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Measurement of canine blood microparticles by flow cytometry: effect of anticoagulants and staining reagents

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ABSTRACT. Microparticles (MPs) are released from budding plasma membranes into body fluids. The use of flow cytometry for the measurement of MP in canines has not been standardized. In this fundamental study, we compared the effect of anticoagulant agents, such as acid-citrate-dextrose (ACD) and heparin on the measurement of canine MPs in platelet-free plasma (PFP) using flow cytometry. In addition, we used annexin V, carboxyfluorescein succinimidyl ester (CFSE), or calcein tetraacetoxymethyl ester (calcein-AM), and explored the characteristics of the staining reagents in MP detection using flow cytometry. We were able to measure canine MPs in PFP prepared from ACD-anticoagulated blood using flow cytometry, in which the highest positive rate for fluorescent staining was observed when CFSE was used.

KEY WORDS: anticoagulant agent, calcein-AM, carboxyfluorescein succinimidyl ester, dog, microparticles

Extracellular vesicles (EVs) are cell fragments present in body fluids, including blood, and are secreted and released from various cells. EVs carry a variety of molecules, such as proteins and RNA species, and play an integral role in cell-cell communication by transporting molecules from the cell of origin under certain physiological and pathophysiological conditions. EVs are broadly categorized into exosomes, microparticles (MPs), and apoptotic bodies, according to their size and production mechanisms. Exosomes, approximately 20–100 nm in size, are secreted into the extracellular space as a result of multivesicular body fusion with the plasma membrane [1, 5, 15, 20, 21]. The vesicles called microparticles (MPs), approximately 200–1,000 nm in size, are released from the budding plasma membrane into body fluids under various physiological and pathophysiological conditions such as cellular activation, apoptosis, or injury of cells [1, 5, 13, 15, 20, 21]. Apoptotic bodies, the largest EVs (approximately 1,000–5,000 nm in size) are released from fragmented apoptotic cells [1, 5, 20, 21].

MPs retain membrane proteins/lipids and cytoplasmic materials of the parent cells and play a variety of physiological roles, such as promotion of the blood coagulation cascade by phosphatidylserine (PS) expressed on the surface of MPs [2, 8, 13, 14, 20], and regulation of the immune system by participating in complement activation [2, 13, 14]. In addition, MPs have been reported to play pathophysiological roles in the promotion of tumor angiogenesis and drug resistance by transporting P-glycoprotein, and autoimmune diseases [2]. An increase in MPs derived from platelets or endothelial cells in peripheral blood has been reported in human patients with stroke, including brain infarction [15], and a relationship between the procoagulant activity of MPs and the onset of brain infarction has been shown [2, 10, 13, 16]. Therefore, MPs have received increasing interest as potential biomarkers of stroke.

Although canine brain infarction occurs frequently in small animal practice, clinical information on canine brain infarction, such as incidence, pathophysiology, clinical signs, and prognosis, is largely unknown [4]. Magnetic resonance imaging (MRI) is widely used for the clinical diagnosis of brain infarction in humans and in veterinary medicine. However, the requirement of anesthesia for MRI examination in small animals is one of the limitations of clinical diagnosis for brain infarction, resulting in poor information about canine brain infarction in veterinary medicine. Therefore, in addition to precise clinical information and MRI examination, a potential biomarker is desired for the diagnosis of canine brain infarction.

Flow cytometry is the most commonly used method for MP measurements in humans. However, the expression of specific marker proteins in canine MPs has not been well studied, and the method for canine MP measurement by flow cytometry has not been standardized. In this fundamental study of the measurement of canine MPs in platelet-free plasma (PFP) using flow cytometry, we compared the effect of anticoagulant agents for PFP preparation, such as acid-citrate-dextrose (ACD) or heparin, on the properties of MPs in PFP from canine blood. In addition, to avoid problems due to the specificity of antibodies used in human

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MP detection, we used annexin V, carboxyfluorescein succinimidyl ester (CFSE), or calcein tetraacetoxymethyl ester (calcein-AM), as a generic fluorescent marker for canine MP detection, and explored the characteristics of the staining reagents in MP detection using flow cytometry.

Blood was collected from healthy beagle dogs or Japanese white rabbits with one-tenth volume of ACD or heparin (1.7 units/ml as a final concentration; Nipro Corp., Osaka, Japan). The experimental protocols were approved by the Animal Care and Use Committee of Kagoshima University. Blood was centrifuged at 2,000 × *g* for 20 min at room temperature (KUBOTA 2800; KUBOTA Corp., Tokyo, Japan), and the supernatant (PFP) was carefully collected. We confirmed the absence of platelets in the PFP using an automatic blood cell counter (Sysmex pocH-100iV; Sysmex Corp., Kobe, Japan).

MPs in the PFP were stained with annexin V-fluorescein isothiocyanate (FITC) (Annexin Assay kit; Medical & Biological Laboratories Co., Ltd., Tokyo, Japan), CFSE, and calcein-AM (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions and previous studies [9, 11]. The final concentrations of CFSE and calcein-AM were 40 µM and 10 µM, respectively.

Negative controls for annexin V-FITC staining were prepared in the presence of ethylenediaminetetraacetic acid (EDTA) in PFP. As negative controls for CFSE or calcein-AM staining, PFP was treated with a vehicle (dimethyl sulfoxide).

BD FACSVerse (BD Biosciences, San Jose, CA, USA) was used for flow cytometric measurement of canine MPs. The optimization of voltage setting and gating of forward scatter (FSC)-A and side scatter (SSC)-A were performed using a mixture solution of 0.35, 1.0, and 3.0 µm control beads (Polybead Microspheres; Polysciences Asia Pacific Inc., Taipei, Taiwan). A threshold of 200 (minimum of the instrument) was set at the FSC and SSC. Measurements were performed until the total number of events reached 10,000. The number of fluorescence 3.0 µm control beads (Polysciences Asia Pacific Inc.) in 100 times diluted PFP containing 10^6 beads/ml was counted until it reached 10,000 (corresponding to 1 µl volume of PFP) to calculate the absolute number of MPs in PFP. Student's *t*-test and Bonferroni correction were used for comparisons between two groups and multiple comparisons, respectively. Statistical significance was set at $P<0.05$. Values are expressed as the mean ± SEM.

The population of MPs was identified by morphological gating, which was determined using control beads (0.35, 1.0, and 3.0 µm) in an SSC versus FSC plot (Supplementary Fig.1). MPs were defined as particles <1.0 µm. Figure 1 shows the number of events in the MP region (MP population) and positively stained MPs for each fluorescent reagent in canine PFP. When the number of MPs was compared between anticoagulants, a significantly higher number of canine MPs was observed in samples anticoagulated with heparin than in those treated with ACD (Fig. 1). In rabbit PFP, we also observed a significantly higher number of MPs in samples anticoagulated with heparin (2,329 ± 284, n=6) than in those treated with ACD (994 ± 105, n=6) (P=0.022). Similar results have been reported in humans [2, 6, 19]. Furthermore, although there was no significant difference, the absolute number of canine MPs in the PFP from heparin-anticoagulated blood ($78.8 \times 10^4 \pm 3.8 \times 10^4$ MPs/µl, n=5) was 2.9 times higher than that from ACD-anticoagulated blood ($27.4 \times 10^4 \pm 10.7 \times 10^4$ MPs/µl, n=5). In addition, the absolute number of rabbit MPs in the PFP from heparin-anticoagulated blood ($8.5 \times 10^4 \pm 2.3 \times 10^4$ MPs/µl, n=6) was significantly higher than that from ACD-anticoagulated blood ($1.6 \times 10^4 \pm 0.2 \times 10^4$ MPs/µl, n=6) (P=0.049). Since platelet activation is known to cause the release of platelet-derived MPs [2, 6, 8], *ex vivo* platelet activation might have occurred during PFP preparation from heparin-anticoagulated blood. These findings suggest that ACD might be more effective in inhibiting the *ex vivo* generation of platelet-derived MP

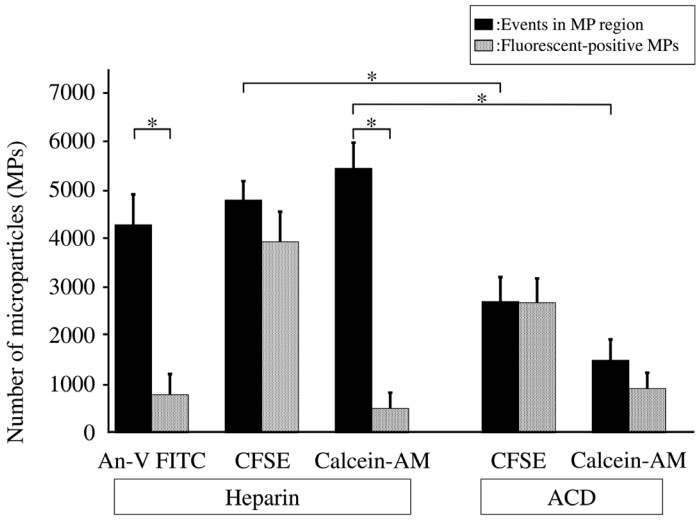

Fig. 1. Effect of anticoagulants and staining reagents on the number of canine microparticles (MPs). *, $P<0.05$; Student's *t*-test with or without Bonferroni correction (n=6). Number of MPs/10,000 events in MP region or fluorescent-positive MPs were evaluated in samples prepared using different anticoagulants, heparin or acid-citrate-dextrose (ACD), and staining reagents, annexin-V Fluorescein isothiocyanate (An-V FITC), carboxyfluorescein succinimidyl ester (CFSE), or calcein tetraacetoxymethyl ester (calcein-AM).

formation through the chelation of free calcium ions in blood. Although the absolute number of human MPs has been measured using various methods, they have generally been reported in numbers ranging from 10^2 to 10^4 MPs/µl [6]. In contrast, Cremer *et al*. reported that the absolute number of canine MPs was 10^5 MPs/µl, which agrees with our observations [8]. In addition, the coefficient of variation (CV; standard deviation divided by the mean) of the absolute canine MP number in the heparin sample (1.07, n=5) or the ACD sample (0.87, n=5) indicates high variability of MP number among the individual canines. Further studies are needed to determine whether the variation is due to artifacts in the preparation method or differences among individual canines.

To distinguish true events from electronic noise and to increase the specificity of MP detection, the canine MP population was further discriminated by fluorescent labeling with annexin V, CFSE, or calcein-AM. Although there was no apparent difference in the number of events in the MP region in heparin-anticoagulated samples, the number of annexin-or calcein-positive canine MPs was significantly lower than the number of events in the MP region (Fig. 1), resulting in a lower positive rate for each staining reagent in the MP population (Table 1). In contrast, the positive rate of CFSE was significantly higher than that of annexin V or calcein (Table 1). Similar results were obtained in ACD-anticoagulated samples, in which the positive rate of CFSE was significantly higher than that of calcein-AM (Table 1).

Circulating MPs have procoagulant activity due to the expression of PS on their surface, which facilitates coagulation. Annexin V exhibits a strong affinity for PS, and binding of annexin V-FITC with PS on the surface of MPs can be detected by flow cytometry [8, 14, 18]. Since the binding of annexin-V to PS is calcium-dependent, when ACD was used as an anticoagulant agent, the MPs were not stained with annexin-V due to the chelating effect of calcium ions by ACD. Therefore, annexin V-FITC was not suitable for the staining of MPs in PFP from ACD-anticoagulated blood. Furthermore, it has been reported that most MPs are PS-negative in unstimulated PFP [7, 8, 20]. Accordingly, the number of annexin-positive canine MPs was significantly lower than that in the MP region in heparin-anticoagulated samples (Fig. 1), indicating that annexin V was not an appropriate staining reagent for detecting generic canine MPs by flow cytometry.

