International Journal of Cardiology 176 (2014) 145–150

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Red blood cell and platelet microparticles in myocardial infarction patients treated with primary angioplasty

Georgios Giannopoulos [a,c,*,1], Georgios Oudatzis [b,1], Georgios Paterakis [b,1], Andreas Synetos [d], Eleni Tampaki [a], Georgios Bouras [a], George Hahalis [e], Dimitrios Alexopoulos [e], Dimitrios Tousoulis [d], Michael W. Cleman [c], Christodoulos Stefanadis [d], Spyridon Deftereos [a,c]

[a] Cardiac Catheterization Laboratory, Department of Cardiology, Athens General Hospital "G. Gennimatas", Athens, Greece
[b] Flow Cytometry Laboratory, Department of Immunology, Athens General Hospital "G. Gennimatas", Athens, Greece
[c] Section of Cardiovascular Medicine, Yale University School of Medicine, New Haven, CT, USA
[d] 1st Department of Cardiology, University of Athens Medical School, Athens, Greece
[e] Department of Cardiology, University of Patras Medical School, Patras, Greece

ARTICLE INFO

Article history:
Received 7 May 2014
Accepted 5 July 2014
Available online 12 July 2014

Keywords:
Microparticles
Erythrocyte
Platelet
Myocardial infarction
Flow cytometry

ABSTRACT

Background: Red blood cell and platelet microparticles (RBCm and PLTm, respectively) have drawn research attention as to their potential prothrombotic and vasoconstrictive effects in experimental settings. However, the relevance of circulating microparticles in clinical settings is largely undetermined.
Methods: Circulating microparticles were quantified with a flow cytometric method in blood samples from consecutive STEMI patients after primary PCI. A matched cohort of healthy volunteers was used to derive reference values for comparison. STEMI patients were followed for 6 months for a composite clinical endpoint.
Results: Fifty-one STEMI patients (age 59.8 ± 8.8 years) and 50 controls (age 56.2 ± 9.2 years; $p = 0.155$) were enrolled. RBCm concentration was 18,198 ± 6062/μl in the reference cohort versus 33,740 ± 21,169/μl in STEMI patients ($p < 0.001$). RBCm count was not correlated to total RBCs (standardized beta 0.018; $p = 0.861$). PLTm did not differ between groups (17,529 ± 16,292/μl in STEMI patients versus 14,372 ± 6211/μl in controls; $p = 0.203$). RBCm *c*-statistic was 0.832 (95% confidence interval 0.720 to 0.944), while PLTm prognostic value was not statistically significant (*c*-statistic 0.614, 95% confidence interval 0.444 to 0.784). In the multivariate analysis, RBCm concentration was independently associated with the occurrence of the clinical endpoint, after adjustment for age, ejection fraction, serum creatinine and presence of diabetes (adjusted $p = 0.034$).
Conclusions: The present study demonstrates for the first time that erythrocyte microparticles are elevated in patients with STEMI treated with primary PCI, with levels approximately double those measured in a reference population of healthy volunteers, and their concentrations appear to be positively associated with adverse clinical events.

1. Introduction

Although coronary artery disease is a pathologic process involving mainly the vascular wall, it has long been known that blood constituents, including coagulation and fibrinolysis factors, inflammatory cells and mediators and, of course, platelets, also play an important role in ischemic heart disease manifestations and their sequelae [1,2]. Among cellular blood constituents, red blood cells (RBCs) have probably received the least attention in the context of acute coronary syndrome pathophysiology, although it is conceivable that this most abundant blood element could well be implicated in the complex crosstalk of processes taking place in the ischemic area vasculature. Indeed, RBC hemolysis and microparticle formation has been shown – in an experimental setting – to cause decreased nitric oxide bioavailability and vascular dysfunction [3], but their role in a clinical setting of cardiovascular disease has not been investigated.

We used flow cytometry to quantify RBC microparticles (RBCm), RBC transitional cells (RBCt; small-sized, less than 3 μm, but larger than microparticles, erythrocyte cell forms), and platelet microparticles (PLTm) in peripheral blood of ST-elevation myocardial infarction (STEMI) patients treated with primary percutaneous coronary intervention (PCI), according to current guidelines [4], with a dual goal: compare them to a reference group of healthy controls and assess potential associations with subsequent clinical events.

