Generation of tissue factor-bearing procoagulant microparticles in dogs with malignant tumors

(イヌの腫瘍性疾患における組織因子含有凝固促進性マイクロパーティクルの産生)

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March 2020

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GENERAL INTRODUCTION

Hypercoagulability disorders, including disseminated intravascular coagulation (DIC) and thrombosis, are common, and are significantly associated with poor prognoses in dogs with malignant tumors [Andreasen *et al*., 2012; Kristensen *et al*., 2008; Lip *et al*., 2002; Maruyama *et al*., 2004; Ralph and Brainard, 2012]. However, the pathogenesis of DIC is not fully elucidated, and available tools for the diagnosis and treatment methods for procoagulant states in dogs are limited. Therefore, I conducted various experiments with the aim to elucidate the pathogenesis of DIC and develop a novel strategy for diagnosis and treatment of DIC in dogs.

Tissue factor (TF), which is the primary initiator of the extrinsic coagulation cascade, is thought to play a key role in the pathogenesis of hypercoagulability disorders. Aberrant TF expression was evaluated in various human tumor tissues [Callander *et al.*, 1992; Han *et al*., 2014] and cells expressing TF, including tumor cells, were thought to produce TF-bearing microparticles (MPs) in general circulation, and induce procoagulant states [Sartori *et al*., 2013; Zwicker *et al*., 2009]. Moreover, TF procoagulant activity (TF-PCA) of plasma was reportedly associated with the presence of DIC and thrombosis in human patients with malignant tumors [Cui *et al*., 2018; Langer *et al*., 2008]. Even in veterinary practice, plasma TF-PCA may be useful as a diagnostic biomarker of procoagulant states. Stokol *et al.* reported and discussed TF expression in tumor cells and the role for procoagulant states in canine malignancy [Gruber *et al.*, 2016; Stokol *et al.*, 2011; Witter *et al.*, 2017]. However, there are no clinical investigations into plasma TF-PCA and the importance in dogs

with hypercoagulability disorders. Therefore, in chapter 1, as a preliminary study, I investigated TF expression, release of MPs, and TF-PCA in canine tumor cell lines, including mammary gland tumors, hemangiosarcoma (HSA), malignant melanoma, and lymphoma cell lines, *in vitro*. Furthermore, I measured plasma TF-PCA in dogs with naturally developed malignant tumors and assessed the association between plasma TF-PCA and the presence of DIC.

DIC also develops in various diseases other than malignant tumors [Ralph and Brainard, 2012]. Thus, in chapter 2, I measured plasma TF-PCA in dogs with various underlying diseases of DIC, including malignant tumors, inflammatory diseases, and infections. Plasma TF-PCA was measured using the method described in chapter 1 with some optimizations. In addition, I discussed the association between the activity and presence of DIC based on evidence from clinical cases. Moreover, in some cases, plasma TF-PCA was assessed during the clinical course of disease.

For the treatment of dogs with DIC, low-molecular-weight heparin, which potentiates antithrombin activity, and rivaroxaban, which directly inhibits activated factor X, are used to prevent excess activation of the coagulation cascade [Mischke *et al*., 2005; Ralph and Brainard, 2012; Yang *et al*., 2016]. Blood transfusions are conducted for the supplementation of coagulation factors. However, gold-standard therapeutic procedures have never been established. Statins, the mevalonate pathway inhibitors, are commonly known as lipid-lowering medications. It is also presumed that statins have beneficial effects on systematic and local coagulation systems. Several *in vitro* studies

have reported that statins decrease TF expression and TF-PCA in endothelial cells [Eto *et al*., 2002] and tumor cells [Aberg *et al.*, 2008a; Aberg *et al.*, 2008b], and increase thrombomodulin expression in endothelial cells [Shi *et al*., 2003]. Fluvastatin suppresses tumor necrosis factor-α-induced MP release from endothelial cells [Tramontano *et al*., 2004]. Several clinical studies have suggested that statins decrease the risk of development of venous thromboembolism in human patients with solid tumors [Khemasuwan *et al*., 2010; Lötsch *et al*., 2014; Matsuo *et al.*, 2019]. Moreover, several pre-clinical studies indicate that statins inhibit cell proliferation and tumor growth in a variety of tumors *in vitro* and *in vivo* [Beckwitt *et al*., 2018; Fujiwara *et al*., 2017; Gbelcová *et al*., 2008; Tsubaki *et al*., 2017]. To date, a large number of human clinical trials have been used to investigate the treatment of cancers with statins [Iannelli *et al*., 2018]. Therefore, I hypothesized that statins have potential for the treatment of malignant tumors with procoagulant activity. In my preliminary study, I found that simvastatin inhibited cell proliferation but not TF-PCA in canine tumor cells. Therefore, in chapter 3, I investigated the anti-proliferative effect of simvastatin in canine tumor cells, including HSA, malignant melanoma, and lymphoma *in vitro*. Moreover, I investigated the cytotoxic mechanism focusing on the Ras signaling pathway, which is one of the most important tumor-driving mechanisms.

CHAPTER 1

Tissue factor procoagulant activity in tumor cell lines and plasma of dogs with various malignant tumors

SUMMARY

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 (HSA), 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 my 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.

INTRODUCTION

Hypercoagulability disorders, such as disseminated intravascular coagulation (DIC) and thrombosis, are often detected according to disease progression in human and canine patients with various malignant tumors and are significantly associated with poor prognosis [Andreasen *et al*., 2012; Kristensen *et al*., 2008; Lip *et al*., 2002; Maruyama *et al*., 2004; Ralph and Brainard, 2012]. Although various factors can contribute to the pathogenesis of hypercoagulability secondary to malignant tumors, tissue factor (TF), which is the primary initiator of the extrinsic coagulation cascade, is considered an essential element.

Aberrant TF expression is reportedly observed in various types of cancers in humans, particularly in cancers of epithelial origin [Callander *et al*., 1992; Han *et al*., 2014]. Moreover, TF is found on microparticles (MPs) released from tumor cells, monocytes, endothelial cells, and possibly platelets into the blood [Hron *et al*., 2007; Owens and Mackman, 2011; Sartori *et al*., 2013; Zwicker *et al*., 2009]. MPs are extremely small (0.1–1.0 μm in diameter) vesicles derived during cellular activation or damage and contain different proteins, phospholipids, and nuclear acids (mRNA and microRNA) from the parent cells; hence, they are considered an essential mediator in various pathological conditions [Herring *et al*., 2013]. Regardless of its cellular origin, circulating TF-bearing MPs (TF-MPs) can initiate blood coagulation via the formation of a complex with factor VII (FVII)/activated FVII (FVIIa) in the plasma. The increased levels of TF procoagulant activity

(TF-PCA), derived from TF-MPs, in the plasma are associated with the risk of venous thromboembolism [Bharthuar *et al*., 2013; Cui *et al*., 2018; Faille *et al*., 2018; Hisada *et al*., 2018; Manly *et al*., 2010; Tesselaar *et al*., 2009], DIC [Dicke *et al*., 2015; Langer *et al*., 2008; Thaler *et al*., 2014], and poor prognosis [Bharthuar *et al*., 2013; Hisada *et al*., 2016] in human patients with various malignant tumors. Even in veterinary practice, plasma TF-PCA may reflect prothrombotic status and aid in early detection, diagnosis, prediction of outcome, and decision of therapeutic intervention in DIC and thrombosis.

In canines, TF expression and TF-PCA have been reported in mammary gland tumor (MGT), pancreatic carcinoma, prostatic carcinoma, bronchoalveolar carcinoma, osteosarcoma, fibrosarcoma, and hemangiosarcoma (HSA) cell lines [Stokol *et al*., 2011; Witter *et al*., 2017]. Moreover, aberrant TF expression in canine intracranial meningiomas, gliomas, and MGTs *in situ* has been reported [Andreasen *et al*., 2016; de la Fuente *et al*., 2014; Font *et al*., 2015]. 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, I 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) [Murai *et al*., 2012; Uyama *et al*., 2006], canine malignant melanoma cell lines (CMeC-1, CMeC-2, KMeC, and LMeC) [Inoue *et al*., 2004], canine HSA cell lines (JuA1, JuB2, JuB4, Re12, Re21, Ud2, and Ud6) [Kodama *et al*., 2009], canine lymphoma cell lines (CLC, Nody-1, and UL-1) [Umeki *et al*., 2013] 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, lymphoma, and T24 cell lines were cultured in RPMI 1640 (Nakalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA), 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 [Stokol *et al*., 2011]. The cells (5×10^4) 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 three times.

MP separation from the culture supernatants of tumor cell lines

MPs were separated from culture supernatants using the protocol described in a previous report [Stokol *et al*., 2011] with slight modifications. In brief, 3×10^5 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×g for 10 min at 4°C. The MPs were separated via centrifugation at 20,600×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. The MP suspensions were immediately stored at −80°C and were analyzed within 2 weeks.

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 [Lacroix *et al*., 2010] (Fig. I-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 three times.

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

Cellular surface and MP-associated TF-PCA were measured using the protocol described

in a previous report [Stokol *et al*., 2011] with slight modifications. The cells were seeded at 3.0×10^4 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 MP suspensions were 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 $CaCl_2$ (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 three times.

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 vein 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 ($<200\times10^3/\mu l$), 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/ml). 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 [Maruyama *et al*., 2004].

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 each well. After incubation at 37°C for 2 hr, the absorbance at 405 nm was measured using a Multiskan FC^{TM} 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. I-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. I-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 MP-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. I-4, cf. Fig. I-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 CHMp had low cellular surface and MP-associated TF-PCA. The TF-PCA on the cellular surface

was similar among CHMp-5b, CHMm, 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 MGTs, 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 shown in Table I. 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, I 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 [Stokol *et al*., 2011; Witter *et al*., 2017]. 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 [Herring *et al*., 2013; Mackman *et al*., 1990]. 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 [Davila *et al*., 2008; Owens et al., 2008; Wang *et al*., 2012; Yu *et al*., 2005]. Further studies are needed to elucidate the role of MP-associated TF released from tumor cells and TF of tumor cells themselves to the activation of coagulation in dogs with tumors.

To the best of my 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. I 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 [Ikezoe, 2015]. It is quite important whether 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, my 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 [Lacroix *et al*., 2011; Lee *et al*., 2012]. 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 [Lee *et al*., 2012]. 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 [Cesarman-Maus *et al*., 2014]. 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 [Sase *et al*., 2005]. 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 [Yenigürbüz *et al*., 2019]. Thus, plasma TF-PCA may be elevated in dogs with lymphoma due to TF-MPs derived from cells other than tumor cells.

In conclusion, I 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.

CHAPTER 2

Plasma tissue factor procoagulant activity

in dogs with disseminated intravascular coagulation

SUMMARY

Disseminated intravascular coagulation (DIC) is a life-threatening complication characterized by systemic activation of coagulation. Plasma tissue factor procoagulant activity (plasma TF-PCA) might play a role in human DIC. The aim of this study was to compare plasma TF-PCA between non-DIC and DIC groups. Ten clinically healthy beagles and 26 diseased dogs (non-DIC, n = 14; DIC, n = 12) were enrolled. Plasma TF-PCA was measured using the Factor Xa generation assay. The reference value of plasma TF-PCA was defined as ≤11.3 AU calculated by mean+2SD for healthy controls. Median plasma TF-PCA was 5.0 AU (range, 1.9-37.2 AU) and 26.6 AU (range, 1.6-88.6 AU) in the non-DIC and DIC groups, respectively. The proportion of dogs with increased plasma TF-PCA was significantly higher in the DIC group than the non-DIC group (P = 0.014). Plasma TF-PCA in the DIC group was significantly higher than the non-DIC group (P = 0.021). Plasma TF-PCA positively correlated with plasma D-dimer concentration (r = 0.42, P = 0.034). Moreover, increased plasma TF-PCA was decreased by the time of recovery in some dogs with DIC. In conclusion, some dogs with an underlying DIC disease had increased plasma TF-PCA. Plasma TF-PCA might reflect hypercoagulability and be associated with DIC development. Larger prospective studies are warranted to determine whether plasma TF-PCA is increased in dogs with DIC and to assess its value as a diagnostic and prognostic biomarker in DIC.

INTRODUCTION

Disseminated intravascular coagulation (DIC) is a life-threatening condition characterized by systemic activation of coagulation. This can lead to widespread intravascular fibrin formation that can cause multiple organ failure and hemorrhage by depleting clotting factors and platelets, and activating fibrinolysis. DIC always occurs secondary to an underlying condition such as malignancy, infection, immune-mediated disease, sepsis, trauma, heatstroke, toxicosis, and other conditions [Ralph and Brainard, 2012].

Tissue factor (TF) is a transmembrane protein that functions as the principal initiator of the extrinsic coagulation cascade [Mackman *et al*., 2007]. TF overexpression related to the underlying disease process triggers excessive thrombin generation. TF expression on monocytes and endothelial cells is induced by several mediators such as bacterial lipopolysaccharide, tumor necrosis factor-α, interleukin-1β, interleukin-6, vascular endothelial cell growth factor, reactive oxygen species, serotonin, thrombin, and thromboxane A2 [Mackman and Bode, 2014; Mitrugno *et al*., 2016]. Some malignant tumor cell types also overexpress TF [Andreasen *et al*., 2016; de la Fuente *et al*., 2014; Font *et al*., 2015; Han *et al*., 2014]. Importantly, TF can be associated with circulating microparticles (MPs) in the blood [Owens and Mackman, 2011]. TF-bearing MPs (TF-MPs) are primarily released from activated monocytes, vascular endothelial cells, platelets, and TF-expressed tumor cells [Davila *et al*., 2008; Owens and Mackman, 2011; Sartori *et al*., 2013; Zwicker *et al*., 2009]. TF-MPs plasma

levels can be measured in humans using flow cytometry and antigen- and activity-based assays [Key and Mackman, 2010]. Activity-based assays, in which TF procoagulant activity (TF-PCA) of MPs is measured using Factor Xa (FXa) generation, are considered more sensitive and reliable than other assays [Hisada *et al*., 2016; Lee *et al*., 2012]. Several studies have been reported TF-PCA measurements in cells and plasma from dogs [Kidd *et al*., 2015; Kobayashi *et al.*, *in press*; Stokol *et al*., 2011].

