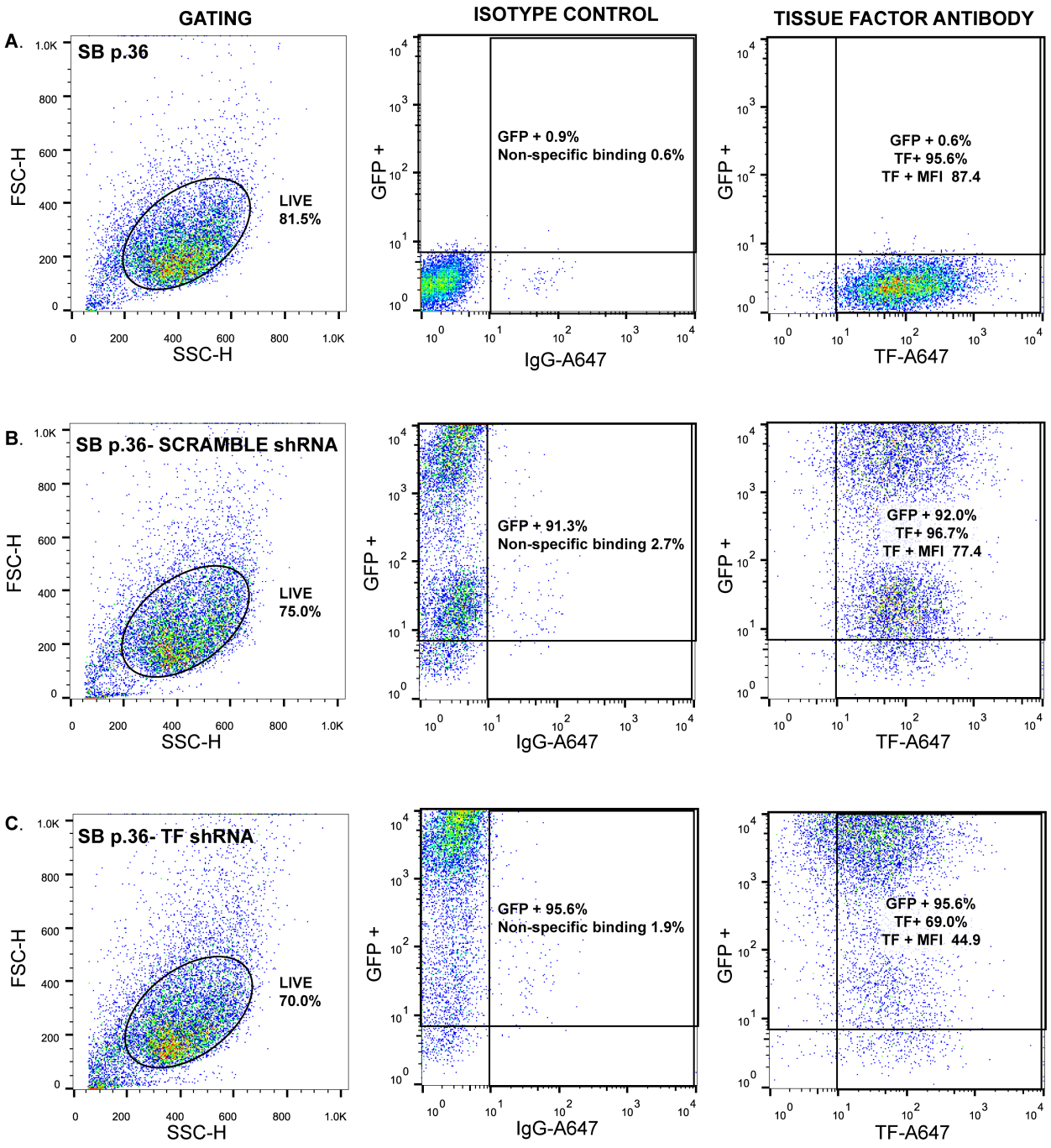
GATING
ISOTYPE CONTROL
TISSUE FACTOR ANTIBODY
A.
SB p.36
LIVE
81.5%
GFP + 0.9%
Non-specific binding 0.6%
GFP + 0.6%
TF+ 95.6%
TF + MFI 87.4
B.
SB p.36- SCRAMBLE shRNA
LIVE
75.0%
GFP + 91.3%
Non-specific binding 2.7%
GFP + 92.0%
TF+ 96.7%
TF + MFI 77.4
C.
SB p.36- TF shRNA
LIVE
70.0%
GFP + 95.6%
Non-specific binding 1.9%
GFP + 95.6%
TF+ 69.0%
TF + MFI 44.9
FSC-H
SSC-H
GFP +
IgG-A647
TF-A647