* Corresponding author at: Athens General Hospital "G. Gennimatas", 154 Mesogeion Ave., 11527 Athens, Greece. Tel.: +30 2132032560.
E-mail addresses: ggiann@med.uoa.gr, georgios.giannopolous@yale.edu (G. Giannopoulos).
[1] Authors Giannopoulos, Oudatzis and Paterakis contributed equally to this work.

http://dx.doi.org/10.1016/j.ijcard.2014.07.022
0167-5273/

2. Methods

2.1. Study design, population and procedures

This study consisted of two parts, one part being a two-group cross-sectional observational study (comparison of STEMI patients with a reference group), while the second part was a prospective, cohort study (prospective follow-up of STEMI patients for clinical events – see below). Consecutive patients referred for primary PCI were screened for inclusion. Eligible patients had at least 0.1-mV ST-segment elevation in three contiguous leads with associated chest or epigastric pain and consistent angiographic findings. Patients receiving intra-aortic balloon counterpulsation were excluded. All patients received standard treatment with antiplatelet agents and anticoagulants, as recommended by current guidelines [4]. All patients received drug eluting stents. Blood was drawn for flow cytometry within 2 h from completion of the primary PCI. Daily routine blood workup was performed in the cardiac care unit.

The clinical endpoint of the study was the composite of death or resuscitated sudden death, hemodynamically significant sustained ventricular tachycardia, nonfatal myocardial infarction, target lesion revascularization, acute heart failure or rehospitalization for cardiac causes within 6 months after the index STEMI. Clinical events were adjudicated by an independent 3-person committee, blinded as to the RBCm or PLTm results.

A group of healthy volunteers, matched for age, sex and diabetic status with no history of heart disease, was used as the reference group.

2.2. Flow cytometry

RBC- and PLT-derived microparticles were counted by flow cytometry. Whole blood, anticoagulated with EDTA, was processed within 1 h after venipuncture. One hundred microliters of blood was diluted in 100 μl normal saline and 10 μl from this suspension was transferred into a TRUCOUNT™ absolute cellular count tube (Becton Dickinson, California, USA). The following monoclonal antibodies (10 μl each) were then added: anti-Annexin V, labeled with fluorescein (FITC) (Cell Lab, Beckman Coulter, Florida, USA), CD41 Platelet Glycoprotein IIb labeled with phycoerythrin (PE), clone 5812 (Dako, Glostrup, Denmark) and CD235a (Glycoforin-A, clone PNA71564, Beckman Coulter, Florida, USA) labeled with phycoerythrin-cyanin 7 (PeCy7). They were incubated for 15 min at room temperature. After incubation no wash or centrifugation step followed. One milliliter diluted Buffer Annexin (Cell Lab, Beckman Coulter, Florida, USA) (1:10 ice 4°C water) was added, as well as 100 μl of Megamix beads (0.5, 0.9 and 3 μm) (Biocytex, France), in order to enable a volumetric gating strategy. The reconstituted sample was immediately counted by an FC-500 Beckman Coulter (Florida USA) flow cytometry analyzer, with CXP Software (Beckman Coulter), without baseline offset setting and with highly amplified photomultiplier gain and voltage in forward logarithmic channel (FSlog). At least 1500 TRUCOUNT™ bead events were acquired for precise calculation of microparticles per microliter.

2.3. Gating strategy

Fig. 1A illustrates the initial gating of erythrocytes (RBC gate) and platelets (PLT gate) in the scattergram of CD41-PE versus CD235a-FITC. The gates of the volumetric Megamix beads are defined as Mx0,5&0.9u and Mx3u, respectively. The count beads gate encompasses the TRUCOUNT™ absolute count bead events. In Fig. 1B, the scattergram of logarithmic forward scatter (FSlog) versus Annexin V-FITC fluorescence is shown. The clusters of the volumetric beads 0.5 μ and 0.9 μ define the FSlog lanes used to standardize the gating of erythrocyte and platelet microparticles. In Fig. 1C, in the scattergram of FSlog versus CD235a-PeCy7, the erythrocyte microparticle gates are defined. Rm encompasses the microparticles with FSlog corresponding to the 0.5 μ volumetric microbead cluster, while the RBCt gate includes erythrocyte microparticles spanning the range of the 0.9 μ lane up to the lower limit of the 3 μ microbead cluster, which are called transitional. The gate RBCm sums the Rm and RBCt events in order to calculate the total microparticle number. Fig. 1D shows that erythrocyte microparticles are positive for Annexin V. Fig. 1E shows all CD41-PE positive events, encompassing platelets and microparticles. Platelet microparticles were discriminated by the same FSlog lanes that were used for erythrocyte microparticles, defined by the clusters of the 0.5 and 0.9 μ volumetric beads (Fig. 1F). In Fig. 1G the PLTm gate corresponds to the 0.5 μ volumetric microbead cluster and defines platelet microparticles. The PLTan gate encompasses all CD41-PE/Annexin-FITC positive events. Fig. 1H shows the histogram of the counting TRUCOUNT™ beads in the unused fluorescence channel of FL4.

2.4. Quantitation of erythrocyte and platelet microparticles

In order to estimate the concentration (C) of erythrocyte or platelet microparticles the following parameters were used: the tested volume in μl (V, equal to 10 μl), the number of counted beads in the corresponding histogram (b), the number of microparticle events in the above defined gates (m), the number of beads provided by the manufacturer on the TRUCOUNT™ tube foil pouch label (N) and the dilution factor (α), which was 2 (1:1 dilution in normal saline). Concentration (absolute count of microparticles per whole blood μl) was computed according to the formula:

$$C = \frac{\alpha m N}{V b}$$

2.5. Statistical analysis

Continuous variables were summarized as mean ± standard deviation and compared using the t-test, after testing for homogeneity of variance using Levene's test of

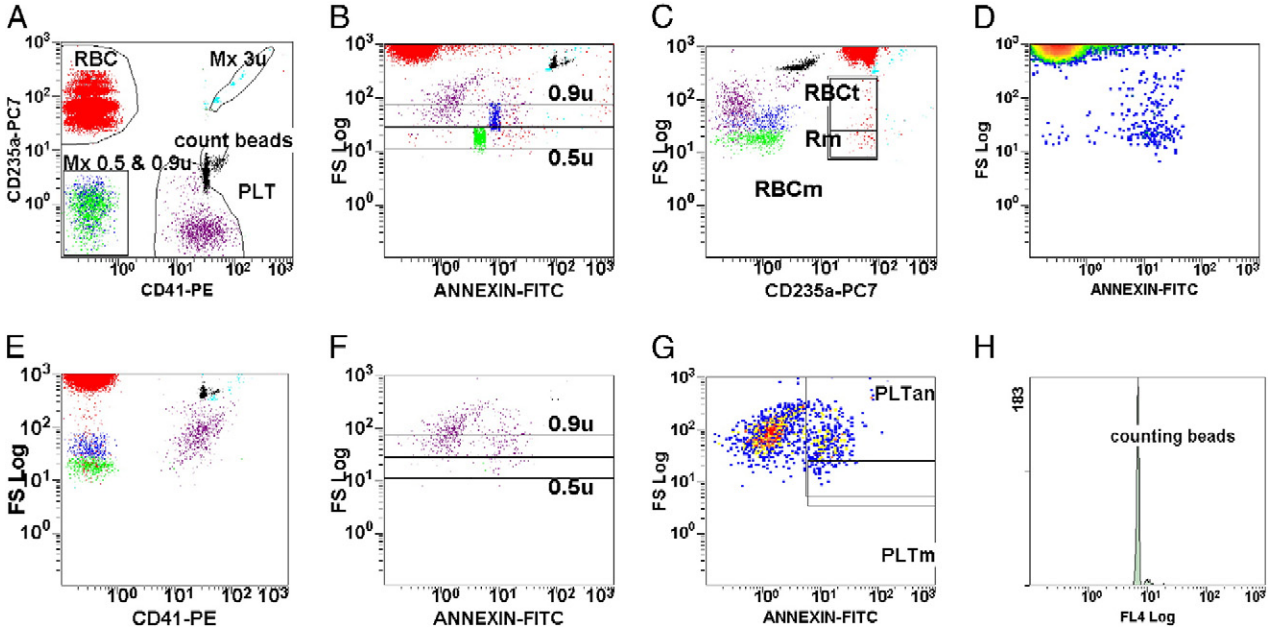

Fig. 1. Gating strategy. A. Initial gating of erythrocytes (RBC gate) and platelets (PLT gate) in the scattergram of CD41-PE versus CD235a-FITC. The gates of the volumetric Megamix beads are defined as Mx0,5&0.9u and Mx3u, respectively. B. Scattergram of logarithmic forward scatter (FSlog) versus Annexin V-FITC fluorescence. The clusters of the volumetric beads 0.5 μ and 0.9 μ define the FSlog lanes used to standardize the gating of erythrocyte and platelet microparticles. C. Scattergram of FSlog versus CD235a-PeCy7. Rm encompasses the microparticles with FSlog corresponding to the 0.5 μ volumetric microbead cluster, while the RBCt gate includes erythrocyte microparticles spanning the range of the 0.9 μ lane up to the lower limit of the 3 μ microbead cluster, which are called transitional. D. Erythrocyte microparticles are positive for Annexin V. E. All CD41-PE positive events, encompassing platelets and microparticles. F. Platelet microparticle segregation by the same FSlog lanes as for erythrocyte microparticles. G. The PLTm gate corresponds to the 0.5 μ volumetric microbead cluster and defines platelet microparticles. The PLTan gate encompasses all CD41-PE/Annexin-FITC positive events. H. Histogram of the counting TRUCOUNT™ beads in the unused fluorescence channel of FL4.