BRIEF REPORT

Circulating microvesicles in snakebite patients with microangiopathy

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Abstract

Background: Venom-induced consumption coagulopathy is a common consequence of snake envenoming that can lead to life-threatening hemorrhage, and is associated with microangiopathic hemolytic anemia (MAHA), acute kidney injury and thrombocytopenia. The role of microvesicles (MV) in snakebite patients has not been previously investigated.

Objective: To compare changes in subsets of circulating MV levels in snakebite patients with venom induced consumption coagulopathy and with or without microangiopathic hemolysis to those of healthy controls.

Methods: This study used samples from patients recruited to the Australian Snakebite Project (ASP) with snake envenoming, including bites by brown snakes, tiger snakes, and taipans. Citrated blood from envenomed patients was collected, processed, and stored according to a national standardized protocol. Full blood count and coagulation parameters were measured as per routine clinical care and blood films were examined for evidence of hemolysis. Baseline coagulation parameters were measured on a Behring Coagulation System. Flow cytometry was performed to detect CD41a (platelet), CD62e (endothelial), and glycophorin (red cell) MV. The results were analyzed using BD software and appropriate statistical tools.

Results and Conclusions: The red cell MV in snakebite patients with MAHA (n = 13) were significantly higher than those without MAHA (n = 17) while there was no significant difference in platelet MV levels between the snakebite patients with and without MAHA. Interestingly, the endothelial MV were reduced in all snakebite patient samples compared to the control samples. Measuring red cell MV at presentation could be useful as a predictive marker for MAHA in patients with snakebites.

KEYWORDS
coagulopathy, extracellular vesicles, microangiopathy, microvesicles, snakebite, venom

Essentials

- Snake bites are associated with venom induced coagulopathy, microangiopathic hemolytic anaemia (MAHA) and thrombocytopaenia.
- The mechanisms of MAHA and role of MV in its pathophysiology in snake bite are not known.
- This results of this study show that red cell MV are increased in snake bites associated with MAHA whilst endothelial MV are reduced in all snakebites.
- Evaluation of circulating endovascular microvesicles provide an important mechanistic tool and have a potential role as predictive markers for studying the effects of snake venom.

1 | INTRODUCTION

Snake venoms are a complex mixture of proteins and polypeptides which cause a broad range of toxic effects when released by the snake into the prey during a bite. Although snake toxins have probably evolved to assist in prey capture and defense, they inadvertently cause a range of systemic effects when humans are bitten. Many snake venoms contain procoagulant toxins that activate the clotting pathway, which results in venom induced consumption coagulopathy (VICC) in human envenoming, and this is the most common important clinical manifestation of snakebites worldwide.[1,2] Australian elapids contain serine proteases, which closely resemble the mammalian prothrombinase complex.[3]

Exposure to these prothrombin activators leads to widespread activation of the coagulation pathway resulting in consumption of the key clotting factors, including fibrinogen, FV, and FVIII.[2,4] More recently, microangiopathic hemolytic anemia (MAHA), acute kidney injury, and thrombocytopenia have also been recognized to be associated with VICC. Although this is thought to be a form of thrombotic microangiopathy, the pathophysiology remains unclear.[2-4]

Circulating microvesicles (MV) or small cell-derived extracellular vesicles have been implicated in hemostatic disorders—an increase in MV associated with pathological thrombosis, and a decrease contributing to potential bleeding.[5-7] Several cell-derived MV are frequently found in the circulation—these include red cell, platelet, leukocyte, and endothelial cell-derived.[8,9] Flow cytometry is a popular and easily accessible technique for measuring MV and determining their cellular origin.[10]

The role of circulating MV in snakebite patients is not known and we present the results of a unique investigation of MV in snakebite related VICC and MAHA. Changes in MV levels after envenoming could indicate direct toxin mediated damage to the endovascular system which has been hitherto difficult to evaluate or measure in a systematic fashion. To explore MV in snake envenoming further, we analyzed the levels of circulating platelet, endothelial, and red blood cell-derived MV in patients recruited to the Australian Snakebite Project (ASP) with confirmed envenoming from brown snake, tiger snake, or taipan.