Figure 3.2- Effect of shRNA lentiviral-mediated silencing of TF surface expression

Representative gating strategy and results for flow cytometric detection of TF surface expression on the canine SB HSA cell line 5 days after transduction with anti-TF shRNA within a lentivirus vector containing a green fluorescent protein (GFP) tag. **Left panel (gating):** Cell events were gated (LIVE) on a forward scatter (FSC-H) and side scatter (SSC-H) dot plot. The LIVE gate was drawn around the densest population of cells that was above 200 arbitrary units on the X-axis (to avoid cell debris). **Middle panel (Isotype control)**: Cells were incubated with mouse IgG to account for non-specific binding. **Right panel (anti-TF antibody)**: Cells were incubated with monoclonal mouse anti-canine TF (clone 133-2), followed by a secondary Alexafluor® 647-conjugated goat anti-mouse IgG (A647). Within the LIVE gate, GFP-positive and TF-positive (TF+) cells were quantified using a quadrant plot based on non-transduced cells (for GFP) and the isotype control (for TF).

A: Non-transduced SB cells: These lacked expression of GFP and most cells expressed TF. **B**: SB cells transduced with scrambled shRNA GFP-lentivirus expressed GFP with a 92% transduction efficiency. Two populations of GFP-expressing cells are seen, with similar fluorescent intensity for surface TF. C: SB cells transduced with canine TF shRNA GFP-lentivirus. Cells were efficiently transduced (96%) with two subpopulations showing low and high GFP expression. Compared to scrambled and non-transduced controls, there is a lower percentage of TF-expressing cells and lower fluorescent intensity, however most of the cells still expressed TF surface antigen.

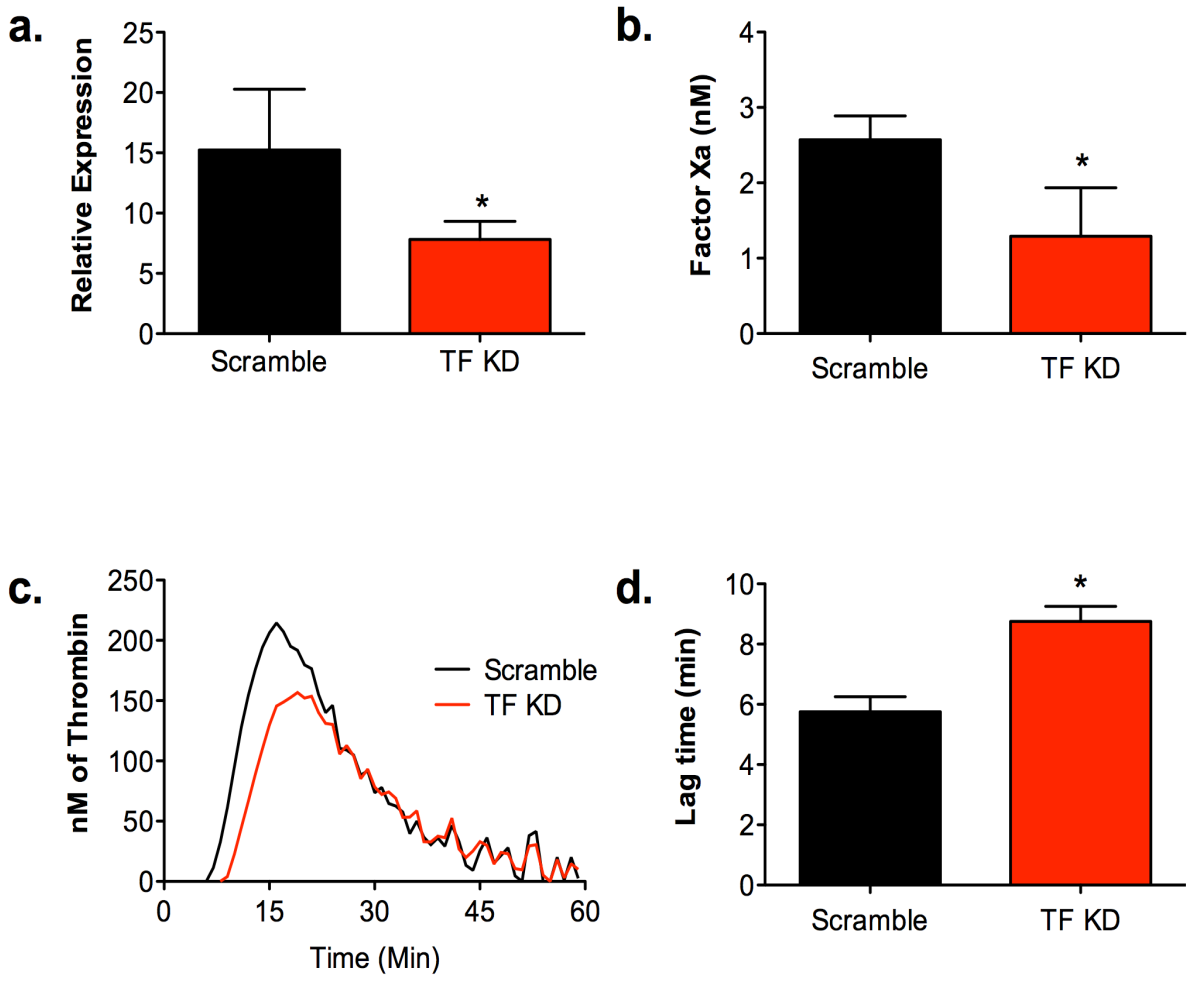

Figure 3.3- Effect of shRNA lentiviral-mediated silencing of TF mRNA expression and procoagulant activity in canine SB HSA cells.

Cells were transduced with a lentiviral vector containing shRNA against TF, with scrambled shRNA and non-transduced controls. **A. mRNA expression:** Quantitative PCR was performed on the cells 5 days after transduction. Results for scrambled shRNA transduced (scramble) and TF shRNA transduced (TF KD) are expressed as the mean ± SD of relative mRNA expression to non-neoplastic canine aortic endothelial cells (given a value of 1) and normalized to expression of the ribosomal protein subunit 5 as a reference gene, using the $2^{-\Delta\Delta Ct}$ method (n=2). The TF KD

cells expressed significantly less TF mRNA than SB cells transduced with the scramble control (P<0.05). **B. Activated factor X generation**: In this assay, purified human FVIIa and FX are added to the cancer $2x10^5$ cells in clear 96-well tissue culture plates for 15 minutes. Then cleavage of a FXa-dependent substrate was monitored kinetically every 30 seconds at an optical density of 405 nM, with the rate of cleavage being compared to that of a standard curve of purified human FXa (in nM). The TF knockdown cells activated significantly less FX than scramble control. **C. Thrombin generation:** Representative thrombograms of scramble control and TF-KD SB cells. TF knockdown SB cells generated significantly less thrombin than the control cells. D. Lag time: The lag time was significantly shorter with TF-knockdown cells (n=2).

*P <0.005 versus non-transduced SB cells.

Currently we are planning on examining how reduced TF expression affects HSA behavior in vitro, including cell viability, anchorage independent growth, and sensitivity to doxorubicin, the chemotherapeutic drug of choice for HSA. Anchorage independent growth is a marker of cancerous transformation. Normal cells respond to contact by other cells by inhibiting their growth. In a soft agar assay, where cells are suspended in media with agar, transformed cells will continue to grow and form colonies where as non-transformed cells remain as single-cells. In pilot studies SB cells have formed colonies in soft agar compared with canine endothelial cells, which do not. We have noticed decreased cell viability and slower growth in the TF KD SB cells. This implies that TF is important for HSA cell viability in SB cells, however the slowly growing and dying cells have made it difficult to examine these changes.

Hemangiosarcoma and AS are notoriously resistant to common chemotherapeutic drugs such as doxorubicin. If TF knockdown of HSA cells in vitro increase tumor sensitivity to doxorubicin, patient morbidity and mortality could be significantly improved using drugs that deplete or target TF on cancer cells. By using canine HSA as a model for AS, we can learn more about the biology and improve treatment and outcome for both canine and human patients. We have cell viability and apoptosis to examine the effect of doxorubicin at concentrations ranging from 0uM to 10uM in SB cells (Figure 3.4). Small populations of SB cells remain alive after incubation with 10uM doxorubicin for 48 hours. We have yet to examine the effect of the TF knockdown on doxorubicin sensitivity in SB cells. A study examining the role of TF in neuroblastoma revealed that TF silencing in high-TF expressing neuroblastoma cells increased cell sensitivity to doxorubicin [26].

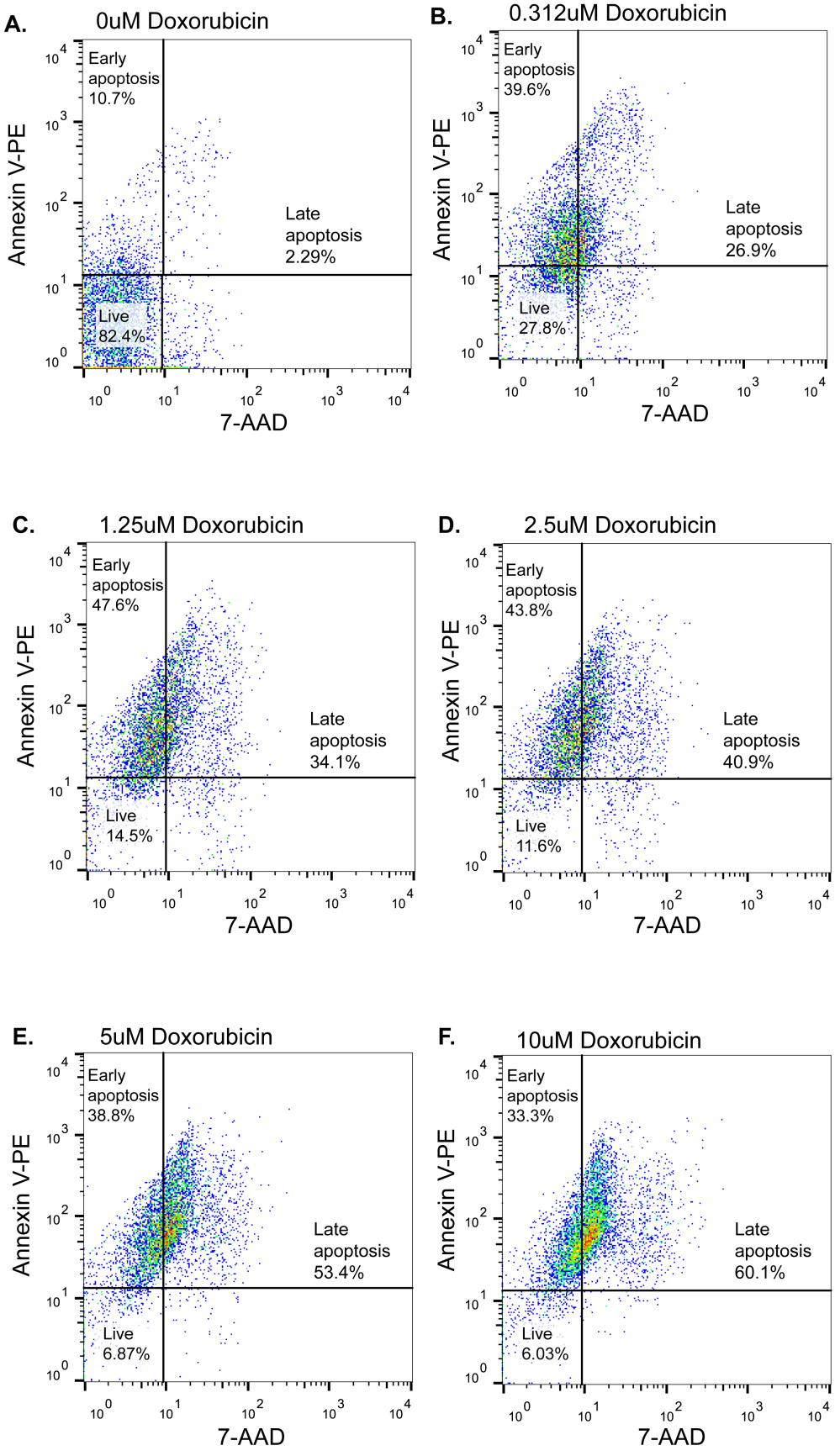
A.
0uM Doxorubicin
Early apoptosis 10.7%
Late apoptosis 2.29%
Live 82.4%
B.
0.312uM Doxorubicin
Early apoptosis 39.6%
Late apoptosis 26.9%
Live 27.8%
C.
1.25uM Doxorubicin
Early apoptosis 47.6%
Late apoptosis 34.1%
Live 14.5%
D.
2.5uM Doxorubicin
Early apoptosis 43.8%
Late apoptosis 40.9%
Live 11.6%
E.
5uM Doxorubicin
Early apoptosis 38.8%
Late apoptosis 53.4%
Live 6.87%
F.
10uM Doxorubicin
Early apoptosis 33.3%
Late apoptosis 60.1%
Live 6.03%
Annexin V-PE
7-AAD

Figure 3.4- Doxorubicin sensitivity of SB HSA cells.

Increasing concentrations of doxorubicin were incubated with SB HSA cells for 48 hours and then analyzed for markers of early and late apoptosis via flow cytometric analysis using an Annexin V apoptosis staining kit (Affymetrix, eBioscience, San Diego, CA). Cell events were gated on a forward scatter (FSC-H) and side scatter (SSC-H) dot plot around the densest population of cells that was above 200 arbitrary units on the X-axis (to avoid cell debris) (not shown). Cells in early apoptosis expose PS, which binds to Annexin-V labeled with phycoerythrin (PE), seen in the y-axis (Annexin V-PE). Cells in late apoptosis will have PS exposed and become permeable to 7-AAD, which binds to DNA strongly seen in the x-axis (7-AAD). SB cells with 0uM of doxorubicin (**a**) show very few cells undergoing apoptosis, whereas those exposed to 0.312uM (**b**), 1.25uM (**c**), 2.5uM (**d**), 5uM (**e**), 10uM (**f**) of doxorubicin have increased numbers of cells in early and late apoptosis. In all conditions with doxorubicin there are still live cells indicating that small populations of HSA cells are resistant to doxorubicin toxicity even at the highest concentrations.

The TF knockdown that we created successfully reduced TF surface expression by 50%. This is comparable to human TF shRNA mediated knockdown in a human melanoma cell line, which showed TF protein reduction to be between 30-50% via ELISA for TF protein [27]. In a study with breast cancer cell line MCF-7 TF protein expression and mRNA was reduced by 80% due to TF shRNA. Positional variation of siRNA targets to human TF can knockdown expression from a 20% reduction to an 80% reduction in TF protein expression in human cancer cell lines [23]. We designed several canine TF target sequences to the extrapolated position from the most effective siRNA in human TF. While the sequence we chose was fairly effective one of these other sequences could prove more effective at knocking down TF in canine cells. Another avenue we are pursuing is using the CRISPR/Cas9 genome editing system to completely knock out TF in canine cells. With our observation that the TF KD is decreasing cell growth and viability this could cause cell death in the SB HSA cell line. This could be promising for future therapeutic strategies in HSA.

Research into the role of TF in tumor biology of HSA would change the way we currently diagnose and treat patients with it. Understanding how TF is upregulated and what benefits it offers HSA in creating a metastatic niche will increase our knowledge of why TF is upregulated in other aggressive cancers like breast, prostate and pancreatic cancer. The results shown in this thesis are a small part in the puzzle of defining the role of TF in HSA. The larger picture remains to be elucidated to create these new diagnostics and treatments that will change how we assess this cancer.

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APPENDIX 1

Materials and Methods

shRNA Design and Plasmids— The 29 base pair siRNA target was designed to maximally silence canine TF mRNA referencing optimal siRNA design in human TF [1]. The shRNA sequence was greater than 50 nucleotides from the start codon and had GC content between 30-50% as per guidelines previously described [2-4]. The scrambled control was designed by scrambling the same nucleotides used in the canine TF siRNA target. The 76 base pair ShRNA oligonucleotides were designed with BamHI 5' overhang, siRNA sense-oriented target, loop sequence (TCAAGAG), antisense oriented siRNA target, termination sequence, and the BamHI 3'overhang [2, 4]. Compliment shRNA was also ordered as desalted oligonucleotides (IDT, Coralville, IA, USA)

Canine TF shRNA:

5'GATCCGCACTGCAGATGTAGTCGTAGCATATAATTCAAGAGATTATATGCTACGA
CTACATCTGCAGTGC TTTTTG

3'GCGTGACGTCTACATCAGCATCGTATATTAAGTTCTCTAATATACGATGCTGATGT
AGACGTCACG AAAAACCTAG

Scramble shRNA:

5’GATCCATGGAAGTCACACGTATAGTATCGTATCGTCAAGAGCGATACGATACTAT
ACGTGTGACTTCCATTTTTTG

3’GTAGGTACCTTCAGTGTGCATATCATAGCATAGCAGTTCTCGCTATGCTATGATAT
GCACACTGAAGGTAAAAAACCTAG

Oligonucelotides were annealed together in annealing buffer (10mM Tris base, 50mM NaCl, and 1mM EDTA, pH 7.5) (Sigma-Aldrich, St. Louis, MO, USA). Equimolar concentrations of complimentary oligonucleotides were heated at 95°C and cooled to room temperature over 60 min and then stored at -20°C until use. shRNA pGFP-C-shLenti shRNA-29 expression vector (Origene) was linearized with BamHI FastDigest kit according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA USA). Annealed oligonucleotides designed with BamHI overhangs were ligated into the BamHI linearized shRNA pGFP-C-shLenti shRNA-29 expression vector using a T4 DNA ligase kit with manufacturer’s directions (Thermo Fisher Scientific).

Chemically competent *E. coli* (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen, kindly provided by the Leifer laboratory) was transformed with shRNA plasmids as previously described [3]. Chloramphenicol-resistant colony plasmids were isolated and purified using endotoxin-free plasmid purification mini preps (Qiagen Sciences). Isolated plasmids were checked via PCR, using primers designed to amplify the expression vector (Forward: 5’ ATCTTGTGGAAAGGACGCGG 3’; Reverse: 5’ ACACACATTCCACAGGGTCG 3’) with the following PCR conditions: 35 cycles of denaturing for 30 sec at 95°C, annealing for 30 sec at

56°C, and extending for 15 sec at 72°C. PCR-positive transformants were confirmed by sequencing using an Origene-specified universal sequencing primer. One canine TF positive plasmid and one scramble control plasmid that were correctly sequenced were amplified.

Cell Culture—Human Embryonic Kidney (HEK) 293 cells (ATCC #CRL-1573, kindly provided by the Leifer laboratory), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine, 50 U ml^{-1} penicillin, 50μg ml^{-1} streptomycin, 10mM HEPES, 1mM sodium pyruvate and 10% low endotoxin FBS. Canine HSA SB cells were cultured in FK-12 media supplemented with 10% fetal bovine serum, 0.05mg/mL endothelial cell growth supplement (BD Bioscience, San Jose, CA, USA), 10mM HEPES (Gibco, Grand Island, NY, USA), 0.01mg/mL heparin (Sigma-Aldrich, St. Louis, MO, USA), and 100ug/mL primocin (InvivoGen, San Diego, CA, USA). All cells were maintained in 5% CO_2 humidified atmosphere at 37°C. Cells were detached with 0.25% trypsin–EDTA, and trypsin was neutralized with growth medium. Cells were used in the log phase of growth between 60-80% confluency and were 90% viable based on trypan blue exclusion.

Lentiviral Transduction— Lentiviral supernatants were generated using Lipofectamine 2000 (Invitrogen) transfected HEK-293 cells. TF shRNA and scramble shRNA plasmids were transfected with lentiviral component plasmids; Tat, Rev, Gag/Pol, and vsv genes. Maximal virus was collected after 48 hours and used for subsequent transfection. Canine HSA SB cells were spinfected (1811 X *g*, 32 °C, 90 minutes) with lentiviral supernatants mixed with 8μg/mL polybrene (Sigma-Aldrich). Following centrifugation, the media was changed and cells were incubated for 48 hours at 37°C. SB cells were selected with 7ug/ml puromycin for 5 days before isolating a 95-99% transfected population.

Quantitative real time PCR—Total RNA was isolated from harvested cells using a commercial kit (Qiagen RNeasy, Valencia, CA, USA) with concentration and purity of total RNA determined by UV measurement at 280nm/260nm (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA). cDNA was synthesized from 1ug total RNA using a commercial kit with oligo$(dT)_{20}$ primers (Superscript® III RT system, Life Technologies, Grand Island, NY, USA). Quantitative real time PCR was performed using the SYBER® Green Master Mix (Applied Biosystems, Austin, TX, USA), with primers specific for canine TF that were designed to span the exon 2 and 3 junction of canine TF DNA (Forward: 5' AGTGGGAACCCAAACCCATC 3'; Reverse: 5' ATGGAGGCTCCCCAGAGTAG 3') (IDT, Coralville, IA, USA). The canine ribosomal protein subunit 5 (RPS5) (Forward: 5' TCACTGGTGAGAACCCCCT 3'; Reverse: 5'GTTCTCATCGTAGGGAGCAAG 3', IDT) was used as a housekeeping gene, using published primer sequences .[5] The following PCR conditions were used: 2 min at 50°C, 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, then 1 min at 57°C (PTC 200 PCR Thermal Cycler, MJ Research, Ramsey, MN, USA). Amplification curves and cycle threshold (Ct) values were collected and analyzed by specific software (StepOne Software v2.3, Life Technologies). Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with canine TF mRNA expression being normalized to the endogenous control RPS5.

Flow cytometry—Detached cells (1 X 10^5 cells/reaction) were resuspended in phosphate-buffered saline (PBS) with 1% bovine serum albumin and 0.05% sodium azide (PBSA) and were incubated with a murine IgG_1 monoclonal anti-canine TF antibody (clone 133-2) that was generated in the laboratory or murine IgG_1 (both at a final concentration of 20 μg/mL) for 30 minutes on ice, followed by a secondary Alexafluor® 647-conjugated goat anti-mouse IgG

(1:200, Life Technologies, Eugene, OR, USA) for 30 min on ice. After washing twice in PBSA, cells were suspended in PBS and analyzed with a flow cytometer (FACSCalibur, BD Bioscience, San Jose, CA, USA). For data acquisition, forward scatter (FSC-H) was set at a voltage of E-1, ampere gain of 4.75, and linear mode. Side scatter (SSC-H) was set at a voltage of 350 V, ampere gain of 1.00, and linear mode. Fluorescence was set on log mode and 10,000 events were counted in a gate set around the majority of cells on a FSC-H versus SSC-H dot plot. For analysis, dot plots of FL1, green fluorescent protein from the plasmid, by FL4, fluorescence from TF-positive cells were examined for positive reactions, being defined as events falling within a quadrant gate established with the isotype control and non-transduced cell line. Less than 5% of cells labeled with the isotype control fell within the positive quadrants. The percentage and median fluorescent intensity of GFP- and TF-positive cells was determined using commercial software (FlowJo V.10, Tree Star Inc., Ashland, OR, USA).

Surface Factor X Activation—Non-transduced SB cells, scramble shRNA-transduced and TF shRNA-transduced SB cells (2×10^5) were washed twice with HEPES buffer (10mM HEPES, 137mM sodium chloride, 5mM calcium chloride, 4mM potassium chloride, 10mM glucose, 0.5% bovine serum albumin, pH 7.4), then procoagulant activity was measured using a standard two-step amidolytic assay based on FXa generation. Purified human activated FVII (final concentration, nM, Hematologic Technologies) and FX (final concentration, 75 nM, Hematologic Technologies) diluted in HEPES buffer were added to the cells for 15 min at 37°C. Chromogenic substrate (Spectrozyme-FXa, final concentration, 167 μM, American Diagnostica) was then added. Color change was measured kinetically every 30 seconds for 10 minutes at an optical density of 405 nm at 37°C with a plate spectrophotometer (Spectramax M3, Molecular

Devices, Sunnyvale, CA, USA). Optical density results were converted to the amount of FXa generated (nM) based on a standard curve created from serial dilutions of purified human FXa (American Diagnostica) that were incubated with the substrate. The standard curve was linear between FXa concentrations of 5.43 and 0.17 nM, the latter yielding optical densities more than twice baseline values. Human recombinant TF (Innovin, Dade-Behring) with added reagents was included as a positive assay control.

Calibrated automated thrombography—Detached cells were washed free of culture media with PBS and suspended 2×10^4 in 20uL HEPES-saline buffer (10mM HEPES, 137uM sodium chloride, 4mM potassium chloride, 10uM glucose, pH 7.05) in a black 96-well plate (FluoroNunc with MaxiSorp, Fisher Scientific, Pittsburgh, PA, USA). Then, 30ul of microparticle-depleted human factor replete plasma was added, followed by 50ul of fluorescent substrate (Technothrombin TGA substrate, Technoclone GmbH, Vienna, Austria). Fluorescence was then measured every minute at 360nm/460nm for 2 hours at 37°C on a 96-well plate fluorescent spectrophotometer (Spectramax M3, Molecular Devices, Sunnyvale, CA, USA). A plasma control containing no cells (only HEPES-saline buffer added) and a positive human control from the substrate manufacturer (TGA CH and RCH, Technothrombin) were included in each run. All samples were run in duplicate. Thrombograms were generated by comparing raw fluorescence output to a standard curve of thrombin calibrator (Technothrombin Calibrator), using specific software (Technothrombin software). Due to a malfunction with the proprietary software that was discussed with the manufacturer the lag time or time to initiation of thrombin generation was measured manually as the first minute when thrombin concentration increased above 4nM.

Statistical analysis—Data was Gaussian and expressed as means ± standard deviations. Comparison of 3 means was performed using a 2-way Analysis of Variance with a Tukey's multiple comparison post-test. Statistical analysis was performed with commercially available software (Prism 5, GraphPad Software, INC, La Jolla, CA). *A P* value set at <0.05.

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