Quantification and characterisation of microvesicles: Applications in hereditary spherocytosis, type-II heparin-induced thrombocytopenia and cancer

F. Mullier, N. Bailly, C. Chatelain, B. Chatelain, J. Dogné

Microvesicles (MVs) are sub-micron-size cellular fragments released by eukaryotic cells following activation or apoptosis. Their diameter ranges between 30 and 1000 nm. Microvesicles are thought to play a major role in cellular cross-talk, inflammation, thrombosis and angiogenesis. As potential disease biomarkers, MV measurement and characterisation in biological fluids could also reveal new diagnostic and/or prognostic information in human disease. In this work:

- **We developed and validated an easy-to-use and useful quality control parameter for MV analysis by flow cytometry (FCM), the most frequently used technique to study MVs.**
- **We developed and validated a reproducible MV quantification method by FCM in whole blood in order to avoid preanalytical concerns of plasma assays (i.e. loss of MVs by centrifugation and lack of standardisation in centrifugation methods).**
- **We showed that this method could contribute to the diagnosis of hereditary spherocytosis (HS), a haemolytic anemia characterised by a release of MVs and unexplained occurrence of venous and arterial thrombosis after splenectomy.**
- **We developed and validated a high sensitive sizing atomic force microscopy (AFM) method.**
- **We characterised tumour cell-derived MVs released by cultured breast cancer cells MDA-MB 231 (Cells) by FCM, Transmission Electron Microscopy, AFM and Thrombin Generation Assay.**
- **Finally, we developed a platelet microparticle generation assay (PMPGA), a test which reproduces the in vivo type II heparin-induced thrombocytopenia (HIT) reaction. We showed that this assay, presented at least similar performances in comparison to the current biological reference, i.e. ^{14}C-Serotonin Release Assay. As flow cytometry is widespread available, PMPGA**

(Belg J Hematol 2012;3:157-160)

Authors: F. Mullier, PharmD, PhD[1,2], N. Bailly[2], C. Chatelain, Pr, MD[3], B. Chatelain, Pr, PharmD[2], J-M. Dogné, Pr, PharmD, PhD[1]. [1]Department of Pharmacy, NARILIS, Namur Thrombosis and Hemostasis Center (NTHC), University of Namur, Namur, Belgium. [2]Laboratory of Hematology, NARILIS, Namur Thrombosis and Hemostasis Center (NTHC), CHU Mont-Godinne, Université Catholique de Louvain, Leuven, Belgium. [3]Department of Hematology, NARILIS, Namur Thrombosis and Hemostasis Center (NTHC), CHU Mont-Godinne, Université Catholique de Louvain, Leuven, Belgium

Please send all correspondence to: F. Mullier, PharmD, PhD, CHU Mont-Godinne, Laboratory of Hematology, Avenue Docteur G. Thérasse 1, 5530 Yvoir, Belgium, tel: 0032 81 423243, fax: 0032 81 423204, email:mullierfrancois@gmail.com.

Conflicts of interest: The authors have nothing to disclose and indicate no potential conflict of interest.

Key words: microvesicles, microparticles, thrombosis, hereditary spherocytosis, type-II heparin-induced thrombocytopenia, cancer

Introduction

Microvesicles (MVs) are sub-micron-size cellular fragments released by eukaryotic cells following activation or apoptosis. They are highly heterogenous both in size (30-1000nm) and in composition.[1] Based on the size and mechanism of synthesis, MVs are currently divided into exosomes and microparticles (MPs). The diameter of exosomes is comprised between 30 and 100 nm whereas MPs have a size comprised between 100 nm and 1 μm.[1]

Microvesicles are thought to play a major role in cellular cross-talk, infection, immunity, inflammation, thrombosis and angiogenesis.[1] As potential disease biomarkers, MV measurement and characterisation in biological fluids could also reveal new diagnostic and/or prognostic information in human disease. Numerous techniques have been described to detect and/or characterise the MVs. However, no single technique is able to provide all MV characteristics. In addition, many pre-analytical variables lead to potential artefacts in MV analysis.

