ABSTRACT. Hypercoagulability is a common paraneoplastic complication in dogs with various malignant tumors. Importantly, tissue factor procoagulant activity (TF-PCA) induced by TF-bearing microparticles (TF-MPs) is associated with hypercoagulability in human patients with cancer. However, TF-PCA in tumor cells and the association between circulating TF-MPs and hypercoagulability in dogs with malignant tumors remain poorly understood. Therefore, the present study was conducted to evaluate the TF-PCA in various types of canine tumor cell lines and plasma in dogs with malignant tumors. Mammary gland tumor, hemangiosarcoma, and malignant melanoma cell lines, but not lymphoma cell lines, expressed TF on their surfaces and showed cellular surface and MP-associated TF-PCA. The plasma TF-PCA was elevated in some dogs that naturally developed such tumors. No significant difference was observed in plasma TF-PCA between the disseminated intravascular coagulation (DIC) group (median: 43.40; range: 3.47–85.19; n=5) and non-DIC group (median: 7.73; range: 1.70–16.13; n=12). However, plasma TF-PCA was remarkably elevated in three of five dogs with DIC. To the best of our knowledge, this is the first study to evaluate plasma TF-PCA in dogs with malignant tumors. Further studies must be conducted to determine the cellular origin of TF-MPs and the efficacy of plasma TF-PCA as a biomarker of DIC in dogs with malignant tumors.

KEY WORDS: hypercoagulability, microparticle, procoagulant activity, tissue factor, tumor
carcinoma, bronchoalveolar carcinoma, osteosarcoma, fibrosarcoma, and hemangiosarcoma (HSA) cell lines [33, 39]. Moreover, aberrant TF expression in canine intracranial meningiomas, gliomas, and MGTs in situ has been reported [1, 8, 11]. However, whether procoagulant TF is expressed in other types of canine malignant tumors remains unknown. Furthermore, there have been no investigations providing plasma TF-PCA measurements in dogs with malignant tumors.

In the present study, TF expression, release of MPs, and TF-PCA in canine tumor cell lines, including MGT, HSA, malignant melanoma, and lymphoma cell lines, were investigated because these tumors are often associated with the occurrence of DIC in dogs. Furthermore, we first measured plasma TF-PCA in dogs that naturally developed malignant tumors and assessed the association between TF-PCA and the presence of DIC.

MATERIALS AND METHODS

Cell lines

Canine MGT cell lines (CHMp, CHMp-13a, CHMp-5b, CHMm, CIPp, CIPm, CTBp, and CTBm) [28, 37], canine malignant melanoma cell lines (CMec-1, CMec-2, KMeC, and LMeC) [17], canine HSA cell lines (JuA1, JuB2, JuB4, Re12, Re21, Ud2, and Ud6) [18], canine lymphoma cell lines (CLC, Nody-1, and UL-1) [36] were all used in the present investigation. D17 (ATCC CCL-183) and T24 (ATCC HTB-4) were also used as negative and positive controls for TF expression and TF-PCA, respectively. MGT, malignant melanoma, and T24 cell lines were cultured in RPMI 1640 (Nakalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, U.S.A.), 100 U/ml of penicillin (Nakalai Tesque), 100 µg/ml of streptomycin (Nakalai Tesque), and 55 µM of 2-mercaptoethanol (Sigma, St. Louis, MO, U.S.A.). The HSA cell lines and D17 were cultured in Dulbecco’s Modified Eagle’s medium (Nakalai Tesque) with 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. All cell lines were maintained at 37°C in a humidified 5% carbon dioxide incubator.

Evaluation of TF expression

To detect TF in the cellular surface, a polyclonal rabbit anti-human TF antibody (Sekisui Diagnostic [former American Diagnostica], Exton, PA, U.S.A.) was used in this study. The reactivity of the antibody to canine TF was previously validated [33]. The cells (5 x 10⁶) were labeled with the anti-TF antibody or normal rabbit IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A.; both at 20 µg/ml) for 30 min at 4°C. The cells were washed and then incubated with Alexa 488-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) for 30 min at 4°C in the dark. After washing, the cells were analyzed using a flow cytometer (BD Accuri™ C6; BD Biosciences, San Jose, CA, U.S.A.) and the FlowJo software version x.0.7 (Tree Star, Ashland, OR, U.S.A.). All experiments were repeated at least thrice.

MP separation from the culture supernatants of tumor cell lines

MPs were separated from culture supernatants using the protocol described in a previous report [33] with slight modifications. In brief, 3 x 10⁶ cells were cultured in 1 ml of culture medium in 12-well plates for 6 hr; then, 950 µl of the supernatant was collected, and 900 µl of cell-free supernatant was obtained via centrifugation at 2,500 x g for 10 min at 4°C. The MPs were separated via centrifugation at 20,600 x g for 20 min at 4°C. The MP pellets were then suspended in 60 µl of Annexin V binding buffer (eBioscience, San Diego, CA, U.S.A.) or 180 µl of Tris-buffered saline (TBS) to measure the number of MPs or TF-PCA, respectively. All cell lines were maintained at 37°C in a humidified 5% carbon dioxide incubator.

Counting of MPs

A total of 25 µl of the MP suspension thawed at 37°C was labeled with 2.5 µl of PerCP-eFluor® 710 dye-conjugated Annexin V (eBioscience) and diluted in 22.5 µl of Annexin V binding buffer for 30 min in the dark at room temperature. Then, 75 µl of Annexin V binding buffer and 25 µl of flow-count beads (Beckman Coulter, Miami, FL, U.S.A.) were added, followed by an immediate analysis using a flow cytometer. The guidelines for canine MP populations have not been established. Therefore, the gating for the MPs was determined using Megamix beads (BioCytex, Marseille, France) in accordance with the International Society of Thrombosis and Hemostasis guidelines for human MPs [20] (Fig. 1A–D). The MP size gate extended from the minimum detection limit of the Accuri™ C6 flow cytometer (0.5 µm) until 0.9 µm diameter. The MPs were defined as Annexin V-positive events within the MP size gate. By counting 2,000 events of the counting beads, the concentration of MPs in the culture medium was calculated. An analysis was conducted using the FlowJo software. All experiments were conducted in triplicate and repeated at least thrice.

Measurements of cellular surface and MPs-associated TF-PCA in tumor cell lines

Cellular surface and MPs-associated TF-PCA were measured using the protocol described in a previous report [33] with slight modifications. The cells were seeded at 3.0 x 10⁴ per well into a 96-well plate. After incubation for 6 hr, the cells were washed twice with TBS, followed by the addition of 40 µl of TBS to each well. To measure TF-PCA in culture supernatants, 40 µl of cell-free supernatant was seeded into each well of a 96-well plate. A total of 60 µl of coagulation factor mix (TBS with recombinant human FVIIa [final concentration of 1 nM]; Haematologic Technologies, Burlington, VT, U.S.A.), recombinant human Factor X (FX [final concentration of 30 nM]; Haematologic Technologies), and CaCl₂ (final concentration of 10 mM) was then added. The same volume of TBS was used as the negative control. After incubation at 37°C for 15 min, S-2765 (final concentration of 0.25 mM; Sekisui Medical, Tokyo, Japan), which is the chromogenic substrate of activated FX, was added to each well. After
further incubation at 37°C for 5 min, the absorbance at 405 nm was measured using the Multiskan FC™ instrument (Thermo Fisher Scientific). A standard curve was generated, where 100 arbitrary units (AU) was defined as a dilution ratio of 1:500 for recombinant human TF (Dade®, Innovin®, Sysmex, Kobe, Japan). All experiments were conducted in triplicate and repeated at least thrice.

**Dogs and plasma samples**

In this study, 11 healthy beagles maintained as blood donors at the Yamaguchi University Animal Medical Center (YUAMEC) were included in the healthy control group. The plasma samples for TF-PCA measurement were stored after being used for a routine medical check-up. Among cases in which complete blood count and coagulation tests were performed for clinical purposes at YUAMEC, 17 dogs that naturally developed malignant tumors (MGT [n=2], HSA [n=3], malignant melanoma [n=6], and high-grade lymphoma [n=6]) were enrolled. Histopathological examination was conducted to diagnose MGT, HSA, and malignant melanoma. The diagnosis of lymphoma was based on cytology, polymerase chain reaction for clonal rearrangements of antigen receptor genes, and/or histopathologic evaluation findings. Venous blood (0.9 ml) was collected from the cervical or saphenous
ven with 0.1 ml of 3.8% sodium citrate using a 23-gauge needle and a 1-ml syringe. If hemolysis or chyle was confirmed via visual observation, the samples were excluded from this study. The blood samples were centrifuged at 2,000 × g for 5 min at room temperature. Platelet poor plasma (PPP) sample was cautiously collected without disturbing the buffy coat and was used to measure hemostatic parameters. The remaining PPP sample was frozen immediately at −80°C. The time from blood collection to centrifugation was less than 1 hr. Informed consent for the storage and subsequent use of clinical specimens for research purposes was obtained from the owners of each dog enrolled during admission. The institutional ethics committee for animal clinical test of the Joint Faculty of Veterinary Medicine at Yamaguchi University approved all procedures in the study (approval no. 007).

Hemostatic parameters and diagnosis of DIC

Blood coagulation tests were conducted to evaluate the coagulation state in each dog enrolled. The platelet count was calculated using the XT-2000iV system (Sysmex) with peripheral blood samples with EDTA or heparin. If platelet aggregations were detected on blood smears, the sample was excluded from this study. Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration, antithrombin III activity (AT III), fibrin degradation products (FDP) concentration, and D-dimer concentration were measured using the CA-500 (Sysmex). In-house reference ranges for each parameter were based on values obtained from healthy dogs. DIC was diagnosed when four or more abnormal findings were observed, which included low platelet count (<200 × 103/µl), prolonged PT (>10.0 sec) or prolonged aPTT (>20.0 sec, >25% of the reference range), low plasma fibrinogen concentration (<200 mg/dl), low plasma AT III activity (<80%), and high plasma FDP level (>10 µg/ml) or high plasma D-dimer level (>3.5 µg/dl). DIC was also diagnosed based on the presence of three or more of the abovementioned abnormal findings if FDP or D-dimer was not measured [27].

Measurement of plasma TF-PCA

All frozen samples were thawed once and analyzed within 6 months of freezing. The samples were thawed at 37°C and then centrifuged at 13,000 × g for 2 min at 4°C. The MPs were pelleted from 100 µl of the supernatant via centrifugation at 20,600 × g for 20 min at 4°C. The MP pellets were washed once with TBS and then suspended in 100 µl of TBS. A total of 40 µl of the MP suspension was seeded into a 96-well plate, and then 60 µl of coagulation factor mixture was added. The same volume of TBS was used as the negative control. After incubation at 37°C for 30 min, the final concentration of 0.25 mM of S-2765 was added to the platelet suspension via centrifugation at 20,600 × g for 20 min at 4°C. The MP pellets were washed once with TBS and then suspended in 100 µl of the MP suspension. The same volume of TBS was used as the negative control. After incubation at 37°C for 30 min, the final concentration of 0.25 mM of S-2765 was added to each well. After incubation at 37°C for 2 hr, the absorbance at 405 nm was measured using a Multiskan FC™ instrument. Each incubation time was modified from the procedure used in cell lines to increase the detection sensitivity. A standard curve was generated, as described above.

Statistical analysis

All statistical analyses were conducted using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). The difference in plasma TF-PCA between the DIC and non-DIC groups was determined using the Mann-Whitney U test. A P-value <0.05 was considered statistically significant.

RESULTS

TF expression on the cellular surface of canine tumor cell lines

TF expression was observed in all MGT cell lines, all HSA cell lines, except Re21, and all malignant melanoma cell lines, except CMeC-2, whereas none of lymphoma cell lines expressed TF (Fig. 2A–E). The expression level of TF varied among the cell lines.

Production of MPs in tumor cell lines

The number of MPs in the culture supernatants was measured via flow cytometry. All tested cell lines had detectable MPs in the culture supernatants (Fig. 3). The number of MPs in the culture supernatants varied among the cell lines. Ud2, CIPm, and CTBp had higher number of MPs in the culture supernatants than other cell lines, whereas CHMp, CHMp-5b, CIPp, KMeC, CLC, and Nody-1 had lower number of MPs in the culture supernatants than other cell lines.

Cellular surface and MPs-associated TF-PCA in tumor cell lines

TF-PCA could be detected on both the cellular surface and MPs in culture supernatants in all cell lines with TF expression (Fig. 4, cf. Fig. 2). However, the values varied among the cell lines. In MGT cell lines, CHMp-13a had high cellular surface and MP-associated TF-PCA, whereas CHHm had low cellular surface and MP-associated TF-PCA. The TF-PCA on the cellular surface was similar among CHMp-5b, CHHm, CIPp, CIPm, CTBp, and CTBm. However, MP-associated TF-PCA in CHMp-5b and CIPp was lower than that in other cell lines. In HSA cell lines, Ud2 and Ud6 had higher cellular surface and MP-associated TF-PCA than other cell lines. In melanoma cell lines, both the cellular surface and MP-associated TF-PCA in CMeC-2 were lower than those of other cell lines. In all three lymphoma cell lines, both the cellular surface and MP-associated TF-PCA could not be detected.

Plasma TF-PCA in dogs with malignant tumors

Seventeen dogs with malignant tumors were enrolled in the study. Among them, two presented with MG Ts, three with HSAs, six with malignant melanomas, and six with high-grade lymphomas (Multicentric, n=4 [case no. 12, 13, 16, and 17]; Gastrointestinal, n=2 [case no. 14 and 15]). The clinical characteristics, plasma TF-PCA values, hemostasis profiles, and diagnoses of DIC are...
Fig. 2. Expression of tissue factor (TF) on canine tumor cell lines. Cell surface expressions of TF were analyzed via flow cytometry; (A) D17 (negative control) and T24 (positive control), (B) mammary gland tumor, (C) hemangiosarcoma, (D) malignant melanoma, and (E) lymphoma cell lines. The shaded region and the region below the solid line in the histograms indicated staining with isotype control and anti-TF antibody, respectively. Results shown are representative of a minimum of three independent experiments.
shown in Table 1. Five dogs, including four with lymphomas and one with splenic HSA (cases no. 3, 12, 13, 16, and 17) were diagnosed with DIC. The reference value of plasma TF-PCA was <4.70 AU based on the measurements in 11 healthy dogs (mean ± 2 standard deviation; 2.58 ± 2.04). The plasma TF-PCA was remarkably elevated in three of five dogs with DIC, whereas it was slightly elevated in 9 of 12 dogs without DIC. The median plasma TF-PCA was 43.40 (range: 3.47–85.19) and 7.73 (range: 1.70–16.13) AU in dogs with and without DIC, respectively. No significant difference was observed between the two groups (P=0.328).

DISCUSSION

In this study, we found that most of MGT, HSA, and malignant melanoma cell lines expressed TF on their surfaces and had TF-PCA on both the cellular surface and released MPs; these results are consistent with those of previous investigations [33, 39]. Interestingly, TF expression, the number of MPs in the culture supernatant, and cellular surface and MP-associated TF-PCA varied among the cell lines. TF expression and MPs production are regulated via transcription factors, such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1), and Flippase/Floppase, respectively [13, 26]. The differences in the activity of these molecules may be responsible for the differences in TF expression and MPs production between each cell line. The cellular surface TF-PCA of the tumor cells mimicked the TF expression in that cells with high TF-PCA on the cellular surface (e.g., CHMp-13a and Ud6) also had high TF expression. However, the MP-associated TF-PCA did not necessarily match the TF expression and the cellular surface TF-PCA of tumor cells. For example, the TF-PCA on the cellular surface was similar among CHMp-5b, CHMm, CIPP, CIPm, CTBp, and CTBm, whereas MP-associated TF-PCA in CHMp-5b and CIPp was lower than that in other cell lines. CHMp-5b and CIPp had lower number of MPs in the culture supernatants than other cell lines. Thus, MP-associated TF-PCA can be affected by the degree of TF expression and cellular surface TF-PCA of the tumor cells as well as its ability to release MPs. Previous studies have indicated that tumor-derived TF-MPs can be released into blood circulation and enhance the activation of coagulation in mice [7, 38, 41]. Further studies are needed to elucidate the role of MP-associated TFs released from tumor cells and TFs of tumor cells themselves to the activation of coagulation in dogs with tumors.

To the best of our knowledge, this is the first study to measure plasma TF-PCA in dogs with malignant tumors and evaluate the association between TF-PCA and the presence of DIC. We demonstrated that plasma TF-PCA was elevated in some dogs with naturally developed tumors, particularly in those with DIC. However, plasma TF-PCA was not elevated in two lymphoma dogs with DIC (case no. 13 and no. 16). One possibility is that TF in local tumor tissues and/or a small number of TF-MPs in circulation cause DIC. Another possibility is that the inflammatory cytokines-mediated activation of leukocytes and downregulation of thrombomodulin in vascular endothelial cells play a central role in the development of DIC [16]. It is quite important whether
### Table 1. Characteristics of dogs with a malignant tumor enrolled in this study

| Case No. | Breed, age, gender | Type of tumor | Primary lesion | Metastasis | TF-PCA (AU) (<4.70) |
|----------|-------------------|---------------|---------------|-----------|-------------------|
| 1        | American Cocker Spaniel, 10Y, F | Mammary gland tumor | Mammary gland | LN | 3.40 |
| 2        | Shiba, 9Y, F | Mammary gland tumor | Mammary gland | Lung, LN | 5.19 |
| 3        | Golden Retriever, 10Y, M | Hemangiosarcoma | Spleen | Absent | 85.19 |
| 4        | Miniature Dachshund, 12Y, M | Hemangiosarcoma | Retropertioneal | Absent | 1.71 |
| 5        | Shih Tzu, 8Y, M | Hemangiosarcoma | Skin | Absent | 10.85 |
| 6        | Miniature Dachshund, 6Y, M | Malignant melanoma | Gingival | Absent | 10.56 |
| 7        | Miniature Dachshund, 9Y, FS | Malignant melanoma | Gingival | Absent | 6.75 |
| 8        | Miniature Dachshund, 6Y, MC | Malignant melanoma | Gingival | Absent | 7.97 |
| 9        | Miniature Dachshund, 11Y, M | Malignant melanoma | Intraoral | Absent | 10.73 |
| 10       | Labrador Retriever, 4Y, M | Malignant melanoma | Intraoral | Absent | 13.15 |
| 11       | Miniature Dachshund, 11Y, FS | Malignant melanoma | Intraoral | Absent | 7.50 |
| 12       | Great Pyrenees, 11Y, FS | Lymphoma | B cell | Stage IVb | 43.40 |
| 13       | Mixed, 6Y, M | Lymphoma | B cell | Stage Vb | 3.47 |
| 14       | Jack Russell Terrier, 6Y, FS | Lymphoma | T cell | Stage Ia | 16.13 |
| 15       | Papillon, 10Y, MC | Lymphoma | T cell | Stage Vb | 3.82 |
| 16       | Mixed, 12Y, MC | Lymphoma | B cell | Stage IVb | 3.72 |
| 17       | Mixed, 8Y, FS | Lymphoma | T cell | Stage Vb | 75.59 |

**T/B cell phenotype**

- T/B cell phenotype WHO stage
  - B cell
  - Stage IVb
  - Stage Vb

**Dic**

- WHO stage
  - Stage IVb
  - Stage Vb

**DIC diagnosis**

- Non-DIC
  - DIC
  - Non-DIC

TF-PCA, tissue factor procoagulant activity; PLT, platelets; PT, prothrombin time; aPTT, activated partial thromboplastin time; Fib, fibrinogen; AT III, antithrombin III; FDP, fibrin degradation products; DIC, disseminated intravascular coagulation; F, female; M, male; FS, spayed female; MC, castrated male; LN, lymph node; WHO, World Health Organization; ND, not determined. a) Reference range. b) DIC was diagnosed by four or more abnormal findings among the following: low platelet count (<200 × 10^3/µl), prolonged PT (>10.0 sec) or prolonged aPTT (>20.0 sec) (>25% of the reference range), low plasma fibrinogen concentration (<200 mg/dl), low plasma AT III activity (<80%), and high plasma FDP (>10 µg/ml) or high plasma D-dimer (>3.5 µg/dl). DIC was also diagnosed based on the presence of three or more of the abovementioned abnormal findings if FDP or D-dimer was not measured.
plasma TF-PCA levels reflect the presence of DIC and the clinical severity in dogs with DIC. However, in this study, no significant difference was observed in plasma TF-PCA between the DIC group and non-DIC groups and no association was observed between the clinical severity and the plasma TF-PCA levels in dogs with DIC. Although further studies are necessary to elucidate the efficacy of plasma TF-PCA as a biomarker of DIC in dogs with malignant tumors, our results provide insights into the pathogenic role of TF-MPs in activating coagulation in dogs with malignant tumors.

The present study had several limitations that should be considered, which were as follows: 1) The statistical power may be insufficient due to the small sample size. 2) Cases of suspected hemostatic abnormality were mainly included because residual plasma samples were used after measuring hemostatic parameters for clinical purposes, which may increase the proportion of cases with high plasma TF-PCA. 3) The diagnosis of DIC might be inaccurate because only a few dogs with DIC had prolonged PT and/or aPTT. Furthermore, thrombin-antithrombin complex was not measured in all dogs in this study. 4) Several preanalytical variables might have affected the formation of MPs and plasma TF-PCA values. The major preanalytical parameters, which might have affected the formation of MPs, included the delay between sample collection and centrifugation, agitation of blood samples, centrifugation protocol, and freezing/thawing [21, 23]. The effects of freezing/thawing were more pronounced when PPP samples rather than platelet free samples were used due to the contamination of cellular debris, platelets, and leukocytes [23]. Freezing/thawing might have affected plasma TF-PCA values because frozen PPP samples were used in this study. 5) The cellular origin of TF-MPs was not determined. In addition, a simple and rapid method for the measurement of plasma TF-PCA is needed for clinical application because the procedure used in this study is complicated.

It is noteworthy that plasma TF-PCA was elevated in three of six dogs with lymphoma, whereas none of the lymphoma cell lines expressed TF in vitro. However, TF expression in lymphoma tissues and cellular origin of plasma TF-MPs was not investigated in this study. A previous study has shown the absence of TF on tumor cells in human lymphoma tissues [5]. Another investigation has shown that TF expression was upregulated in peripheral leukocytes in patients with stage IV lymphoma and natural killer (NK) cell lymphoma and was found in vascular endothelial cells around the tumor tissue in NK cell lymphoma, indicating that malignant environment induced TF expression in cells other than tumor cells [32]. More interestingly, a recent study has shown that children with acute lymphoblastic leukemia have increased levels of platelet-derived, endothelial-derived, and TF-positive MPs [40]. Thus, plasma TF-PCA may be elevated in dogs with lymphoma due to TF-MPs derived from cells other than tumor cells.

In conclusion, we revealed TF expression and TF-PCA on both cellular surface and MPs released into their culture supernatant in canine MGT, HSA, malignant melanoma, but not lymphoma, cell lines. Plasma TF-PCA was elevated in some dogs that naturally developed malignant tumors, particularly in those with DIC, and this is the key novel finding of this investigation. These findings emphasized the critical pathogenic role of TF and/or TF-MPs in activating coagulation in dogs with malignant tumors. Further studies must be conducted to determine the cellular origin of plasma TF-MPs and to evaluate the efficacy of plasma TF-PCA as a biomarker of DIC in dogs with malignant tumors.

CONFLICTING INTERESTS. None of the authors have any financial or personal relationships with people or organizations that could inappropriately influence or bias the results of this study.

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