In people, increased TF-MPs levels have been found in various conditions associated with hypercoagulability including endotoxemia, infectious diseases, inflammatory diseases, cardiovascular diseases, liver injury, cirrhosis, and malignant tumors [Bharthuar *et al*., 2013; Hellum *et al*., 2013, Hisada *et al*., 2016; Hisada *et al*., 2018; Ou *et al*., 2017; Owens and Mackman, 2011; Rautou *et al*., 2014; Stravitz *et al.*, 2013; Tesselaar *et al*., 2007; Woei-A-Jin *et al*., 2012]. Several studies have also reported that plasma TF-PCA is associated with increased risk of thrombosis and DIC [Bharthuar *et al*., 2013; Cui *et al*., 2018; Dicke *et al*., 2015; Langer *et al*., 2008; Thaler *et al*., 2014]. On the contrary, there have been few studies of plasma TF-PCA in dogs with a spontaneous disease. A previous study reported that some dogs with immune-mediated hemolytic anemia (IMHA), which includes hypercoagulability as a well-known symptom, have higher plasma TF-PCA than clinically normal dogs [Kidd *et al*., 2015]. Recently, I demonstrated that some dogs with a malignant tumor, including hemangiosarcoma (HSA), malignant melanoma, and high-grade lymphoma, have

higher plasma TF-PCA than clinically normal dogs [Kobayashi *et al*., *in press*]. However, to the best of my knowledge, there have been no studies of the association between plasma TF-PCA and DIC in dogs. Thus, the aim of this study was to compare plasma TF-PCA among non-DIC and DIC groups consisting of dogs with various underlying diseases. In some cases, plasma TF-PCA was assessed during the clinical course.

MATERIALS AND METHODS

Study population

A retrospective study was conducted with dogs referred to the Yamaguchi University Animal Medical Center (YUAMEC) from January 2015 to May 2018. Informed written consent for the storage and subsequent use of clinical specimens for research purposes was obtained from the owners at admission. All procedures were approved by the institutional ethics committee for animal clinical tests at the Joint Faculty of Veterinary Medicine in Yamaguchi University (approval No. 007).

Ten clinically healthy beagles maintained as blood donors at YUAMEC comprised the healthy control group. The criteria for healthy dogs included an unremarkable routine physical examination and no abnormalities detected on complete blood count (CBC), serum biochemistry, X-ray examination, and coagulation-fibrinolysis tests consisting of platelet count, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration, antithrombin III (AT III) activity, and D-dimer concentration. Client-owned diseased dogs that underwent coagulation-fibrinolysis tests at presentation were included in this study and divided into two groups (non-DIC and DIC) depending on the number of abnormal findings observed in the tests [Goggs *et al.*, 2018]. Abnormal findings in coagulation-fibrinolysis tests were defined as follows: low platelet count ($<20\times10^4/\mu l$), prolonged PT (>10.0 sec), prolonged aPTT (>20.0 sec), low plasma fibrinogen

concentration (<200 mg/dl), low plasma AT III activity (<80%), and high plasma D-dimer concentration (>3.5 μg/ml). Diseased dogs were diagnosed as DIC if abnormalities in 3 or more of the abnormalities. Dogs that did not have results for all of six coagulation-fibrinolysis parameters were excluded from this study. The dogs were also excluded if platelet aggregations on blood smears and hemolysis of plasma samples were observed because they might be due to artificial errors in blood sampling, and hemolysis might increase plasma TF-PCA [Kidd *et al.*, 2015]. The dogs were also excluded if chyle of plasma samples was observed because lipid precipitates were contaminated into isolated MPs. A total of 26 cases fulfilled the inclusion criteria.

Laboratory tests

Client-owned diseased dogs underwent coagulation-fibrinolysis tests in addition to clinically relevant examinations including CBC, serum biochemistry, urinalysis, fecal examination, X-ray, ultrasonography, computed tomography (CT), and magnetic resonance imaging. Platelet count was measured using the XT-2000iV system (Sysmex, Kobe, Japan). PT, aPTT, fibrinogen concentration, AT III, FDP concentration, and D-dimer concentration were measured using the CA-500 (Sysmex).

Samples

Venous blood (0.9 ml) was collected from the cervical or saphenous vein with 0.1 ml of 3.8% sodium citrate using a 23-gauge needle and a 1-ml syringe. The blood samples were centrifuged at 2,000×g for 5 min at room temperature. Platelet poor plasma (PPP) samples were cautiously collected without disturbing the buffy coat and subjected to coagulation-fibrinolysis tests. The remainder of the PPP samples were stored at 4°C. To measure plasma TF-PCA, 125 µl of the PPP was centrifuged at 13,000×g for 2 min at 4°C within 24 hr from the storage at 4°C to remove platelets and cellular fragments; 100 µl of the supernatant (platelet free plasma: PFP) was frozen at -80°C. All frozen samples were thawed once and analyzed within a year of freezing.

Measurement of plasma TF-PCA

Plasma TF-PCA was measured using the FXa generation assay according to previously described procedures [Kobayashi *et al*., *in press*] with slight modifications; 100 µl of PFP samples were thawed at 37°C, and the MPs were isolated by centrifuging at 20,600×g for 20 min at 4°C. The supernatants were removed and were used as a negative control. Then, 900 µl of tris-buffered saline (TBS) was added to both the MPs-containing pellet and the supernatant, followed by another centrifugation under the same conditions. After the supernatants were removed, the pellets were resuspended with 50 µl of TBS. A total of 40 µl of the MP suspension and the control were seeded into a 96-well plate, and then 60 µl of coagulation factor mixture [TBS with recombinant human

activated Factor VII (final concentration of 1 nM; Haematologic Technologies, Burlington, VT, U.S.A.), recombinant human FX (final concentration of 30 nM; Haematologic Technologies), and $CaCl_2$ (final concentration of 10 mM)] was added into each well. After incubating at 37°C for 30 min, the final concentration of 0.25 mM of S-2765 (Sekisui medical, Tokyo, Japan) was added. After incubating at 37°C for 2 hr, the absorbance at 405 nm was measured using a Multiskan FC™ microplate reader (Thermo Fisher Scientific, Yokohama, Japan). A standard curve was generated, and 100 arbitrary units (AU) were defined as a dilution ratio of 1:5000 for recombinant human TF (Dade® Innovin®, Sysmex).

Statistics

All statistical analyses were performed with BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). Difference of plasma TF-PCA between the non-DIC and DIC groups was analyzed using Mann–Whitney U test. Difference of the proportion of dogs with increased plasma TF-PCA between the non-DIC and DIC groups was analyzed using Fisher's exact test. Correlation between plasma TF-PCA and plasma D-dimer concentration was analyzed by Spearman's rank correlation coefficient. *P* value of <0.05 was considered statistically significant.

RESULTS

Study population

Ten clinically healthy beagles and 26 diseased dogs were enrolled in this study. Diseased dogs were divided into non-DIC (n = 14) and DIC (n = 12) groups. The signalment, diagnosis, and plasma TF-PCA of healthy beagles and 26 diseased dogs are shown in Table II-1. Dogs with high-grade lymphoma were included in the non-DIC (5 dogs; 35.7%) and DIC (5 dogs; 41.7%) groups. The immunophenotype and occurrence sites varied among cases. Dogs with HSA were included in DIC groups (2 dogs; 16.7%) but not in the non-DIC group. Dogs with IMHA were included in the non-DIC (1 dog; 6.7%) and DIC (2 dogs; 16.7%) groups. Dogs with acute pancreatitis (AP) were included in the non-DIC (1 dog; 6.7%) and DIC (1 dog; 8.3%) groups. Dogs with pyometra were included in the non-DIC (2 dogs; 14.3%) groups but not in the DIC group. Splenic spindle cell carcinoma and leptospirosis (each 1 dog; 8.3%) were also included in the DIC group but not in the non-DIC groups.

Plasma TF-PCA in healthy and diseased dogs

The mean and median plasma TF-PCA in 10 healthy control beagles was 5.3±3.0 AU (mean±SD) and 5.0 AU (range; 0.0-10.0 AU), respectively. The reference value of plasma TF-PCA was defined as ≤11.3 AU (mean+2SD). Ten of 26 dogs (38.5%) had plasma TF-PCA greater than the

reference value (HSA: 2 dogs, lymphoma: 3 dogs, AP: 2 dogs, IMHA: 1 dog, leptospirosis: 1 dog, spindle cell sarcoma: 1 dog). The proportion of dogs with increased plasma TF-PCA was significantly higher in the DIC group (8/12 dogs, 66.7%) than the non-DIC group (2/14 dogs, 14.3%) ($P = 0.014$). Median plasma TF-PCA was 5.0 AU (range, 1.9-37.2 AU) and 26.6 AU (range, 1.6-85.2 AU) in the non-DIC and DIC groups, respectively (Fig. II-1). Plasma TF-PCA in the DIC group was significantly higher than the non-DIC group ($P = 0.021$). Plasma TF-PCA was positively correlated with plasma D-dimer concentration ($r = 0.42$, $P = 0.034$) (Fig. II-2).

Follow-up study of three cases

Repeated samples were available from 3 dogs during treatment, including dogs with splenic HSA (case no. 16), AP (case no. 23), and IMHA (case no. 25). The detailed results of coagulation-fibrinolysis tests in these dogs are shown in Table II-2. Plasma TF-PCA in these dogs were followed-up during the clinical course (Fig. II-3A). In case no. 16, plasma TF-PCA was decreased from 85.2 AU at presentation to 19.6 AU after splenectomy. In case no. 23, the dog was diagnosed with AP depending on the results of serum biochemistry, SNAP cPL test (IDEXX, Tokyo, Japan), and abdominal ultrasonography. The dog gradually recovered with intensive care, and improvements of coagulation-fibrinolysis parameters were observed on day 9. Plasma TF-PCA decreased from 62.5 AU at presentation to 5.1 AU on day 9. In case no. 25, the dog was diagnosed

with primary IMHA depending on the findings of severe regenerative anemia, autoagglutination, and the other clinical examinations. The dog was gradually recovered with intensive care, and DIC cessation was confirmed on day 14. Plasma TF-PCA was decreased from 34.3 AU at presentation to 6.2 AU on day 14. D-dimer concentrations also decreased in all the dogs when they were recovered (Fig. II-3B).

DISCUSSION

In the present study, I found that some diseased dogs had increased plasma TF-PCA. I did not determine the cellular origin of circulating TF-MPs in these dogs. Recent studies demonstrated that TF expression and TF-PCA were detected in some canine HSA cell lines but not lymphoma cell lines [Kobayashi *et al*., *in press*; Witter *et al*., 2017]. Although TF expression in canine HSA *in situ* was not clarified, tumor cells-derived TF-MPs might directly contribute to the plasma TF-PCA in dogs with HSA. On the contrary, several studies of human patients with lymphoma suggest that hypercoagulability in patients with lymphoma is likely not secondary to tumor-derived TF [Cesarman-Maus *et al*., 2014; Sase *et al*., 2005]. The hypothesis suggested in human lymphoma also seems plausible in dogs with lymphoma.

IMHA is an important cause of DIC and thrombosis in dogs [Carr *et al*., 2002]. TF mRNA expression is increased, and the concentration of cytokines associated with monocyte activation is elevated in blood from dogs with IMHA [Kjelgaard-Hansen *et al*., 2011; Piek *et al*., 2011]. Moreover, recent studies demonstrated that the number of TF-positive thrombocytes and plasma TF-PCA were increased in dogs with IMHA [Hennink *et al*., 2018; Kidd *et al*., 2015]. Thus, increased plasma TF-PCA might be associated with thrombosis and DIC development in dogs with IMHA. In this study, plasma TF-PCA was increased in a dog (case no. 25) but not in the other dog (case no. 24) among dogs with DIC secondary to IMHA. A previous report indicates that PCA with

phosphatidylserine (PS)-positive MPs (PS-MPs) is increased in some dogs with IMHA in the absence of plasma TF-PCA [Kidd *et al*., 2015]. Therefore, PS-MPs might also contribute to DIC development in dogs with IMHA.

Two dogs with AP had increased plasma TF-PCA, which was remarkably high (62.5 AU) in the dog with DIC compared to slightly high (13.3 AU) in the other non-DIC dog. A recent investigation demonstrated that the number of TF-MPs is highly increased in both human severe AP patients and a severe AP mouse model [Ou *et al*., 2017]. This is accompanied by blood coagulation system dysfunction, and TF expression is up-regulated in the Kupffer cells of the mouse model. In addition to Kupffer cells, activated monocytes, damaged endothelial and pancreatic ductal epithelial cells may be a source of TF and TF-MPs in dogs with AP because inflammation and endothelial and pancreatic ductal damage are associated with AP.

In the present study, plasma TF-PCA in the DIC group was significantly higher than the non-DIC group. Plasma TF-PCA was also positively correlated with D-dimer concentration. These results suggest that increased plasma TF-PCA reflects hypercoagulability and is associated with DIC development in dogs with various diseases. However, plasma TF-PCA may have increased as a consequence of DIC rather than a cause of DIC due to a potential problem with retrospective study. Prospective cohort studies are needed to determine whether plasma TF-PCA causes DIC in dogs. Furthermore, plasma TF-PCA and D-dimer concentration decreased by the time of recovery in some

dogs with DIC, suggesting that decreased plasma TF-PCA reflects the withdrawal from the hypercoagulable state in dogs. Prospective studies with comprehensive monitoring for DIC development over a defined follow-up period are needed to determine whether plasma TF-PCA is a predictive DIC biomarker.

Plasma TF-PCA was not increased in 4 of 12 dogs (33.3%) with DIC. This could be caused if my assay has a low sensitivity to plasma TF-PCA. It could also mean that hypercoagulability occurs in the absence of plasma TF-PCA, leading alternative cellular and molecular pathways to primarily contribute to hypercoagulability. In this regard, several factors like anionic phospholipids, polyphosphates, and nucleic acids have been indicated as key intrinsic contact pathway activation mediators [Mackman, 2012]. On the other hand, plasma TF-PCA was increased in 2 of 14 dogs (14.3%) in non-DIC group. Several reasons could be considered, which were as follows: First, increased plasma TF-PCA might reflect pre-DIC state before onset of DIC. Second, natural anticoagulant pathways, such as tissue factor pathway inhibitor, antithrombin, protein C, protein S, and thrombomodulin, might prevent onset of DIC in the presence of increased plasma TF-PCA [Papageorgiou *et al*., 2018]. Third, several preanalytical variables might affect the formation of MPs in samples, including the delay from blood collection to centrifugation, blood sample agitation, centrifugation protocol, and freezing/thawing [Lacroix *et al*., 2011; Lee *et al*., 2012]. The handling and delay from blood collection to centrifugation varied among samples due to

the retrospective nature of this study. However, I identically treated all samples after the second centrifugation to obtain PFP and limited the effect of these preanalytical variables.

There are several additional limitations to my study. First, there was a very small number of dogs in each group. Second, several factors like sample size, age, gender, dog breed, and disease types were not completely matched among the groups. Third, DIC diagnosis could be imprecise because there is no validated gold standard for DIC diagnosis in dogs. A recent study indicated that thrombin-antithrombin complex (TAT) is a useful marker for DIC diagnosis in dogs [Rimpo *et al*., 2018]. In future studies, TAT concentration should be included for DIC diagnosis. Finally, concurrent diseases, medications, and other treatments were not considered in this study.

In conclusion, this study is the first to suggest that increased plasma TF-PCA reflects hypercoagulability and is associated with DIC development in dogs with various diseases. However, a conclusion cannot be made regarding the causal relationship between plasma TF-PCA and DIC development. Larger prospective studies are warranted to determine whether plasma TF-PCA causes DIC in dogs and to assess its value as a diagnostic and predictive DIC biomarker.

CHAPTER 3

Effect of simvastatin on cell proliferation and Ras activation in canine tumor cells

SUMMARY

Statins are inhibitors of the mevalonate cascade that is responsible for cholesterol biosynthesis and the formation of intermediate metabolites, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) used in the prenylation of proteins. Although statins are widely used in the treatment of hypercholesterolemia, recent studies suggest that they also inhibit proliferation of tumor cells by reducing prenylation of small GTP-binding proteins, such as Ras. This study aimed to evaluate the effect of simvastatin on cell proliferation and Ras activation in various canine tumor cell lines, including hemangiosarcoma (HSA), melanoma, and lymphoma cell lines. Simvastatin inhibited cell proliferation of all cell lines tested in a concentration- and time-dependent manner, but the susceptibilities were different amongst the cell lines. Simvastatin induced apoptotic cell death via activation of caspase-3 and cell cycle arrest. The cytotoxic effects of simvastatin were attenuated by GGPP and FPP. Simvastatin decreased the amount of prenylated Ras and GTP-bound Ras in HSA and melanoma cell lines, but not in lymphoma cell lines. However, there were no substantial changes in the level of phosphorylated Erk1/2 and Akt as downstream effectors of Ras in the simvastatin-treated cells. These results indicate that simvastatin induces cytotoxic effects through the depletion of GGPP and FPP in a variety of canine tumor cells. However, it is clear that multiple mechanisms are involved in. Further study is required to elucidate the underlying mechanisms of simvastatin-induced cytotoxic effects in a variety of canine tumor cells.

INTRODUCTION

The mevalonate (MVA) pathway is required for the synthesis of cholesterol and isoprenoids, which are the intermediate products of the MVA pathway. These intermediate products also include isopentenyl pyrophosphate, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), dolichol, ubiquinone, and isopentenyl adenine [Goldstein and Brown, 1990]. Statins inhibit 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), which is the rate-limiting enzyme in the MVA pathway, resulting in prevention of the synthesis of cholesterol and isoprenoids. Statins have historically been used as cholesterol-lowering agents in the treatment of hypercholesterolemia [Endo, 1992]. Several lines of evidence indicate that statins exhibit pleiotropic effects other than cholesterol-lowering activities, such as improving endothelial function, enhancing the stability of atherosclerotic plaques, prevention of platelet aggregation, antioxidant effects, anti-inflammatory effects, immunomodulatory effects, and anti-proliferative effects [Liao and Laufs, 2005; Zhou and Liao, 2009]. In cancers, statins appear to exert a variety of pleiotropic effects, including inhibition of cell proliferation, enhanced apoptosis, cell cycle arrest, and modulation of angiogenesis, inflammation, and endothelial function. Pre-clinical studies have reported that statins inhibit cell proliferation and tumor growth in a variety of tumors *in vitro* and *in vivo* [Beckwitt *et al*., 2018; Fujiwara *et al*., 2017; Gbelcová *et al*., 2008; Tsubaki *et al*., 2017]. Epidemiological studies have suggested that statins improve prognosis in human cancers, including endocrine-related

gynecological cancers, esophageal adenocarcinomas, prostate cancers, breast cancers, multiple myelomas, colorectal cancers, and lung cancers [Alexandre *et al*., 2016; Cardwell *et al*., 2017; Hung *et al*., 2017; Iannelli *et al*., 2018; Liu *et al*., 2017; Murtola *et al*., 2017; Sanfilippo *et al*., 2016; Yokomichi *et al*., 2017; Xie *et al*., 2017; Zhong *et al*., 2015]. This evidence indicates the potential of statins as anti-cancer agents.

The intermediate products of the MVA pathway, FPP and GGPP, act as lipophilic anchors on the cell membrane for small GTPases, such as Ras. After prenylation with FPP and GGPP, small GTPases translocate from the cytoplasm to the membrane, and then play their role in cell signal transduction [Thurnher *et al*., 2012]. The Ras signaling pathway is frequently activated in many human cancers and the blockade of Ras signaling is an attractive therapeutic strategy for cancers with aberrant Ras signaling [Gray-Schopfer *et al*., 2007]. Previous studies demonstrated that lipophilic statins, such as atorvastatin, lovastatin, and simvastatin, induced caspase-dependent apoptosis and/or cell cycle arrest at G1 phase in tumor cells. This was achieved by inhibiting Ras prenylation and the downstream effectors, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K), through the depletion of FPP and GGPP [Beckwitt *et al*., 2018; Fujiwara *et al*., 2017; Peng *et al*., 2017; Tsubaki *et al*., 2017]. Thus, while inhibition of Ras prenylation is one of the most important mechanisms for statin-induced cytotoxic effects, the precise mechanisms have not been fully elucidated.

Little has been reported on the anti-tumor effects of statins in veterinary medicine. It has been reported that simvastatin attenuates the proliferation of canine mammary cancer stem-like cells, promoting their chemosensitization and apoptosis [Cruz *et al.*, 2018; Torres *et al*., 2015]. This evidence in human and veterinary medicine encouraged me to explore the cell types and mechanisms by which statins induce anti-tumor effects in canine tumors.

In the present study, I investigated the effect of simvastatin on cell proliferation and Ras signaling activation in canine hemangiosarcoma (HSA), melanoma, and lymphoma cells *in vitro*. I hypothesized that simvastatin induces apoptotic cell death and/or cell cycle arrest by inhibiting the prenylation and activation of Ras in these cells.

MATERIALS AND METHODS

Cell cultures and reagents

Canine HSA (JuB2, Re12, and Ud6) [Kodama *et al*., 2009], melanoma (CMeC-1, CMeC-2, KMeC, and LMeC) [Inoue *et al*., 2004], and lymphoma (T cell origin: CLC, Nody-1, and UL-1; B cell origin: CLBL-1 and 17-71) [Rütgen *et al.*, 2010; Steplewski *et al*., 1987; Umeki *et al.*, 2013] cell lines were used in this study. HSA cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, U.S.A.), 100 U/ml penicillin (Nacalai Tesque, Kyoto, Japan) and 100 μg/ml streptomycin (Nacalai Tesque). Melanoma and lymphoma cell lines were cultured in RPMI-1640 medium (Wako) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 55 μM 2-mercaptoethanol (Sigma, St. Louis, MO, U.S.A.). All cell lines were maintained at 37°C in a humidified 5% carbon dioxide (CO_2) incubator. Dimethyl sulfoxide (DMSO) was purchased from Nacalai Tesque. Simvastatin was purchased from Sigma. Doxorubicin, FPP, and GGPP were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Z-VAD-fmk was purchased from R&D Systems (Minneapolis, MN, U.S.A.). Rabbit anti-human cleaved caspase-3 antibody (#9661), anti-human phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (#9101), anti-human Erk1/2 antibody (#9102), anti-human phospho-Akt (Ser473) antibody (#9271), and anti-human Akt antibody (#9272) were purchased from Cell Signaling Technologies (Danvers, MA,

U.S.A.). Mouse anti-human β-actin antibody (clone AC-15) was purchased from Sigma. Horseradish peroxidase (HRP)-conjugated secondary antibodies specific for rabbit and mouse IgG were purchased from Cell Signaling Technologies and Chemicon (Temecula, CA, U.S.A.), respectively.

Cell proliferation assay

Cell proliferation was assessed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cells (1×10^3 cells of CLC/well or 5×10^3 cells of the other cell lines/well in 96-well plates) were treated with different concentrations of simvastatin (0, 0.5, 1, 3, and 5 μM). At the indicated time points (24, 48, and 72 hr), the absorbance was determined at 450 nm using a microplate reader (Multiskan FC^{TM}; Thermo Fisher Scientific). The percentage absorbance was calculated against DMSO-treated cells. To evaluate the role of FPP and GGPP depletion in the cytotoxic effect of simvastatin on tumor cells, 20 μM FPP, 20 μM GGPP, and/or methanol (control) were added to culture medium 2 hr before treatment with 5 μM simvastatin. After culturing the cells for 48 hr, cell proliferation was assessed.

To evaluate simvastatin-mediated apoptosis, cells were pre-treated with 50 μM Z-VAD-fmk 1 hr before treatment with 5 μM simvastatin. After culturing for 48 hr, cell proliferation was assessed. Each experiment was performed in triplicate and repeated at least three times.

Immunoblotting

Cells were treated with 5 μM simvastatin, doxorubicin (1 μM in JuB2, Re12, CLBL-1, and 17-71, 10 μM in the other cell lines), or DMSO for 24 hr. In some experiments, cells were pre-treated with 20 μM FPP and 20 μM GGPP 2 hr before treatment with simvastatin. Cells were lysed with NP40 lysis buffer [1% NP40, 10 mM Tris hydrogen chloride (HCl) (pH 7.5), 150 mM sodium chloride (NaCl) and 1 mM ethylenediamine tetra-acetic acid (EDTA)], which was supplemented with an EDTA-free protease inhibitor cocktail (Nacalai Tesque) and a phosphatase inhibitor cocktail (Nacalai Tesque). Twenty μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Merck Millipore, Darmstadt, Germany). The membranes were blocked for 1 hr with 5% low-fat milk in Tris-buffered saline [TBS; 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl] containing 0.01% Tween 20 and then incubated with each primary antibody overnight at 4°C followed by incubation with HRP-conjugated secondary antibody for 1 hr at room temperature. The blots were developed with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and visualized using a LAS-3000 (Fuji Film, Tokyo, Japan). Each experiment was repeated at least three times.

Trypan blue exclusion assay

Cells were treated with 50 μM Z-VAD-fmk or DMSO for 1 hr before treatment with 5 μM simvastatin. After culturing for 48 hr, equal volumes of cell suspension and 0.5% trypan blue solution were gently mixed. The viable cells and dead cells were counted on a hemocytometer. Each experiment was performed in triplicate and repeated at least three times.

Cell cycle analysis

Cells were seeded in a 12-well plate at 1×10^5 cells/well. The cells were then treated with 5 μM simvastatin or DMSO for 24 hr. Adherent cells were harvested by trypsinization and combined with cells floating in the medium. Treated cells were washed twice with ice-cold phosphate buffered saline (PBS) and fixed in 70% ethanol overnight at -20°C. After washing twice with ice-cold PBS, the fixed cells were treated in 75 μl of PBS supplemented with 0.1% triton-X and 0.2 mg/ml RNase A (Sigma) for 15 min at 37°C, and then 75 μl of PBS containing 40 μg/ml propidium iodide (PI) solution (Sigma) was added. After incubation for 30 min at 4°C in the dark, cells were washed twice with PBS and analyzed for DNA content by flow cytometry (BD Accuri™ C6; BD Biosciences, San Jose, CA, U.S.A.). Analyses were conducted with FlowJo software ver. 10.6.1 (Tree Star, Ashland, OR, U.S.A.). Each experiment was performed in triplicate and repeated at least three times.

Ras pull-down assay

Ras activation was analyzed using the Ras Activation Assay Kit (Merck-Millipore) according to the manufacturer's instructions. Briefly, 3×10^6 cells were treated with 5 μM simvastatin or DMSO for 24 hr and lysed with Mg^{2+} Lysis Buffer. The lysates were stored at -80°C until required. Three hundred μg of cell lysate was incubated with 7.5 μg of GST-fused Raf1 RBD agarose beads for 1 hr at 4°C while agitating. Beads were isolated by centrifugation and the precipitated Ras-GTP was subjected to SDS-PAGE followed by immunoblotting with the anti-Ras antibody (clone RAS10, 05-516, Merck-Millipore). As a positive control, total cell lysate was included in the immunoblotting assays.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical comparisons between control and treatment groups were performed by one-way ANOVA with Dunnett's multiple comparison post hoc test or by unpaired Student's t-test using BellCurve for Excel (Social Survey Research Information Co., Ltd, Tokyo, Japan). Differences with a *p*-value less than 0.05 were considered statistically significant.

RESULTS

Simvastatin inhibits cell proliferation of canine HSA, melanoma, and lymphoma cell lines

I initially examined the effect of simvastatin on cell proliferation of canine HSA, melanoma, and lymphoma cell lines. Simvastatin inhibited cell proliferation of these cell lines in a concentration- and time-dependent manner (Fig. III-1). The anti-proliferative effect of simvastatin was apparent 24 hr after incubation with 0.5 μM and 48 hr after incubation with 3 μM in Re12 and Ud6, respectively (Fig. III-1A, B). JuB2 appeared less susceptible to simvastatin than Re12 and Ud6 (Fig. III-1C). The anti-proliferative effect of simvastatin was apparent 24 hr after incubation with 3 μM (CMeC-1) and 5 μM (KMeC), and 48 hr after incubation with 3 μM (CMeC-2 and LMeC) (Fig. III-1D-G). CMeC-2 showed the lowest susceptibility to simvastatin among melanoma cell lines tested. The anti-proliferative effect of simvastatin was apparent 24 hr after incubation with 0.5 μM in CLC, UL-1 and Nody-1 (Fig. III-1H-J). The anti-proliferative effect of simvastatin was also observed in CLBL-1 and 17-71, but was not remarkable (Fig. III-1K, L). In summary, these results indicate that simvastatin inhibits cell proliferation of a variety of canine tumor cell lines in a concentration- and time-dependent manner, whereas the susceptibilities are different amongst cell lines regardless of tumor type.

Simvastatin induces caspase-dependent apoptotic cell death

To determine whether simvastatin induces apoptotic cell death in a variety of canine tumor cell lines, I examined activation of caspase-3 in cells incubated with simvastatin. Cleaved caspase-3 increased 24 hr after incubation with 5 μM simvastatin in Re12, Ud6, CMeC-1, KMeC, LMeC, CLC, Nody-1, and UL-1, but not in JuB2, CMeC2, CLBL-1, and 17-71 (Fig. III-2A-C). These results corresponded to the susceptibility of each cell lines to simvastatin, as shown in Figure III-1. To further confirm simvastatin-induced apoptosis, cells were pre-incubated with a pan-caspase inhibitor, Z-VAD-fmk, and then cell viability and cell proliferation were measured. Cell viability was significantly ($p < 0.01$) recovered by pre-incubation with Z-VAD-fmk in Re12, CMeC-1, KMeC, and UL-1 but not in Ud6 and Nody-1 (Fig. III-3A). Cell proliferation was also significantly ($p < 0.01$) recovered by pre-incubation with Z-VAD-fmk in Re12 ($p < 0.05$), CMeC-1, KMeC, and UL-1 but not in Ud6 and Nody-1 (Fig. III-3B). However, neither cell viability nor cell proliferation were fully recovered by pre-incubation with Z-VAD-fmk in all cell lines tested. These results indicate that simvastatin-induced inhibition of cell proliferation is partially mediated by caspase-dependent apoptotic cell death, and other mechanisms may be involved.

Simvastatin induces cell cycle arrest

To investigate the effect of simvastatin on cell cycle progression in a variety of canine tumor cell lines, cells were treated with 5 μM simvastatin and then subjected to cell cycle analysis.

As shown in Figure III-4, the proportion of G0/G1 phase cells was significantly increased 24 hr after treatment with simvastatin in Re12, Ud6, Nody-1, CMeC-1 and KMeC, indicating that simvastatin induces cell cycle arrest at G0/G1 phase in these cell lines (CMeC-1 and KMeC, $p < 0.001$; Re12, Ud6, and Nody-1, $p < 0.01$). Simvastatin significantly ($p < 0.01$) increased the proportion of G2/M phase in UL-1. These results indicate that simvastatin has anti-proliferative effects by inducing cell cycle arrest, but the underlying mechanisms may be different among cell lines.

Simvastatin-induced inhibition of cell proliferation is dependent on depletion of FPP and GGPP

To assess whether simvastatin-induced inhibition of cell proliferation was dependent on the MVA pathway, the effect of FPP and GGPP on cell proliferation and caspase-3 activation in simvastatin-treated cells was assessed. The simvastatin-induced inhibition of cell proliferation was significantly attenuated by pre-treatment with FPP and GGPP in all cell lines tested (Fig. III-5A-C). Simvastatin-induced inhibition of cell proliferation was more efficiently attenuated by GGPP compared with FPP in all cell lines tested. Simvastatin-induced activation of caspase-3 was also attenuated by GGPP and FPP in Re12, KMeC, and UL-1 (Fig. III-6). These results indicate that simvastatin induces cytotoxic effects via suppression of the synthesis of isoprenoids, mainly GGPP.

Simvastatin inhibits Ras activation in canine HSA and melanoma cell lines, but not in lymphoma

cell lines

GGPP and FPP play an important role in Ras activation via prenylation, and thus I hypothesized that the Ras signaling pathway is involved in simvastatin-induced cytotoxic effects. To test the hypothesis, Ras protein prenylation and the activation in cells treated with simvastatin were investigated. GTP-bound Ras was detected in all cell lines tested, excluding UL-1 and 17-71 (Fig. III-7). Simvastatin decreased both the amount of prenylated Ras and GTP-bound Ras in HSA and melanoma cell lines, excluding JuB2, which was less susceptible to simvastatin-induced cytotoxic effects. On the other hand, the amounts of prenylated Ras and GTP-bound Ras were not affected by simvastatin in all lymphoma cell lines tested, whereas simvastatin-induced suppression of Ras prenylation was observed in UL-1.

The effects of simvastatin on the activation of downstream factors of Ras vary amongst cell lines

Next, I investigated the phosphorylation status of Erk1/2 and Akt, which are well-known downstream factors in the Ras signaling pathway, in cells treated with simvastatin. Results are shown in Figure III-9. Simvastatin decreased Erk1/2 phosphorylation in Nody-1 and UL-1 cells, while simvastatin increased Erk1/2 phosphorylation in CMeC-1 and LMeC cells. There was no substantial change in the level of Akt phosphorylation in simvastatin-treated cells, besides the decrease in Akt phosphorylation in Re12 cells. The effect of simvastatin on the Ras signaling

pathway is summarized in Table III.

DISCUSSION

The present study demonstrated that simvastatin had an anti-proliferative effect in canine tumor cells derived from different origins, including HSA, melanoma, and lymphoma cells *in vitro*. Simvastatin exhibited this effect by inducing caspase-dependent apoptosis and cell cycle arrest at G0/G1 or G2/M phase via limiting isoprenoid biosynthesis. Moreover, I revealed that simvastatin prevented the prenylation and activation of Ras in canine HSA and melanoma cell lines.

In this study, caspase-3 cleavage was induced by simvastatin in most cell lines with high or moderate sensitivity, but not in low-sensitive cell lines. However, cell viability and cell proliferation were not completely restored by pan-caspase inhibitor Z-VAD-fmk in these cells. Simvastatin also induced G0/G1 arrest in most of the cell lines tested, whereas G2/M arrest was observed in UL-1 only. These results indicate that both caspase-dependent apoptosis and disturbance of cell cycle progression are involved in simvastatin-induced cytotoxicity in these cells. A number of studies have reported that statins induce caspase-dependent or mitochondrial apoptosis and cell cycle arrest at G0/G1, S, or G2/M phase in a variety of human tumor cells [Cafforio *et al*., 2005; Fujiwara *et al*., 2017; Hoque *et al*., 2008; Relja *et al*., 2010; Saito *et al*., 2008; Sánchez *et al*., 2008; Tu *et al*., 2011]. Thus, simvastatin may widely affect apoptotic pathways and cell cycle progression depending on cell type. Therefore, the mechanism of simvastatin-induced cytotoxicity may be different amongst tumor cells.

In the present study, isoprenoids, including GGPP and FPP, restored cell proliferation and caspase-3 activation in cells treated with simvastatin. This finding indicates that simvastatin induces anti-proliferative effects by limiting isoprenoid biosynthesis in these cell lines. Isoprenoids are required for various cell functions, including mitochondrial respiration and biosynthesis of glycoproteins [Thurnher *et al*., 2012]. They are also needed for prenylation of small GTPases, including Ras, and have an impact on various downstream factors associated with cell proliferation [Thurnher *et al*., 2012]. For this reason I investigated the effect of simvastatin on the Ras signaling pathway. Simvastatin decreased both the prenylation and activation of Ras in HSA cell lines with the exception JuB2, which has low sensitivity to simvastatin, and all melanoma cell lines tested. On the other hand, Ras activation was not affected by simvastatin in all lymphoma cell lines tested. Based on these results, I examined whether Ras and its downstream signaling pathway were involved in simvastatin-induced cytotoxic effects in canine HSA and melanoma cells. Tsubaki *et al*. has reported that simvastatin-induced cytotoxicity is associated with the expression level of Ras in human head and neck carcinoma cells [Tsubaki *et al*., 2017]. Furthermore, several reports indicate that statins, including simvastatin, induce the cytotoxicity via inhibiting Ras and its downstream pathways, such as MAPK/Erk and PI3K/Akt [Beckwitt *et al*., 2018; Fujiwara *et al*., 2017; Wang *et al*., 2016]. In this study, there is no substantial suppression in the level of Erk1/2 and Akt phosphorylation in simvastatin-treated cells, excepting the Akt phosphorylation in Re12 cells. This result indicates that

these Ras downstream factors do not play a central role in the anti-proliferative effect of simvastatin in these cells.

Previous studies have reported that statin-induced anti-proliferative effects are associated with the Rho family of proteins, which are a type of small GTPase molecule in human tumor cells [Eto *et al*., 2002; Kamel *et al*., 2017; Zhu *et al*., 2013]. Further investigation is required to elucidate whether small GTPase molecules, other than Ras, are involved in simvastatin-induced cytotoxic effects in canine tumor cells.

In this study, simvastatin was used at a concentration range of 0.5 to 5 μM. Previous investigations examined the anti-proliferating effect of simvastatin at a concentration range of 0.5 to 150 μM in tumor cells *in vitro* [Alizadeh *et al*., 2017; Fujiwara *et al*., 2017; Ingallina *et al*., 2018; Oliveira *et al*., 2008; Relja *et al*., 2010; Torres *et al*., 2015; Tsubaki *et al.*, 2017; Turrell *et al*., 2017; Wang *et al*., 2016]. Simvastatin is clinically used at 20-80 mg/day for treatment of hyperlipidemia in humans, and the blood concentration is 10-34 ng/ml (24-81 nM) if it is taken at 40 mg/day [Bellosta *et al*., 2004]. A clinical study has reported that higher dosage (15 mg/kg/day) of simvastatin was safe and tolerable in human myeloma and lymphoma patients [van der Spek *et al*., 2006]. However, the concentration of 0.5 to 5 μM used in this study may be very high compared to that in conventional clinical usage of simvastatin. Despite this discrepancy in dosage, simvastatin successfully reduced tumor volume in a xenograft mouse model of several types of human tumor at doses of 0.5-1.0

mg/kg/day, which were similar to the dose used to treat humans [Gbelcová *et al*., 2008; Zanfardino *et al*., 2013]. Thus, simvastatin may elicit an anti-tumor effect at lower dosage than that commonly used *in vitro*. Furthermore, tumor targeted drug delivery systems may enhance the therapeutic effect if simvastatin is clinically applied as an anti-tumor drug because its bioavailability is very low (<5%) compared with another statins, including cerivastatin, pitavastatin, and rosuvastatin [Bellosta *et al*., 2004]. Recent studies have reported that the elevated concentration of simvastatin in tumor tissues can be achieved by a novel drug delivery system, such as liposomal nanoparticles, resulting in an enhanced anti-tumor effect [Jin *et al*., 2019; Safwat *et al*., 2017].

The present study demonstrated anti-proliferative effects of simvastatin in several types of canine tumor cells. These findings suggest that inhibition of the MVA pathway is an attractive therapeutic strategy for canine malignancy. Further studies are warranted to elucidate the cytotoxic mechanism of simvastatin and its therapeutic efficacy in dogs with malignant tumors.

CONCLUSION

Hypercoagulability is often developed and significantly associated with poor prognosis in dogs with malignant tumors [Andreasen *et al*., 2012; Kristensen *et al*., 2008; Lip *et al*., 2002; Maruyama *et al*., 2004; Ralph and Brainard, 2012], and therefore the establishment of an accurate diagnostic strategy and efficient treatment is very important to small animal practice.

In chapter 1, I demonstrated that functional tissue factor (TF) expression and production of TF-bearing microparticles (TF-MPs) in canine tumor cell lines, including mammary gland tumors, hemangiosarcoma (HSA), and melanomas, but not in lymphomas. Furthermore, I demonstrated that TF procoagulant activity of plasma MPs (plasma TF-PCA) was elevated in some dogs with malignant tumors. Given the findings in chapter 1, in chapter 2, I compared plasma TF-PCA between non-DIC and DIC groups, consisting of dogs with various underlying diseases, including malignant tumor, inflammatory diseases, and infections. I demonstrated that plasma TF-PCA was significantly higher in dogs with DIC than non-DIC dogs. Moreover, I found that plasma TF-PCA decrease was associated with the time to recovery in some dogs with DIC. These results suggest that TF-MPs play an important role in the pathogenesis of DIC in dogs and plasma TF-PCA may be useful as a biomarker in assessing procoagulant states. The pathogenesis of DIC is very complicated and multiple factors are involved in activating the coagulant cascade. Therefore, measurement of plasma TF-PCA may be helpful for the diagnosis of DIC by combining it with the existing coagulation-fibrinolysis tests. This hypothesis should be examined further.

Given the anti-coagulant property of statins targeting TF and its anti-tumor potential, I hypothesized that statins are useful for the treatment of malignant tumors with procoagulant activity. In my preliminary study, however, I found that simvastatin inhibited cell proliferation but not TF-PCA in canine tumor cells. Therefore, in chapter 3, I investigated the anti-proliferative effect of simvastatin and the possible mechanisms at play in various types of canine tumors, including HSA, malignant melanoma, and lymphoma. I demonstrated that simvastatin inhibited cell proliferation in several tumor cell lines. It is indicated that the anti-proliferative effect is caused by inducing caspase-dependent apoptosis and cell cycle arrest by limiting the biosynthesis of isoprenoids. These results suggest that the inhibition of mevalonate (MVA) pathway is a candidate for a novel therapeutic strategy for the successful resolution of these tumors. Moreover, I demonstrated that simvastatin inhibited Ras activation in several tumor cell lines. However, there were no substantial changes in the level of phosphorylated Erk1/2 and Akt as downstream effectors of Ras in the simvastatin-treated cells. Thus, though precise mechanisms for the anti-proliferative effects of simvastatin have not been elucidated, the findings in my study warrant further investigation of the therapeutic potential of statins for the treatment of canine tumors, particularly those with procoagulant activity.

In conclusions, I demonstrated that procoagulant TF-MPs were frequently produced in various types of canine tumor cells, and plasma TF-PCA was elevated in dogs with DIC, including

those with tumors. Given the findings in my study, further investigations are warranted to elucidate the role of TF-MPs in the pathogenesis of DIC and its potential usefulness as a diagnostic and/or biomarker for monitoring disease progression in dogs. Furthermore, I demonstrated the anti-proliferative effect against canine tumor cells *in vitro*. The findings in my study warrant further investigation on the therapeutic potential of statins for canine tumors and I hope that my thesis helps to develop a novel diagnostic and therapeutic strategy for DIC and malignant tumors in dogs.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to Prof. Masaru Okuda (Laboratory of Veterinary Internal Medicine, Yamaguchi University) and Assoc. Prof. Kenji Baba (Laboratory of Veterinary Internal Medicine, Yamaguchi University). Their constant encouragements and stimulating suggestions have greatly helped to me in carrying my study and writing this dissertation.

I would like to thank Prof. Mizuno Takuya (Laboratory of Molecular Diagnostics and Therapeutics, Yamaguchi University), Assis. Prof. Satoshi Kambayashi (Laboratory of Veterinary Internal Medicine, Yamaguchi University) and Assis. Prof. Masaya Igase (Laboratory of Molecular Diagnostics and Therapeutics, Yamaguchi University) who supported my study with helpful advices and technical instructions. I am grateful to Prof. Kenji Tani (Laboratory of Veterinary surgery, Yamaguchi University), Prof. Yasuyuki Endo (Laboratory of veterinaly internal medicine, Kagoshima University), Assoc. Prof. Shunsuke Noguchi (Laboratory of Veterinary Radiology, Osaka Prefecture University), Assoc. Prof. Takako Shimokawa-Miyama (Faculty of Veterinary Medicine, Okayama University of Science), Assis. Prof. Yuki Nemoto (Faculty of Veterinary Medicine, Azabu University,), Assoc. Prof. Takayuki Nakagawa (Laboratory of Veterinary Surgery, University of Tokyo), and Assoc. Prof. Hiroki Sakai (Laboratory of Veterinary Pathology, Gifu University) for all

their help and kindness.

I wish to thank the laboratory members for their kindly assisting. I would like to express my gratitude to my family for their understanding and support.

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Tables

Table I. 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)[a]	PLT (×10³/μl) (200-750)[a]	PT (sec) (6.0-8.0)[a]	aPTT (sec) (10.0-16.0)[a]	Fib (mg/dl) (200-400)[a]	AT III (%) (80.0≤)[a]	FDP (μg/ml) (≤10.0)[a]	D-dimer (μg/ml) (≤3.5)[a]	DIC diagnosis[b]
1	American Cocker Spaniel, 10Y, F	Mammary gland tumor	Mammary gland	LN	3.40	486	6.5	10.3	232	83.0	ND	ND	non-DIC
2	Shiba, 9Y, F	Mammary gland tumor	Mammary gland	Lung, LN	5.19	409	6.1	13.5	405	73.9	2.0	ND	non-DIC
3	Golden Retriever, 10Y, M	Hemangiosarcoma	Spleen	Absent	85.19	150	8.1	15.4	79	73.8	11.7	ND	DIC
4	Miniature Dachshund, 12Y, M	Hemangiosarcoma	Retroperitoneal	Absent	1.71	653	6.4	15.4	214	80.5	ND	ND	non-DIC
5	Shih Tzu, 8Y, M	Hemangiosarcoma	Skin	Absent	10.85	158	7.5	11.7	224	68.5	ND	2.4	non-DIC
6	Miniature Dachshund, 6Y, M	Malignant melanoma	Gingival	LN	10.56	462	5.8	12.3	277	101.7	ND	ND	non-DIC
7	Miniature Dachshund, 9Y, FS	Malignant melanoma	Gingival	Absent	6.75	272	5.8	10.9	302	>125	ND	ND	non-DIC
8	Miniature Dachshund, 6Y, MC	Malignant melanoma	Gingival	Absent	7.97	388	6.6	10.4	155	87.6	ND	ND	non-DIC
9	Miniature Dachshund, 11Y, M	Malignant melanoma	Intraoral	Absent	10.73	263	7.9	12.2	307	34.0	ND	ND	non-DIC
10	Labrador Retriever, 4Y, M	Malignant melanoma	Intraoral	Absent	13.15	269	7.3	11.2	445	62.4	5.1	ND	non-DIC
11	Miniature Dachshund, 11Y, FS	Malignant melanoma	Intraoral	LN	7.50	319	8.2	20.5	288	72.5	ND	1.2	non-DIC
			T/B cell phenotype	WHO stage									
12	Great Pyrenees, 11Y, FS	Lymphoma	B cell	Stage IVb	43.40	102	7.6	20.4	75	37.7	44.8	ND	DIC
13	Mixed, 6Y, M	Lymphoma	B cell	Stage Vb	3.47	23	7.8	14.1	91	70.3	ND	ND	DIC
14	Jack Russell Terrier, 6Y, FS	Lymphoma	T cell	Stage Ia	16.13	269	5.6	9.9	349	113.0	ND	ND	non-DIC
15	Papillon, 10Y, MC	Lymphoma	T cell	Stage Vb	3.82	194	7.7	16.8	176	80.3	ND	ND	non-DIC
16	Mixed, 12Y, MC	Lymphoma	B cell	Stage IVb	3.72	65	7.1	12.6	180	74.6	54.7	>65.8	DIC
17	Mixed, 8Y, FS	Lymphoma	T cell	Stage Vb	75.59	38	14.2	26.3	45	43.7	ND	14.0	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, deseminated intravascular coagulation; F, female; M, male; FS, spayed female; MC, castrated male; LN, lympho 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 × 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 (>10 μg/ml) or high plasma D-dimer (>3.5 μg/ml). 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.

Table II-1. Signalment, diagnosis, and plasma tissue factor procoagulant activity (TF-PCA) of healthy and diseased dogs

Cases	Breed	Age	Gender	Diseases	Plasma TF-PCA (AU)
Healthy controls (n = 10)	Beagles	4-10y	M (n =5), F (n = 5)	Clinically healthy	5.3±3.0 (mean)
Non-DIC					
1	Miniature Dachshund	11y0m	F	ALL	2.8
2	Doberman	7y9m	FS	Lymphoma (high-grade, T cell, renal)	1.9
3	Chihuahua	6y2m	MC	Lymphoma (high-grade, B cell, gastrointestinal)	4.1
4	Shiba	10y11m	MC	Lymphoma (high-grade, B cell, multicentric)	5.6
5	Polish Lowland Sheepdog	3y10m	MC	Lymphoma (high-grade, B cell, gastrointestinal)	6.7
6	Miniature Dachshund	16y2m	FS	Lymphoma (high-grade, B cell, multicentric)	**37.2**
7	French Bulldog	8y3m	FS	MCT	1.9
8	Labrador Retriever	10y9m	F	Mammary gland carcinoma, Hyperadrenocorticism	3.7
9	Jack Russell Terrier	9y7m	MC	Thyroid medullary carcinoma, MCT	6.4
10	Chihuahua	10y3m	F	AP, DM, Bilateral adrenomegaly	**13.3**
11	Shiba	4y5m	F	IBD	9.3
12	Chihuahua	5y10m	M	IMHA	10.8
13	Chihuahua	11y6m	F	Pyometra	2.5
14	Miniature Dachshund	11y7m	F	Pyometra	4.4
DIC					
15	Miniature Dachshund	9y10m	FS	HSA (splenic)	**28.5**
16	Flat-coated Retriever	9y0m	FS	HSA (splenic)	**85.2**
17	Shiba	8y7m	MC	Lymphoma (high-grade, T cell, hepatosplenic)	4.3
18	Mixed	8y8m	MC	Lymphoma (high-grade, T cell, gastrointestinal)	5.0
19	Shiba	6y1m	M	Lymphoma (high-grade, T cell, gastrointestinal)	8.1
20	Shiba	2y2m	M	Lymphoma (high-grade, T cell, mediastinal)	**24.8**
21	Miniature Dachshund	10y6m	FS	Lymphoma (high-grade, B cell, splenic)	**30.3**
22	Chihuahua	12y0m	MC	Splenic spindle cell sarcoma	**16.9**
23	Papillon	7y7m	M	AP	**62.5**
24	Pekingese	9y10m	FS	IMHA	1.6
25	Pomeranian	6y5m	M	IMHA	**34.3**
26	French Bulldog	0y6m	M	Leptospirosis	**36.7**

F, female; FS, spayed female; M, male; MC, castrated male; ALL, acute lymphocytic leukemia; MCT, mast cell tumor; SCC, squamous cells carcinoma; AP, acute pancreatitis; DM, diabetes mellitus; IBD, inflammatory bowel disease; HSA, hemangiosarcoma; IMHA, immune-mediated hemolytic anemia.

Plasma TF-PCA above the reference range (≤11.3 AU) calculated by the mean±2SD for healthy controls are indicated by boldface.

Table. II-2. Disease, parameters of coagulation and fibrinolysis, and disseminated intravascular coagulation (DIC) category of three dogs in follow-up study

Case no.	Disease	Day	PLT ($\times 10^4$/μl)	PT (sec)	aPTT (sec)	Fibrinogen (mg/dl)	AT III (%)	D-dimer (μg/ml)	DIC category
16	HSA	1	4.1	7.1	15.6	243.5	53.4	20.6	DIC
		21	63.2	7.1	9.5	127.6	81.0	0.3	non-DIC
23	AP	1	4.6	10.0	130.4	477.8	65.9	11.3	DIC
		9	17.2	5.9	11.9	158.7	119.3	3.8	DIC
25	IMHA	1	5.4	9.1	16.6	143.5	49.7	53.7	DIC
		14	8.7	6.6	9.3	352.5	91.0	4.9	non-DIC

Abnormal findings in coagulation-fibrinolysis tests are defined as follows: low platelet count (<20 × 10^4/μl), prolonged PT (>10.0 sec), prolonged aPTT (>20.0 sec), low plasma fibrinogen concentration (<200 mg/dl), low plasma AT III activity (<80%), and high plasma D-dimer concentration (>3.5 μg/ml).
HSA. Hemangiosarcoma; AP, acute pancreatitis; IMHA, immune mediated hemolytic anemia

Table III. Summary of the effects of simvastatin on Ras and its signaling effectors in canine tumor cell lines

Tumor type	Cell line	Sensitivity*)	Ras prenylation	Ras activation	Erk1/2 phosphorylation	Akt Phosphorylation
Hemangiosarcoma	JuB2	+	↓	→	→	→
	Re12	+++	↓	↓	→	↓
	Ud6	++	↓	↓	→	→
Melanoma	CMeC-1	+++	↓	↓	↑	→
	CMeC-2	++	↓	↓	→	ND
	KMeC	+++	↓	↓	→	→
	LMeC	++	↓	↓	↑	→
Lymphoma	CLC	++	→	→	→	ND
	Nody-1	+++	→	→	↓	ND
	UL-1	+++	↓	ND	↓	ND
	CLBL-1	+	→	→	→	→
	17-71	+	→	ND	ND	ND

*) Sensitivity: +, 50-80%; ++, 20-50%; +++, <20% of cell proliferation when cells were treated with 5 μM simvastatin for 72 hr
↓, decreased; ↑, increased; →, not changed compared with control (DMSO)
ND, not detected

Figures

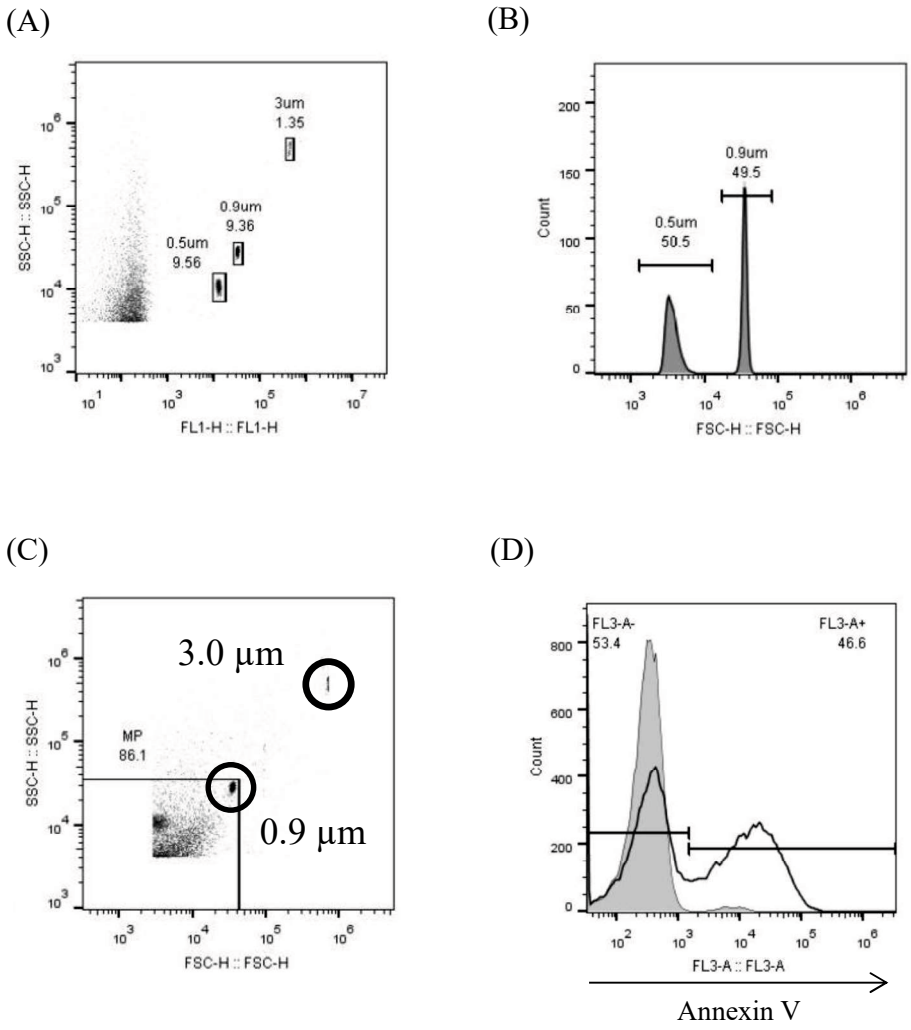
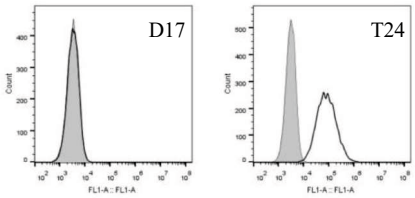
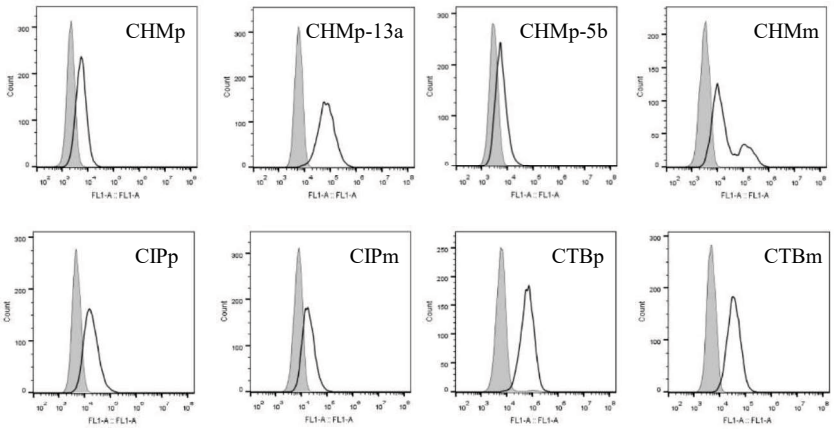


Fig. I-1. Setting of flow cytometry to detect microparticles (MPs). MPs were defined as both events of 0.5–0.9 µm diameter and Annexin V-positive events. (A) Bead subsets (0.5, 0.9, and 3.0 µm beads in diameter) were first recognized based on their side scatter (SS) and FL1 fluorescence properties. (B) In the forward scatter (FS) histogram with distributions of 0.5 and 0.9 µm beads, the threshold level of FSC was set to achieve a percentage of 49.0%–51.0% for 0.5-µm beads (M-0.5µm). (C) On an FSC log × SSC log cytogram, the MP gate was considered a region under 0.9 µm beads. (D) A representative result in Ud2 is shown. The region below the solid red line and the shaded region in the histograms indicated staining with and without (negative control) PerCP-eFluor® 710 dye-conjugated Annexin V, respectively.

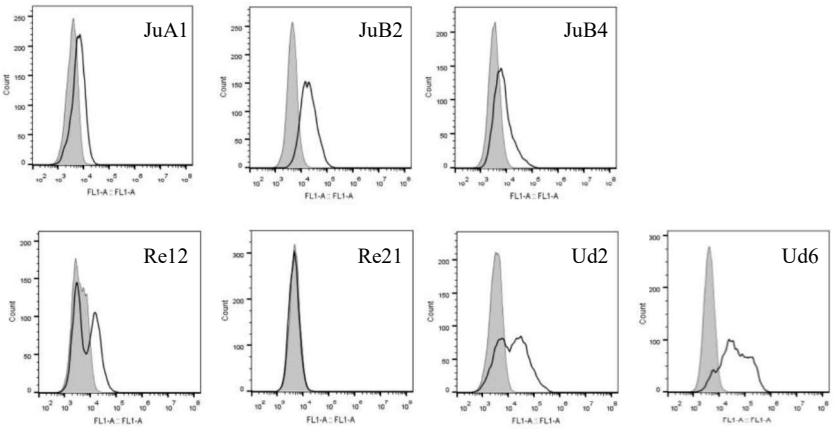
(A) Negative and positive control



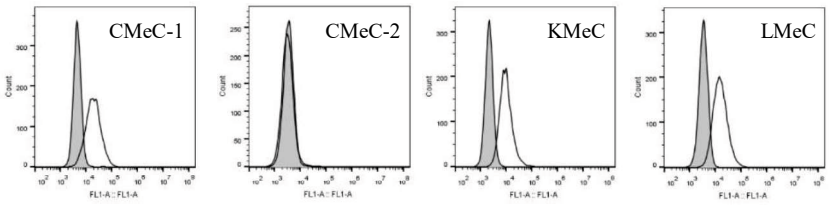
(B) Mammary gland tumor



(C) Hemangiosarcoma



(D) Malignant melanoma



(E) Lymphoma

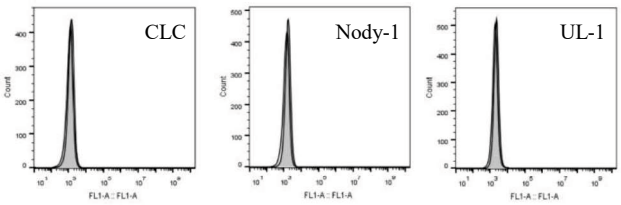


Fig. I-2. Expression of tissue factor (TF) on canine tumor cell lines. Cell surface expressions of TF were analyzed via flow cytometry; (A) D17 (negative control) and T24 (positive control), (B) mammary gland tumor, (C) hemangiosarcoma, (D) malignant melanoma, and (E) lymphoma cell lines. The shaded 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.

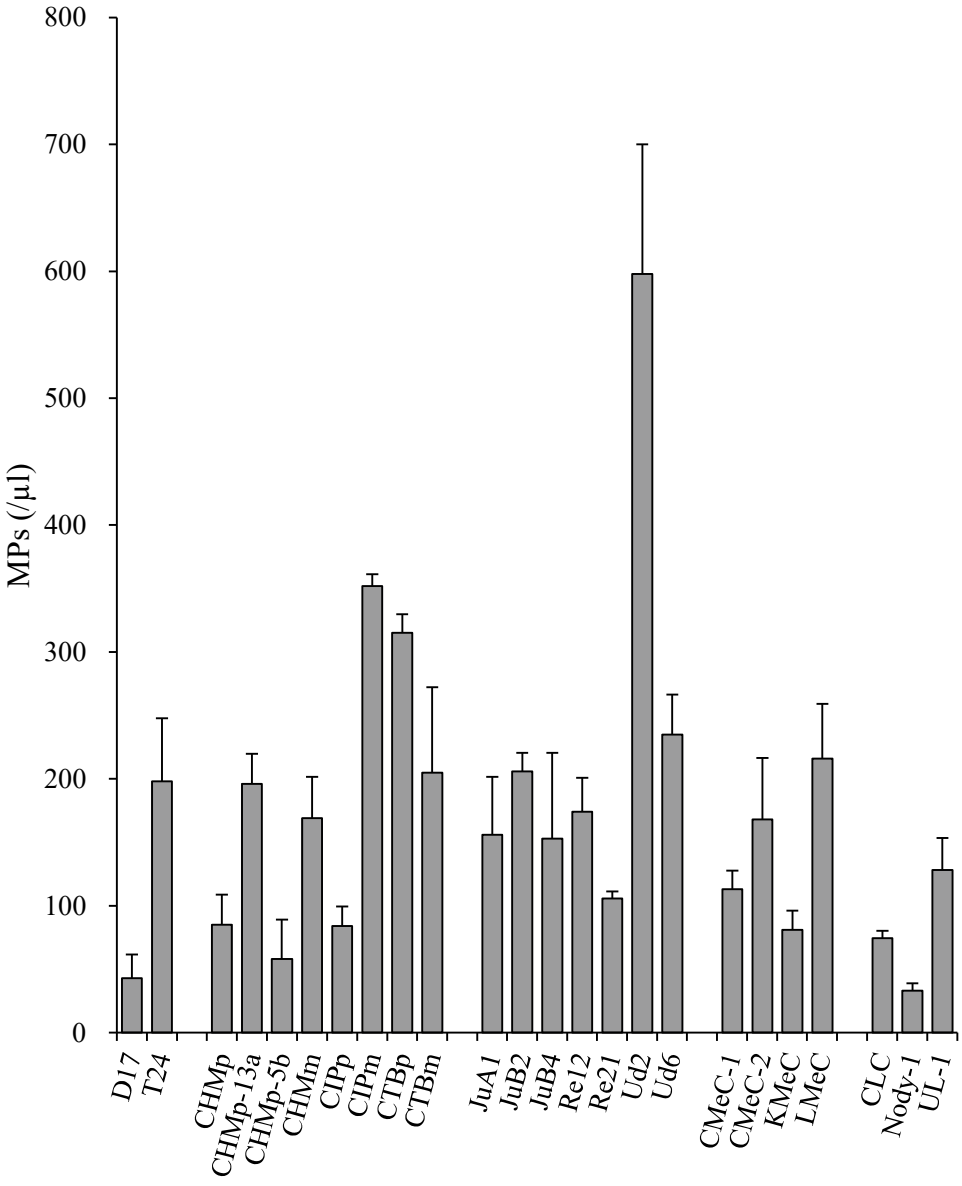


Fig. I-3. Numbers of microparticles (MPs) in the culture supernatants of canine tumor cell lines. MPs in the culture supernatants of canine mammary gland tumor, hemangiosarcoma, malignant melanoma, and lymphoma cell lines were measured via flow cytometry. The MP counts are shown as mean values and standard deviation. Results shown are representative of at least three independent experiments.

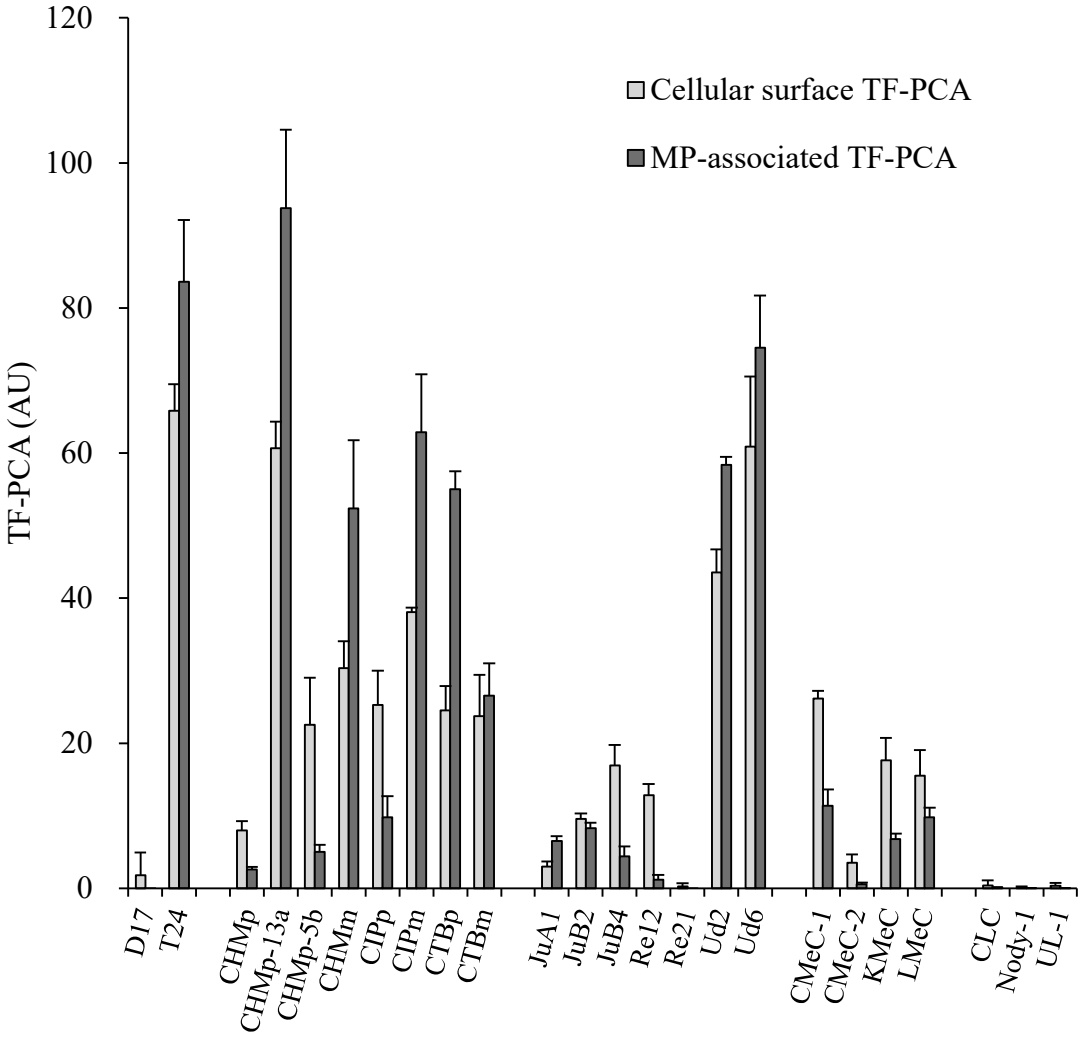


Fig. I-4. Tissue factor procoagulant activity (TF-PCA) of cellular surface and microparticles (MPs) in the culture supernatants of canine tumor cell lines. TF-PCA of the cell surface and MPs in the culture supernatants of canine mammary gland tumor, hemangiosarcoma, malignant melanoma, and lymphoma cell lines are shown. D17 and T24 cells were used as negative and positive controls, respectively. TF-PCA was presented as mean values and standard deviation. Results shown are representative of at least three independent experiments.

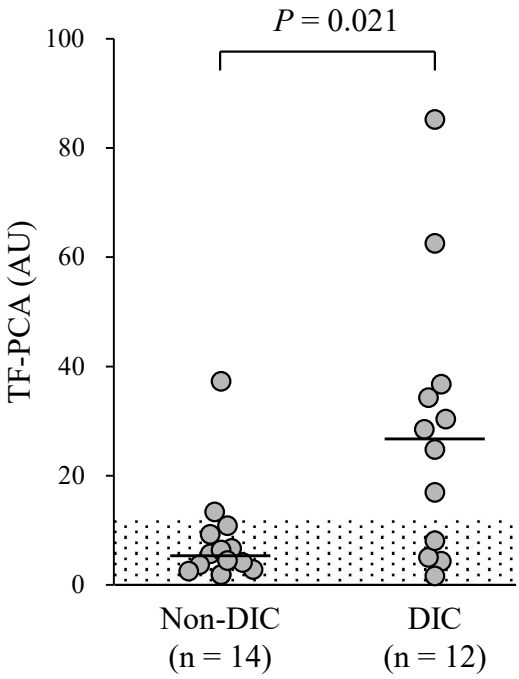


Fig II-1. Tissue factor procoagulant activity (TF-PCA) in diseased dogs categorized into the non-DIC (n =14) and DIC (n =12) groups. The shaded region represents the reference range determined by the results measured in healthy controls (n = 10). The line in each group indicated the median TF-PCA value. *P* value is according to Mann–Whitney U test.

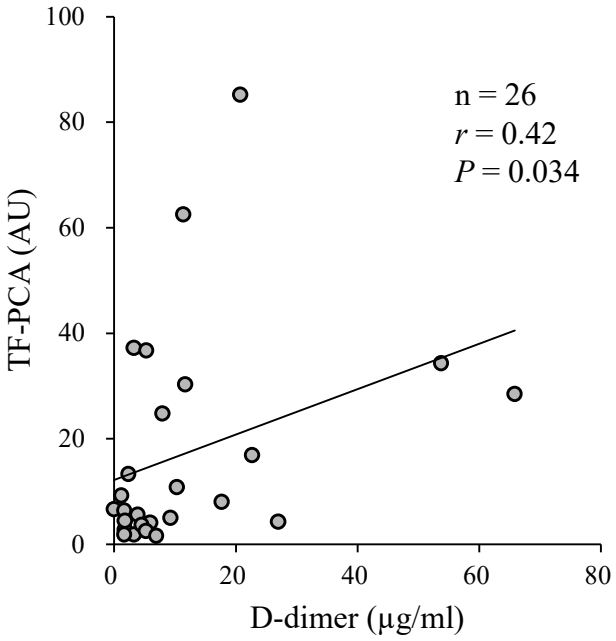
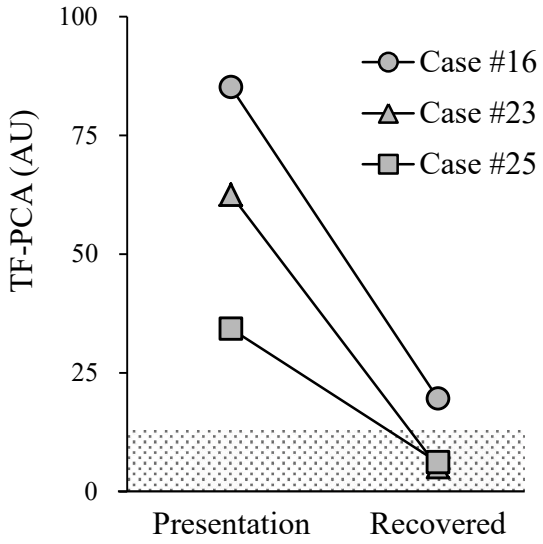


Fig. II-2. Correlation between tissue factor procoagulant activity (TF-PCA) and D-dimer concentration in 26 diseased dogs. Correlation between TF-PCA and D-dimer concentration was analyzed by Spearman's rank correlation coefficient.

(A)



(B)

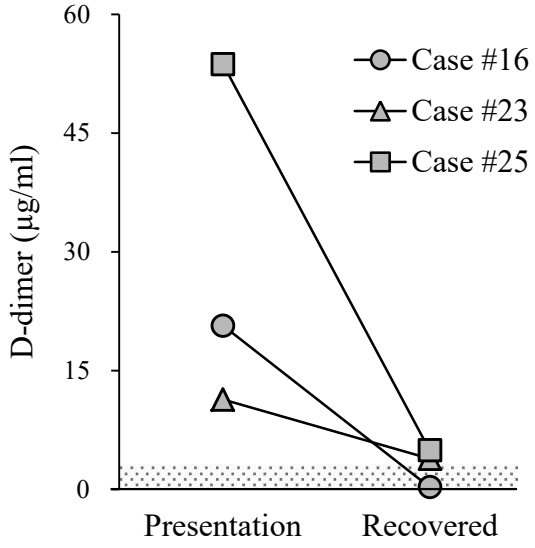


Fig. II-3. Follow-up of three dogs with increased tissue factor procoagulant activity (TF-PCA). (A) TF-PCA at presentation and the time of recovery in dogs with splenic hemangiosarcoma (cases no. 16), acute pancreatitis (case no. 23), and immune-mediated hemolytic anemia (case no.25). (B) Plasma D-dimer concentrations at presentation and the time of recovery in three dogs. The shaded region represents the reference range of each parameter.

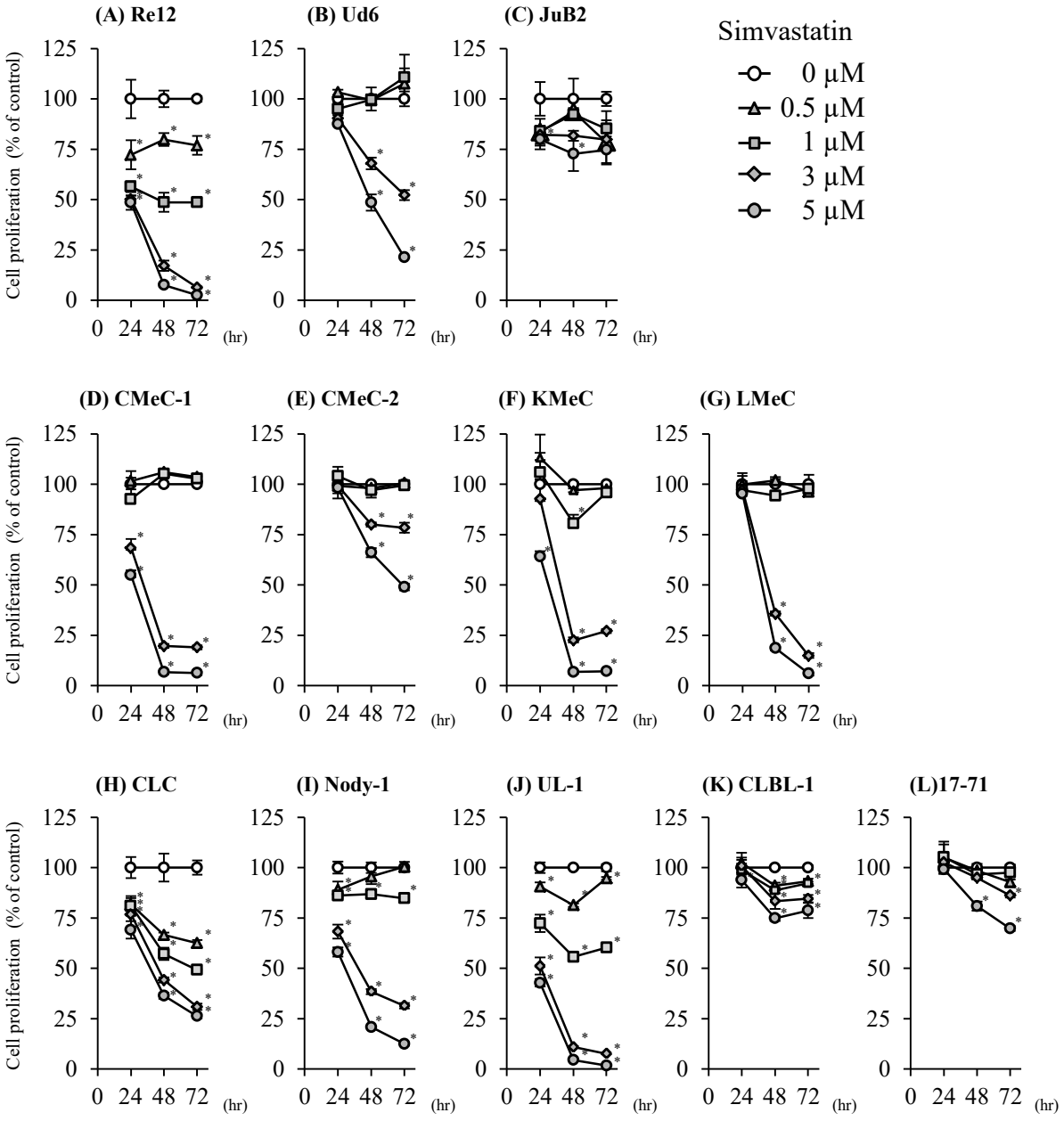


Fig. III-1. Anti-proliferative effect of simvastatin in canine hemangiosarcoma, lymphoma, and lymphoma cell lines. Tumor cell lines in triplicate wells were treated with DMSO (control) or simvastatin (0.5, 1, 3 or 5 μM) for 24, 48, and 72 hr and their cell proliferations were measured by Cell Counting Kit-8. Results are represented as percentage at each time point (mean±SD). Results are representative of three independent experiments. *$p < 0.01$ versus control.

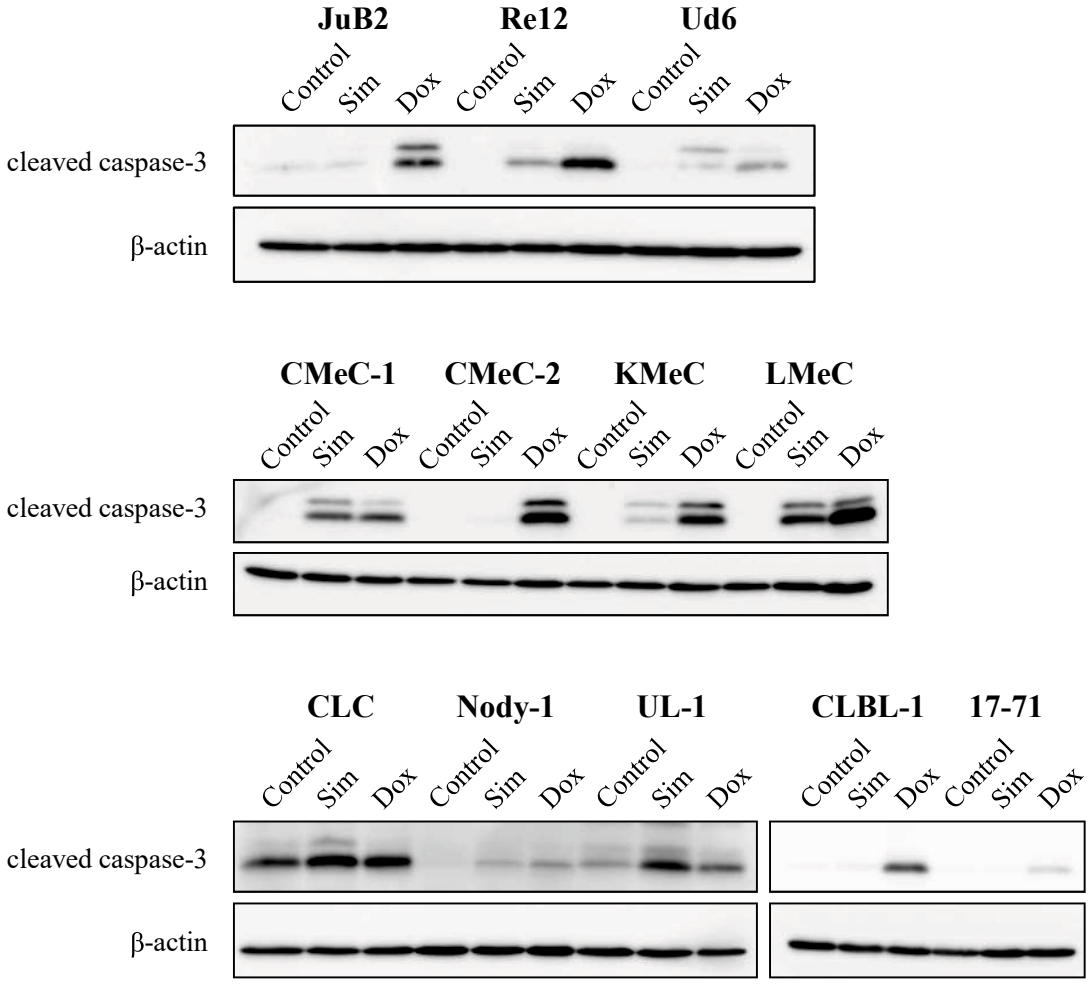


Fig. III-2. Caspase-3 cleavage in canine tumor cells treated with simvastatin. Protein levels of cleaved caspase-3 were assessed by immunoblotting. Cells were treated with DMSO (control), 5 μM simvastatin (Sim), or doxorubicin (Dox; 1 μM in JuB2, Re12, CLBL-1, and 17-71, 10 μM in the other cell lines) for 24 hr. β-actin was used as a protein loading control. Results are representative of three independent experiments.

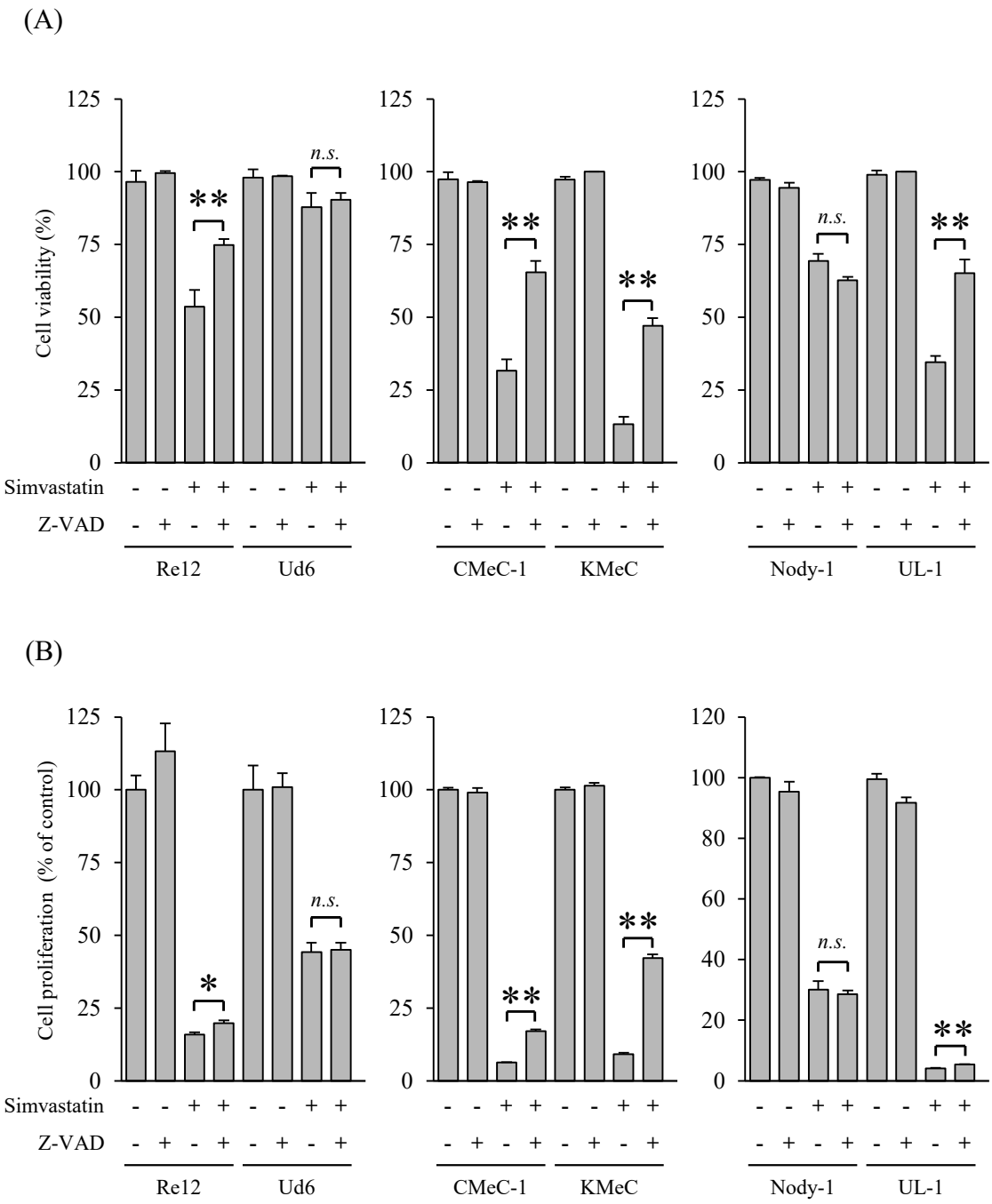
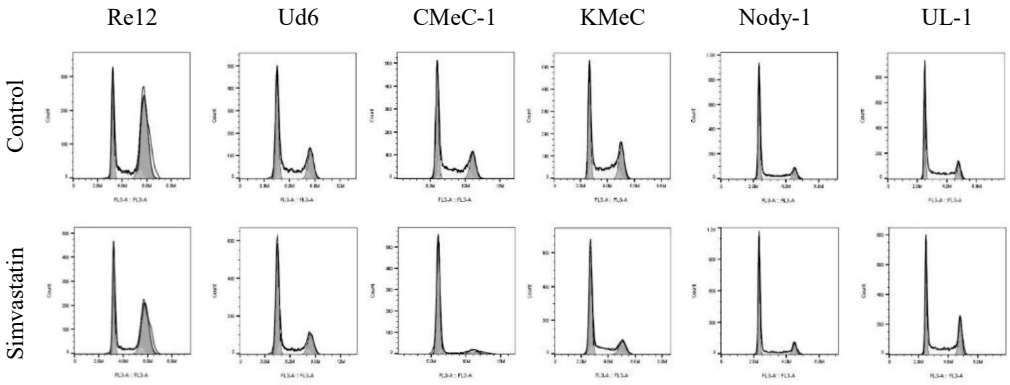


Fig. III-3. Effect of pan-caspase inhibitor Z-VAD-fmk on simvastatin-induced cytotoxicity in canine tumor cells. Canine hemangiosarcoma (Re12 and Ud6), melanoma (CMeC-1 and KMeC), and lymphoma (Nody-1 and UL-1) cells were pretreated with DMSO or 50 μM Z-VAD-fmk for 1 hr, and then treated with DMSO or 5 μM simvastatin in triplicate wells. After 48 hr incubation, (A) cell viability and (B) cell proliferation ratio were assessed by trypan blue exclusion assay and cell proliferation assay, respectively. Results are represented as a percentage of control (mean±SD). Results are representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; *n.s.*, not significant.

(A)



(B)

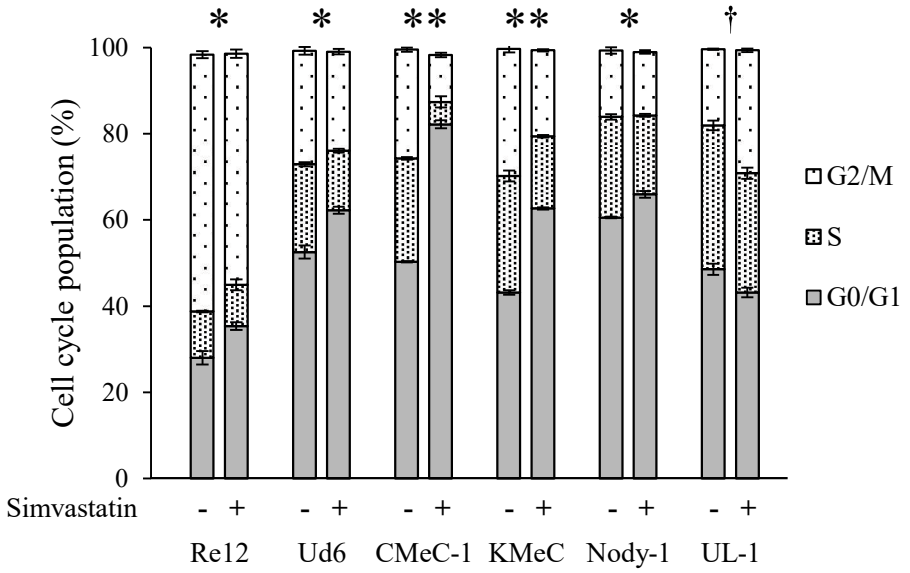
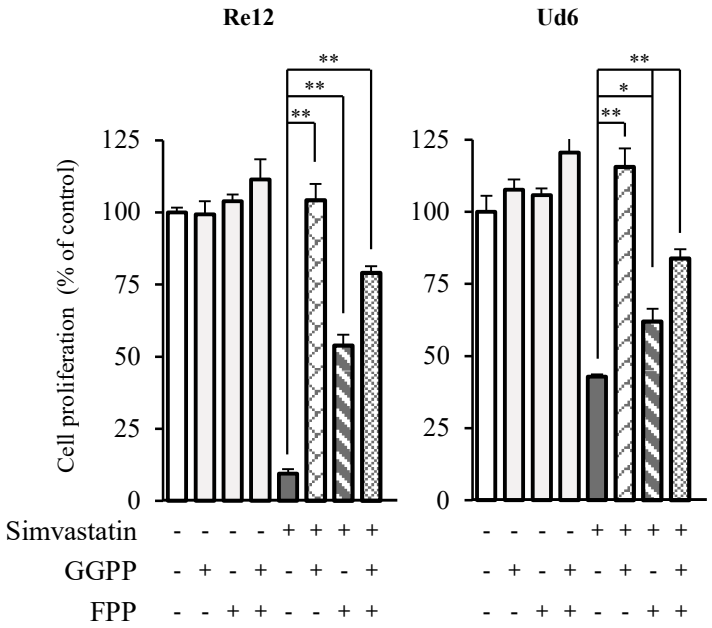
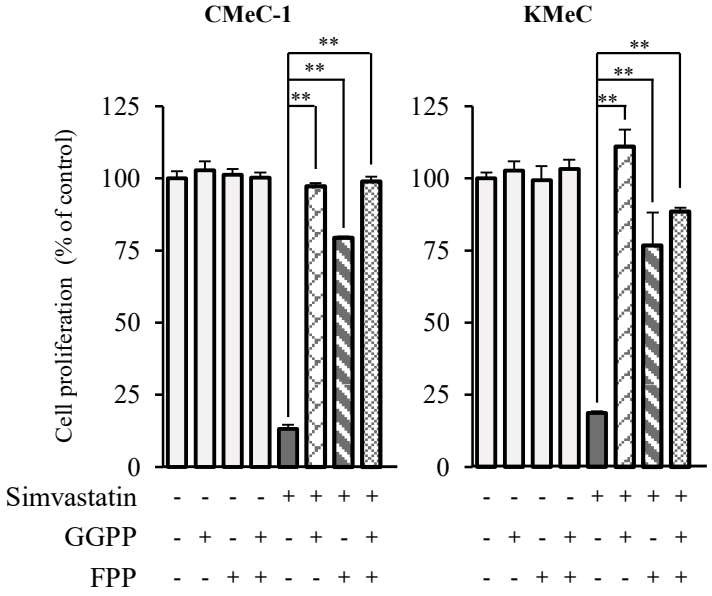


Fig. III-4. Cell cycle distribution in canine tumor cells treated with simvastatin. Canine hemangiosarcoma (Re12 and Ud6), melanoma (CMeC-1 and KMeC), and lymphoma (Nody-1 and UL-1) were treated with DMSO or 5 μM simvastatin for 24 hr. The cell cycle distribution was assessed by flow cytometry. (A) Representative histograms in each cell line are shown. (B) Percentages of each cell cycle phase were shown. Results are representative of three independent experiments. *$p < 0.01$, **$p < 0.001$ versus control (G0/G1 pase); †$p < 0.01$ versus control (G2/M phase).

(A)


Re12
Ud6
Cell proliferation (% of control)
125
100
75
50
25
0
**
*
Simvastatin - - - - + + + +
GGPP - + - + - + - +
FPP - - + + - - + +

(B)


CMeC-1
KMeC
Cell proliferation (% of control)
125
100
75
50
25
0
**
Simvastatin - - - - + + + +
GGPP - + - + - + - +
FPP - - + + - - + +

(C)

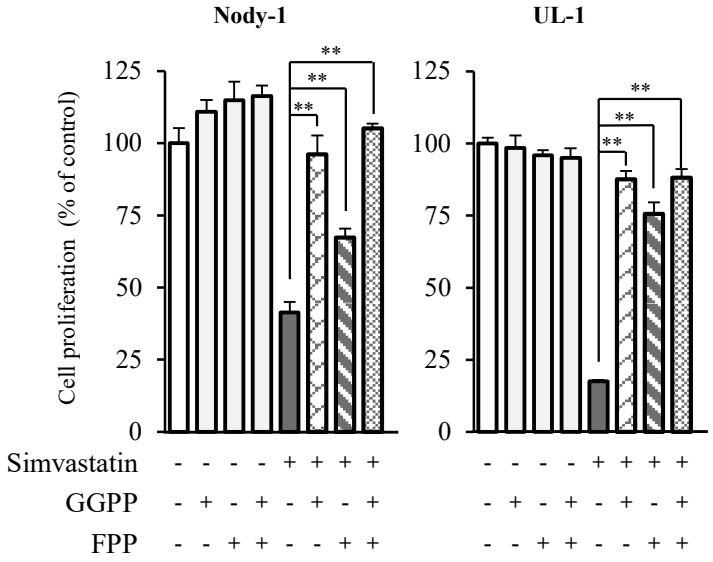


Fig. III-5. Restoration of simvastatin-induced cytotoxicity by isoprenoids in canine tumor cells. (A) Hemangiosarcoma (Re12 and Ud6), (B) melanoma (CMeC-1 and KMeC), and (C) lymphoma (Nody-1 and UL-1) cells were pretreated with methanol, 20 μM GGPP, and/or 20 μM FPP for 2 hr, and then treated with DMSO or 5 μM simvastatin. After 48 hr incubation, cell proliferation was measured by cell proliferation assay. Results are represented as a percentage of controls (mean±SD). Results are representative of three independent experiments. *$p < 0.01$, **$p < 0.001$ versus control.

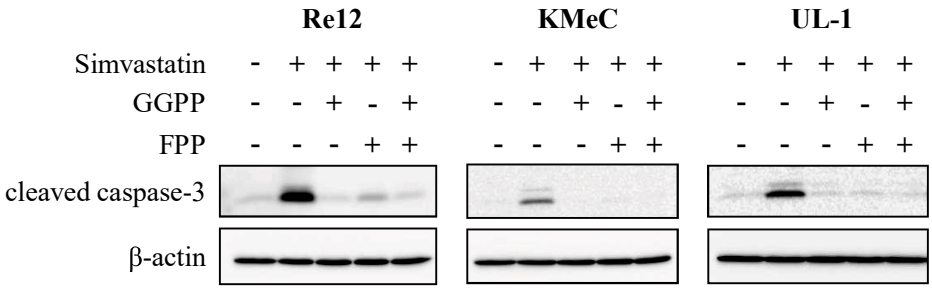


Fig. III-6. Restoration of simvastatin-induced caspase-3 cleavage in canine tumor cells. Hemangiosarcoma (Re12), melanoma (KMeC), and lymphoma (UL-1) cells were pretreated with methanol, 20 μM GGPP, and/or 20 μM FPP for 2 hr, and then further incubated with DMSO or 5 μM simvastatin for 24 hr. Protein levels of cleaved caspase-3 were assessed by immunoblotting. β-actin was used as a protein loading control. Results are representative of three independent experiments.

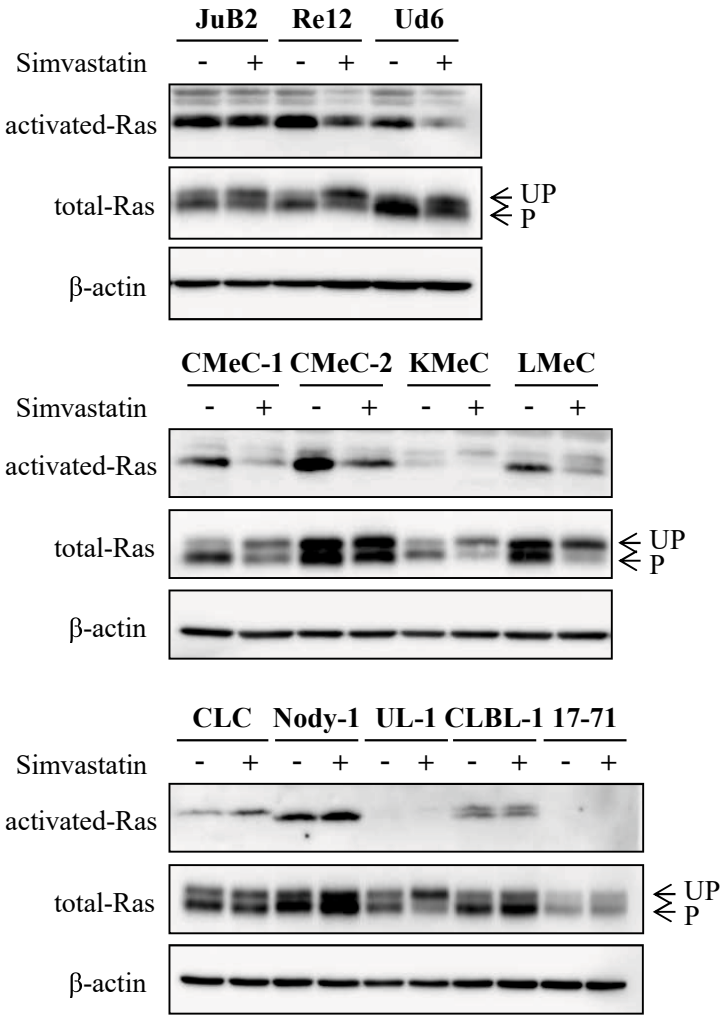


Fig. III-7. Ras prenylation and activation status in canine tumor cells treated with simvastatin. Cells were incubated with DMSO or 5 μM simvastatin for 24 hr. Total Ras protein and GTP-bound Ras, were detected by immunoblotting and Ras pulldown assay, respectively. Results are representative of three independent experiments. UP, unprenylated form; P, prenylated form.

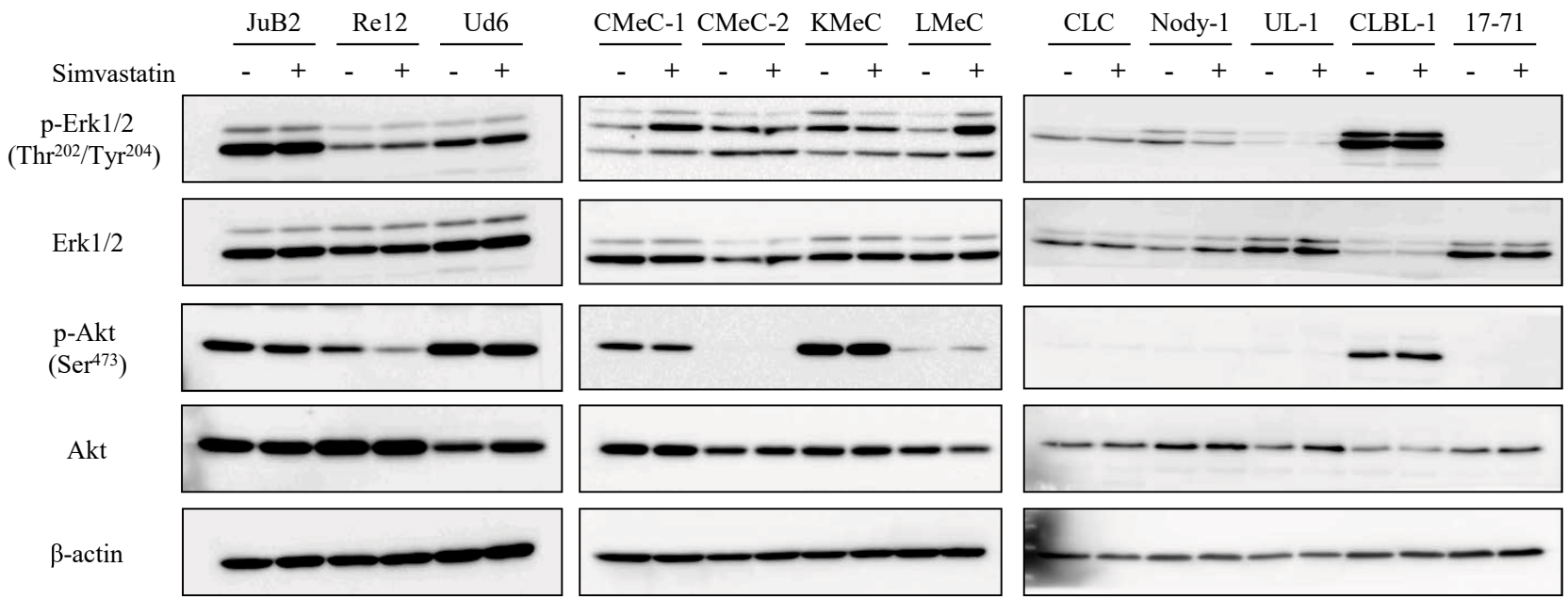


Fig. III-9. Activation status of Erk1/2 and Akt in canine tumor cells treated with simvastatin. Protein levels of phosphorylated (p-) and total forms of Erk1/2 and Akt were assessed by immunoblotting in cells treated with DMSO (control) or 5 μM simvastatin for 24 hr. β-actin was used as a protein loading control. Results are representative of three independent experiments.