The validation and standardisation of techniques that could be used to determine the MV characteristics are needed before studying the diagnostic and prognostic impacts of MVs in retrospective and prospective clinical trials.

Quantification and characterisation of microvesicles

Development of quality control tools for microvesicle count determination by flow cytometry

Despite its lack of sensitivity, FCM is able to quantify MVs, to determine the cellular origin with a practicable technique of low cost with a quite low complexity of instrumentation. In addition, development of new flow cytometers (FCMrs) with increased sensitivity is ongoing. However, the variety of pre-analytic and analytic variables, results in a wide range of large platelet MVs (PMVs) values in platelet-free-plasma (PFP) of healthy subjects (100–4000 PMVs/μl).[2] Thus, the development of quality control tools is mandatory. We proposed the use of the separation index (SI) as an easy-to-use quality control for MV analysis.[3]

We also demonstrated that a decrease of the resolution in the MV range objectivised by the separation index leads to an underestimation of the MV count. We showed that whatever the instrument type, optimal scatter resolution may vary in-time and between individual instruments and should therefore be accurately checked on a systematic basis (submitted for publication).

Comparison of techniques available to characterise microvesicles

The combination of different techniques is required for a complete description of MVs.

Atomic force microscopy (AFM), a high-sensitive technique able to image biological samples in aqueous fluids was recently proposed for the detection and quantification of CD41-positive MVs but has never been developed for MVs bearing tissue-factor (TF), the main determinant of the procoagulant activity (PCA) of MVs.[4]

Consequently, we developed and validated a high sensitive sizing AFM method for TF-MVs and we compared FCM, TEM, AFM and TGA to characterise MVs released by cultured breast cancer cells (MDA-MB231). TGA is a useful technique to study the PCA of tumour cell-derived MVs whatever their size. It should be combined by high-sensitive sizing and counting techniques such as AFM in order to determine TF-MVs. We concluded that TGA and AFM should be validated and used to study TF-MVs in plasma from healthy subjects and cancer patients (submitted for publication).

Applications in haematological diseases

Hereditary spherocytosis

One common feature of the erythrocytes (Ery) in HS is weakened vertical linkages between the membrane skeleton and the lipid bilayer and its integral proteins, leading to destabilisation of the lipid bilayer and the release of MVs.[5]

This has been shown by few teams and only qualitatively as sensitive techniques were not available.

Consequently, we first developed and validated a reproducible MV quantification method by FCM in whole blood in order to avoid pre-analytical concerns of plasma assays (i.e. loss of MVs by centrifugation and lack of standardisation in centrifugation methods). Then, we used this new MV quantification method by FCM in WB to a limited series of HS patients. We showed that this new method could contribute to the diagnosis of HS, an haemolytic anemia (HA) characterised by unexplained occurrence of venous and arterial thrombosis after splenectomy.[6] In the present work, we confirmed quantitatively that HS induced the