2 | METHODS

Samples and clinical information were sourced from patients recruited to the ASP with elapid envenoming (including brown snake, tiger snake, and taipan envenoming), as previously described.[11] Citrated plasma was collected from each snakebite patient recruited to the study. Peripheral blood was collected in 0.109 mol/L tri-sodium citrate and platelet-free plasma was prepared by double centrifugation of whole blood for 15 minutes at 2500×g. All samples were processed within 2 hours of collection, and aliquots were stored at −80°C for further batched analysis. Samples were then thawed at 37°C, 15 minutes before testing. Control samples were obtained from healthy volunteer blood donors after appropriate consent—these were processed and stored in an identical fashion to the patient samples.

Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, and D-dimer concentrations were measured on a Behring Coagulation System (BCS; Dade Behring, Marburg, Germany) at a centralized laboratory. Full blood count was measured as per local routine laboratory protocol and blood films were examined by an independent pathologist for presence of red cell fragmentation for confirmation of MAHA. VICC was defined as an international normalized ratio (INR) >3 with undetectable fibrinogen, and MAHA as the occurrence of thrombocytopenia and red cell fragmentation.[11]

Flow cytometry was done on a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) with combinations of antibodies specific for platelet marker (CD41a-PE; Clone HIP8, BD Biosciences), red cell marker (CD235a-APC; Clone GA-R2, BD Biosciences), endothelial cell marker (CD62e-APC; Clone 68-5H11) and appropriate isotype controls in a final volume of 100 μL of PBS. Megamix beads (Biocytex, Marseille, France) were used to gate events less than 1 μm in diameter and analysis was undertaken using FACSDiva software. Control samples were obtained from healthy volunteer blood donors from the Australian Red Cross blood service. They ranged in age from 21 to 60 years and had a mix of both sexes. These subjects were screened for any significant medical conditions and samples obtained were processed within 4 hours from collection in a citrated tube in a manner similar to the snakebite patients' samples.

Microsoft Excel 2007 and SPSS, (IBM SPSS Statistics for Macintosh, Version 19.0., IBM Corp., Armonk, NY, USA) were used to collate and evaluate the data. Prism 7.0a (Graphpad Software, La Jolla, CA, USA) was used for graphical representations and nonparametric analysis was undertaken to evaluate differences given the large standard deviations in the MV levels. The normal cohort, VICC without MAHA (VICC no MAHA) and VICC with MAHA

TABLE 1 Comparison of levels of microvesicles (MV) in healthy controls and snakebite patients with VICC (no MAHA) and with VICC + MAHA

Samples/variable	Healthy controls (n = 28)	VICC (no MAHA) (n = 17)	VICC + MAHA (n = 13)
Age	30 ± 14	38 ± 9.8	38 ± 19.5
Gender (male, %)	54	76	77
Hb (g/L)	144 ± 9	136 ± 15	85 ± 16
Platelet count (10^9/L)	265 ± 85	141 ± 75	35 ± 13
Platelet MV/μL	1073 (614-1630)	1509 (1055-2387)	1147 (541-5168)
Endothelial MV/μL	359 (289-431)	<50	<50
Red cell MV/μL	<50	78 (7-180)	626 (393-1019)

Data presented as mean ± SD for parametric distribution (age, Hb and platelet counts) and median (interquartile range) for non-parametric distribution (MV levels).

(VICC + MAHA) groups were compared. One way ANOVA (Kruskal-Wallis) test was used for non-parametric comparisons between the groups and a level of 0.05 was set for statistical significance. The study was approved by the local ethics committee (Hunter New England ethics committee approval number 06/12/13/5.05) and by all major State and Territory human research ethics committees for the hospitals involved in the ASP.

3 | RESULTS AND DISCUSSION

A cohort of snakebite patient samples who had envenoming and VICC, and had baseline samples collected on admission to hospital, were evaluated. There were 17 patient-samples with VICC alone (no evidence of MAHA; designated as VICC no MAHA) and 13 patient-samples with VICC with MAHA (designated as VICC + MAHA); and 28 normal healthy controls. The age range for all snakebite patients was 24-73 years and there were more males than females (see Table 1). However, the differences between the age and gender between the groups compared was not statistically significant (Kruskal-Wallis test for age and z-test for gender). All envenomation was by brown snake bites apart from five patients (two tiger snake and three where the type could not be determined due to insufficient pre-antivenom samples). results for baseline blood counts and circulating MV levels in the three groups are shown in the table and illustrated in Figure 1. As expected, snakebite patients with MAHA had lower hemoglobin and platelets compared to controls and those without MAHA ($P < 0.001$). Platelet MV were significantly elevated in snakebite patients without MAHA (VICC no MAHA) compared to controls ($P = 0.009$), but there were no differences between the snakebite patients with and without MAHA ($P = 0.21$). Interestingly, the endothelial MV were significantly reduced in all snakebite samples compared to the controls ($P < 0.001$). The levels of red cell MV in snakebite subjects with MAHA (VICC + MAHA group), however, were significantly higher than those without MAHA ($P = 0.002$) and when compared to controls (see Figure 1).

A markedly elevated red cell MV level may be used as a surrogate marker for MAHA whereas thrombocytopenia alone may
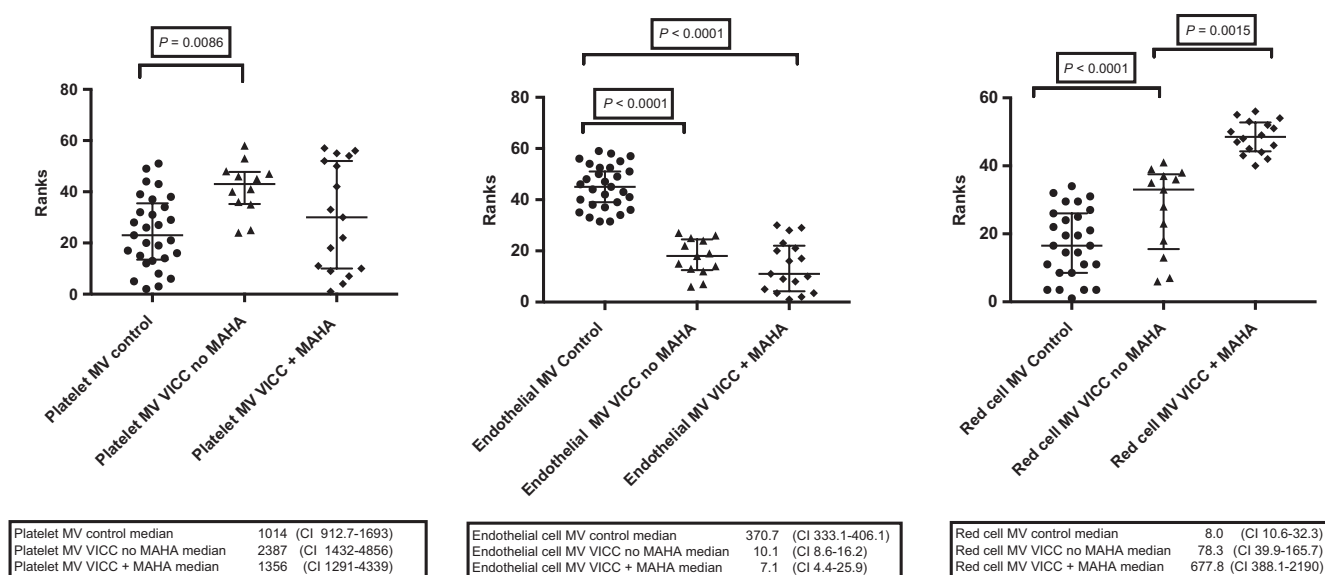

Platelet MV control median	1014 (CI 912.7-1693)
Platelet MV VICC no MAHA median	2387 (CI 1432-4856)
Platelet MV VICC + MAHA median	1356 (CI 1291-4339)

Endothelial cell MV control median	370.7 (CI 333.1-406.1)
Endothelial cell MV VICC no MAHA median	10.1 (CI 8.6-16.2)
Endothelial cell MV VICC + MAHA median	7.1 (CI 4.4-25.9)

Red cell MV control median	8.0 (CI 10.6-32.3)
Red cell MV VICC no MAHA median	78.3 (CI 39.9-165.7)
Red cell MV VICC + MAHA median	677.8 (CI 388.1-2190)

FIGURE 1 Median and 95% confidence interval for platelet, endothelial, and red cell microvesicles between the snakebite patients with VICC (no MAHA) and with VICC + MAHA. The significant *P* values are shown for differences between the groups for one way ANOVA (Kruskal-Wallis test)

not be able to discriminate between those snakebite patients with evolving MAHA and those without. The correlation between INR, PT, and aPTT and MV levels could not be quantified because most subjects had un-recordable parameters due to VICC, and there was likely to be no difference in these parameters between the two groups.

Increased levels of red cell MV in snakebite patient samples with MAHA is consistent with red cell breakdown and hemolysis. If this is a consistent finding then measuring MV at presentation could be useful as a predictive marker for MAHA in snakebite patients. This suggests an alternative mechanism for the observed microangiopathy other than endothelial damage alone due to the snake toxin. The rapid generation of red cell MV may subsequently lead on to a consumptive coagulopathy and microvascular thrombosis, which is often accompanied by thrombocytopenia that is typical of MAHA. It is also possible that some of the "vesicles" may represent red cell fragments rather than "microvesicles" and it may be considered technically challenging to separate the two populations by flow cytometry alone. However, majority of these fragments as seen on a blood film are usually in the size range of 1 μm or above, and in the range of the size of large platelets.[12] In previous studies evaluating the detection of fragmented red cells, using an automated hematology analyzer, have also used the principle of light scatter. The average dimension of the fragments was about 30 fL with a unidimensional size of a few microns when compared to a red cell which ranges between 80 to 100 fL with a diameter of about 8 μm.[13,14] The gating strategy used in our experiments excluded events larger than 1 μm in the MV gate therefore ensuring only red cell MV were enumerated.

The reason for apparent low levels of circulating endothelial MV could be due to changes in the size or surface antigen expression of the MV which may be altered in response to envenomation, resulting in MV that are undetectable via our method of flow cytometry. This was an unexpected finding that requires further investigation.

Limitations of this study include that we did not perform any functional studies in MV isolates or use alternative methods to evaluate MV apart from flow cytometry. Inter-laboratory as well as inter-instrument variability in flow cytometry is a concern despite the fact that it is an easily and widely available technique. Also, MV smaller than 200 nm are usually not detectable by flow cytometry. The use of isotype controls, correlation with functional testing and participation in global standardization initiatives may reduce this variability.[15] Given the small sample size, we did not stratify the results according to gender or comorbidity status but this may be important to explore in a larger cohort.

There may be some variability in how snake venom interacts with the human coagulation system. However, all of the snake venoms in the current study are well known to contain procoagulant toxins that closely resemble part or all of the mammalian prothrombinase complex and produce identical clinical sequelae. The toxin in brown snake is the most potent, being most similar to the FXa-Va complex, and able to directly cleave prothrombin to produce activated thrombin. The procoagulant toxin in tiger snake resembles FXa and

requires completion of the complex from human plasma FVa to enable thrombin activation.[1]

In summary, we provide the first report of changes in endovascular MV in snakebite patients and their possible role in the pathophysiology of snakebite-related MAHA. This study provides proof of principle that MV levels could be a marker for direct toxin mediated damage to red cells, platelets, and endothelial cells, that has been hitherto difficult to define. We established that flow cytometric assessment of MV could be used as an important technique for measurement of direct toxin mediated damage to red cells in snakebite associated MAHA.

RELATIONSHIP DISCLOSURES

AUTHOR CONTRIBUTIONS

AKE, LL, MS, and GI conceived the project; GI provided access to samples and interpretation of data; MS provided access to coagulation testing and flow cytometry; AKE carried out the project and data analysis; AKE, LL, MS, and GI were responsible for data interpretation and manuscript.

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