Calcein-AM [3] and CFSE [12] are membrane-permeable and fluorogenic esterase substrates, respectively. Upon cleavage by intracellular esterase, they become a fluorescent and relatively membrane-impermeable dye within the cytoplasm-containing cell/vesicle. These fluorescent reagents can be used as generic fluorescent markers for MPs without the concomitant staining of debris. Although calcein and CFSE stained MPs through a similar mechanism, a clear difference was observed in the positive rates (Table 1). When the CV of fluorescence intensity for CFSE or calcein in ACD-anticoagulated samples was compared to observe the variability of fluorescence staining among canine MPs, CFSE showed a significantly higher value (80.7 ± 5.5, n=6) than calcein-AM (51.8 ± 3.0, n=6) (P=0.027) (Fig. 2). Rond *et al*. [18] reported that calcein-AM had lower sensitivity for EV detection than CFSE, which may be due to insufficient brightness of calcein or insufficient esterase activity in EVs for calcein-AM. Furthermore, CFSE covalently attached to intracellular proteins via its succinimidyl group, allowing long-term tracking with little leakage from the cells [17]. Therefore, it is likely that the higher positive rate of CFSE than that of calcein-AM is due to the higher staining rate of the MPs, rather than non-specific staining of non-EV components.

In conclusion, we were able to measure canine MPs in PFP prepared from ACD-anticoagulated blood using flow cytometry. The highest positive rate for fluorescent staining was observed when CFSE was used as a generic MP marker. Heparin, an anticoagulant, was not able to prevent the activation of platelets, which might result in the *ex vivo* generation of MPs during PFP preparation. In addition, annexin V or calcein-AM caused only partial staining of canine MPs, regardless of the type of anticoagulant used.

Table 1. Comparisons of percentage (%) of positive canine microparticles (MPs) for fluorescent staining reagent in samples

Anticoagulant	Staining reagent	Positive rate
Heparin	An-V FITC	0.18 ± 0.072*
	CFSE	0.79 ± 0.088
	Calcein-AM	0.10 ± 0.062*
ACD	CFSE	0.98 ± 0.005
	Calcein-AM	0.64 ± 0.099†

P<0.05 vs. carboxyfluorescein succinimidyl ester (CFSE) in samples anticoagulated with heparin (*) or acid-citrate-dextrose (ACD) (†); Student's *t*-test with or without Bonferroni corrections (n=6). FITC, fluorescein isothiocyanate.

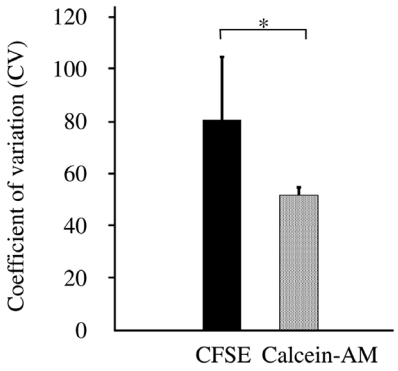

Fig. 2. Differences in coefficient of variation (CV) of fluorescence intensity for carboxyfluorescein succinimidyl ester (CFSE) or calcein tetraacetoxymethyl ester (calcein-AM) in acid-citrate-dextrose (ACD)-anticoagulated canine samples. Variability of fluorescence staining among microparticles (MPs) in ACD-anticoagulated samples was compared between CFSE (black column) and calcein-AM (dotted column). *, P<0.05; Student's *t*-test (n=6).

POTENTIAL CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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