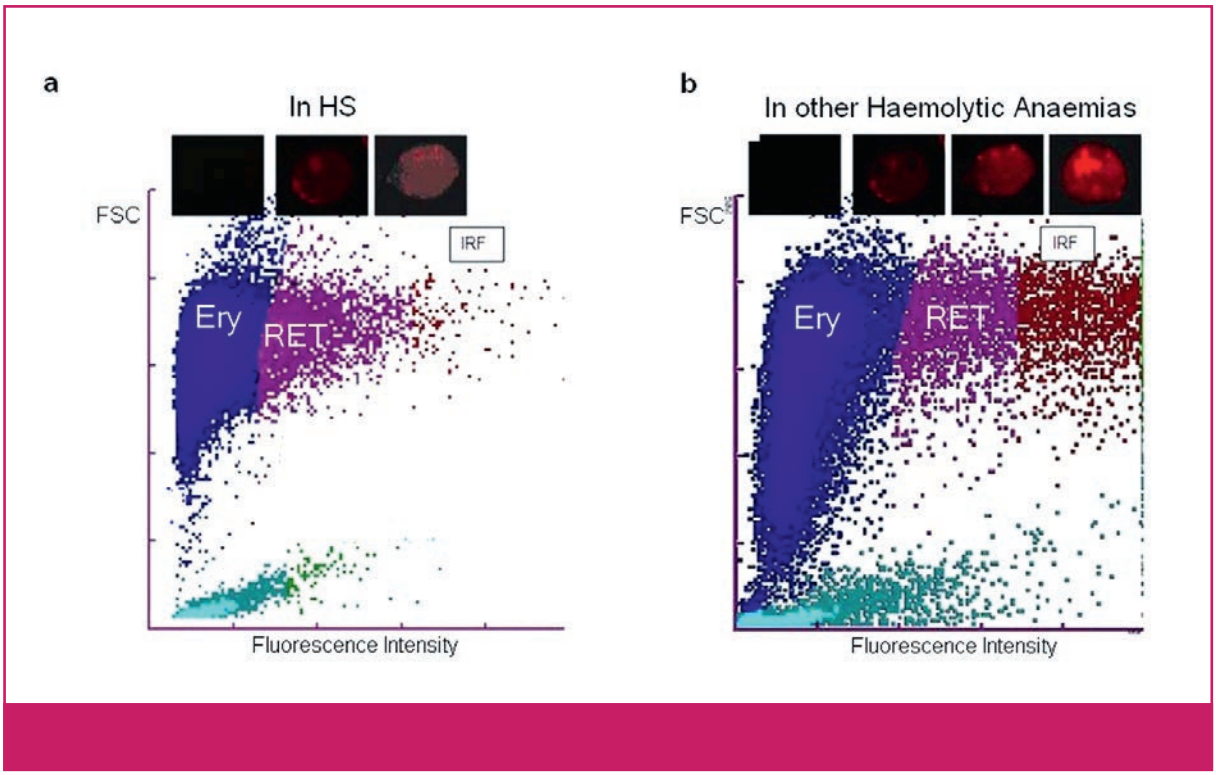

Figure 1. Reticulocytes channel on Sysmex XE-5000CM **(a)** Hereditary Spherocytosis: reticulocytosis with decreased Immature Reticulocyte Fraction (IRF). **(b)** Other Haemolytical Anaemias: Reticulocytosis with normal IRF.
Footnote: FSC: Forward Scatter, IRF: Immature Reticulocyte Fraction, Ery: Erythrocytes, Ret: Reticulocytes.

release of significantly more EryMVs than healthy subjects and other HA (in preparation).

Furthermore, one previous study showed that different mechanisms lead to reduced membrane surface area in HS and some forms of autoimmune hemolytic anemia (AIHA). Indeed, in HS, but not in AIHA, the surface area loss is already present at the circulating reticulocyte stage.[7] We thus decided to study reticulocyte parameters to develop a diagnostic tool on two haematological analysers: XE-2100 and XE-5000 (Sysmex, Japan). The algorithm is mainly based on an abnormal immature reticulocyte fraction (IRF), as illustrated in *Figure 1*. This diagnostic tool could be used routinely as an excellent screening method for the diagnosis of HS.[8] This is in line with recommendations proposed by some authors in recent reviews.[5] This rapid method is also validated on mild HS and in neonates, and overcomes the lack of sensitivity of electrophoresis in ankyrin deficiencies. This tool in combination with clinical data could make the diagnosis of HS easier by reducing the number of expensive and time-consuming confirmation tests.

Type-II heparin-induced thrombocytopenia

The release of procoagulant PMVs triggered by the presence of pathogenic anti-heparin-platelet factor 4 (PF4) antibodies serves as a catalytic surface for enhanced thrombin generation and is considered as a major component of type-II heparin-induced thrombocytopenia.[9] Although early diagnosis of HIT is essential to improve clinical outcomes, such a diagnosis is challenging. The reference functional test is ^{14}C Serotonin Release Assay (^{14}C-SRA) but it cannot be considered as a gold standard to diagnose HIT.[10,14] The validation of a new gold standard assay would be useful to avoid missed diagnosis and overdiagnosis. We developed a platelet microparticle generation assay (PMPGA), a test which reproduces the in vivo type II heparin-induced thrombocytopenia (HIT) reaction.[11]

During the incubation of a type II HIT patient's plasma or serum with citrated 109 mM whole blood from a healthy donor, PMVs expressing phosphatidylserine (PS) are generated at low heparin concentration (1IU heparin/ml) due to the formation of immune complexes (i.e. IgG-PF4-heparin). On the contrary, PMV rate decreases in presence of higher

Key messages:

1. Microvesicles play a major role in cellular cross-talk, infection, inflammation, hemostasis, thrombosis and angiogenesis.
2. Microvesicle measurement and characterisation in biological fluids may provide diagnostic and/or prognostic information in several major human diseases.
3. The validation and standardisation of techniques used to determine the MV characteristics in retrospective and prospective clinical trials are currently ongoing.
4. The study of microvesicles in clinical settings should currently be limited to centres involved in the process of standardisation.

heparin concentration (500 IU heparin/ml). This high concentration leads to a dissociation of the complex Ig-Heparin-PF4 and is therefore used to enhance the specificity of all functional tests (9). Consequently, we used a combination of ratio between PMV annexin V positive concentration generated with 1 IU heparin/ml and 500 IU heparin/ml and the concentration of PMPs annexin V positive generated at 1 IU heparin/ml to define one positive type-II HIT. We showed that this assay, presented at least similar performances in comparison to ^{14}C-SRA (in revision).[14] As FCM is widespread available, PMPGA may become a new promising biological reference to diagnose type-II HIT.

Conclusions

Microvesicles are thought to play a major role in cellular cross-talk, infection, inflammation, hemostasis, thrombosis and angiogenesis.

Microvesicle measurement and characterisation in biological fluids could reveal new diagnostic and/or prognostic information in human disease.

The validation and standardisation of techniques that could be used to determine the MV characteristics in retrospective and prospective clinical trials are currently ongoing.

References

1. Gyorgy B, Szabo TG, Pasztoi M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci. 2011 Aug;68(16):2667-88.
2. Lacroix R, Robert S, Poncelet P, et al. Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost. 2010 Nov;8(11):2571-4.
3. Mullier F, Bailly N, Chatelain C, et al. More on: calibration for the measurement of microparticles: needs, interests, and limitations of calibrated polystyrene beads for flow cytometry-based quantification of biological microparticles. J Thromb Haemost. 2011 Aug;9(8):1679-81.
4. Yuana Y, Oosterkamp TH, Bahatyrova S, et al. Atomic force microscopy: a novel approach to the detection of nanosized blood microparticles. J Thromb Haemost. 2010 Feb;8(2):315-23.
5. Perrotta S, Gallagher PG, Mohandas N. Hereditary spherocytosis. Lancet. 2008 Oct 18;372(9647):1411-26.
6. Schilling RF, Gangnon RE, Traver MI. Delayed adverse vascular events after splenectomy in hereditary spherocytosis. J Thromb Haemost. 2008 Aug;6(8):1289-95.
7. Da Costa L, Mohandas N, Sorette M, et al. Temporal differences in membrane loss lead to distinct reticulocyte features in hereditary spherocytosis and in immune hemolytic anemia. Blood. 2001 Nov 15;98(10):2894-9.
8. Mullier F, Lainey E, Fenneteau O, et al. Additional erythrocytic and reticulocytic parameters helpful for diagnosis of hereditary spherocytosis: results of a multicentre study. Ann Hematol. [Multicenter Study]. 2011 Jul;90(7):759-68.
9. Greinacher A. Heparin-induced thrombocytopenia. J Thromb Haemost. 2009 Jul;7 Suppl 1:9-12.
10. Tardy B, Presles E, Akrour M, et al. Experts' opinion or the serotonin release assay as a gold standard for the diagnosis of heparin-induced thrombocytopenia (HIT)? J Thromb Haemost. 2011 Aug;9(8):1667-9.
11. Mullier F, Bailly N, Cornet Y, et al. Contribution of platelet microparticles generation assay to the diagnosis of type II heparin-induced thrombocytopenia. Thromb Haemost. 2010 Jun;103(6):1277-81.

Acknowledgments

The authors would like to thank Julie Hardij, Damien Gheldof and Philippe Devel for their contribution in the comparison of techniques available to characterise microvesicles.

This work was supported by a grant from the Le Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA).