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Research Article

Circulating Microvesicles Mediate the Inflammatory Response and Coagulopathy in Coronary Artery Bypass Graft Surgery

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Abstract

Coronary Artery Bypass Grafting (CABG) is inevitably associated with some level of activation of the coagulation and inflammation systems. The purpose of this research was to access the Circulating Microvesicles (cMVs) possible association with inflammatory response, coagulopathy in patients with on- and off- pump Coronary Artery Bypass Graft Surgery (CABG). Firstly, microvesicles obtained from the patients' plasma before (A0/B0) and 24 hours after (A1/B1) CABG with or without the use of Cardiopulmonary Bypass (CPB) (n=10 on-pump (A) , n=10 off-pump (B), before surgery (A0/B0), 24 hours after surgery (A1/B1)). The microvesicles were analyzed by Flow cytometry. iTRAQ (isobaric tags for relative and absolute quantitation) analysis was performed at the levels of proteins. In result, the level of cMVs showed parallel changes with the levels of Brain Natriuretic Peptide (BNP), C-Reactive Protein (CRP), and Myoglobin (Mb) in the groups. The microvesicles from the endothelial (EMP), platelets (PMP), white blood cells (LMP) were all detected by makers of CD144, CD41a, CD45, but CD41a PMP and CD144 EMP levels enhanced significantly after on-and off-pump CABG, especially in the on-pump group. Further, iTRAQ analysis showed that the changing proteins contained in the microvesicles were mostly associated with the responses of inflammation and coagulation. Further, GO function classification and the KEGG pathway also revealed the same results. In conclusion, the microvesicles played a key role in the processes of inflammation and coagulation in CABG patients.

Keywords: Circulating microvesicles; Coronary artery bypass grafting; Cardiopulmonary bypass; Inflammation; Coagulation; Myocardical dysfunction

Abbreviations

cMVs: Circulating Microvesicles; CABG: Coronary Artery Bypass Grafting; CPB: Cardiopulmonary Bypass; BNP: Brain Natriuretic Peptide; CRP: C-reactive protein; Mb: Myoglobin; PS: Phosphatidylserine

Introduction

Cardiovascular disease remains the leading of death in the world. Coronary Artery Bypass Grafting (CABG) has become the commonly performed surgical procedures, and reduced mortality in patients with severe coronary artery disease [1-3]. Early, Coronary Artery Bypass Grafting (CABG) with the use of cardiopulmonary bypass ("on pump") can provide a controlled condition under cardioplegic arrest. However, increasing numbers of researches reported that many deleterious effects were observed in patients undergoing on-pump technique, such as "systemic inflammatory response, coagulopathy and end-organs dysfunction". In the past decades, Coronary Artery Bypass Grafting (CABG) without the use of cardiopulmonary bypass ("off pump") has been developed to reduce the postoperative complications. Recently, accumulating studies have demonstrated a reduction in additional complications and the mortality in patients undergoing off-pump compared to on-pump approach [4-10].

In clinical, we often see more inflammatory response, coagulopathy and Myocardical dysfunction after Coronary Artery Bypass Grafting (CABG) with/without the use of cardiopulmonary bypass. And these effects are responsible for most of the morbidity after surgery. But there are no accurate prediction makers to access the post-CABG clinical outcomes [1,11].

Microvesicles are released from the plasma membrane of cells by outward budding of membrane vesicles, following disruption of the natural asymmetrical distribution of membrane phospholipids, under conditions of cell activation, mechanical stress, and apoptosis. They retain surface antigens specific to their parent cell, and contain protein, RNA, miRNA, and lipids derived from the parent cell. Now microvesicles are widely recognized as key players in cell-to-cell communication. Moreover, it is ascribed important role to the process of coagulation and inflammation [12-15]. Here, we focused on the role of microvesicles and discussed their emergent role in mediating activation and response to inflammation, coagulation and Myocardical dysfunction during the Coronary Artery Bypass Grafting (CABG).

Materials and Methods

Patient population and plasma collection

Twenty patients undergoing cardiac surgery, either conventional CABG with Cardiopulmonary Bypass (CPB) (n=10) or CABG without CPB (n=10), at the cardiac center of Air force General Hospital were included in this study. This clinical study was conducted in accordance

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with the Ethics Guidelines for Research Involving Human Subjects or Human Tissue from the Air force General Hospital. All of the patients provided written informed consent. Exclusion criteria were as follows: Patients having unstable conditions (recent acute coronary syndrome with peak Creatine kinase-MB (CK-MB) >2 times the upper limit of normal), acute heart failure or left ventricular ejection fraction <45%, requiring immediate surgery, severe hepatic and renal dysfunction, additional valve replacement surgery, or any evidence of infection. All involved patients were taking aspirin and/or clopidogrel/Ticagrelor, which were discontinued at least one week before the surgery, and instead, low molecular weight heparin was continued until 24 hours before surgery. Table 1 summarized the characteristics of the main study groups. Cardiopulmonary bypass duration was 85.3 min ± 7.8 min. Midline sternotomy was performed in all patients.

EDTA tubes (1 mg/ml EDTA) were used to collect venous blood from each group of patients at least 12 h before the induction of anesthesia (before surgery, on-pump A0/off-pump B0), and at 24 hours after surgery (on-pump A1/off-pump B1). Samples were kept in a refrigerator at 4°C. To obtain platelet-free plasma samples, the samples were first centrifuged at 1, 600 × g for 10 min and then at 16, 000 × g for 30 min, which was followed by filtration through a 0.22 μm Super Membrane (Pall Life Sciences, New York, USA) to remove apoptotic bodies; after that, the supernatant was isolated and stored at -80°C to be used in this study.

Table 1: Demographic Data of patients.

	on-pump	off-pump
Age (y)	66.2 ± 11.5	67.6 ± 8.4
Gender (M/F)	1/9	6/4
Ejection Fraction (LVEF%)	54.5 ± 10.7	56.2 ± 12.4
Creatinine (μmol/L)	48.6 ± 9.6	51.2 ± 8.9
Diabetes (%)	70	60
Weight(kg)	67.3 ± 12.7	64.5 ± 10.8

Data are expressed as mean ± SEM or as % of population.

Microvesicle isolation and identification

Circulating Microvesicles (cMVs) were harvested as per the instructions on the ExoQuick serum exosome precipitation solution. The surface makers were evaluated with the bead-based flow cytometry technique. Microvesicles were reacted with a monoclonal antibody that targeted PS, all platelets, epithelial cells, and leukocyte-derived microvesicles (CD41a, CD144, and CD45). A Linotype control mixture matching the HRP conjugated anti-rabbit antibody was used (Cell Signaling Technology, Boston, USA, cat #7074). All of the antibodies were purchased from BD Biosciences, USA (CD41a, CD144, Annexin-V and CD45, cat #340931, #562242, #556420, #340943). We collected the data of microvesicles of different origins.

Protein preparation

Microvesicles were collected as per the method described above. The samples were solubilized in lysis buffer (8 M urea, 30 mM HEPES, 1 mM PMSF, 2 mM EDTA, and 10 mM Dithiothreitol (DTT)) using 5 min of sonication (pulse-on 2 s, pulse-off 3 s, power 180 W), followed by centrifugation at 20,000 rpm for 30 min at 4°C. The protein concentration of the clear supernatant was quantified with 2-D Quant kit. All of the proteins were kept at -80°C for further analysis.

Protein digestion and iTRAQ labeling

iTRAQ (isobaric tags for relative and absolute quantitation) analysis was performed at Beijing Proteome Research Center (Beijing, China). Total protein (100 μg) was isolated from each sample solution and then the protein was digested with Trypsin Gold (Promega, Madison, WI, USA) with the 30:1 ratio of protein: trypsin at 37°C for 16 h. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plexiTRAQ reagent (Applied Bio systems, Carlsbad, CA, USA).

Strong cation exchange (SCX) fractionation

Each fraction was resuspended in buffer A (25 mM NaH2PO4 in 25% ACN, pH 2.7) and loaded onto a 4.6 mm × 250 mm Ul tremex SCX column containing 5 μm particles (Phenomenex). The peptides were eluted at a flow rate of 1 ml/min using buffer A for10 min, 5% - 60% buffer B (25 mM NaH2PO4, 1 M KCl in 25% ACN, pH 2.7) for 27 min, and 60%-100% buffer B for 1 min. The eluted peptides were desalted with a Strata X C18 column (Phenomenex).

MS Analysis by Q-Exactive

The eluted peptides were reconstituted with Mobile phase A (2% acetonitrile, 0.1% FA). The mixture of peptides was loaded onto a C18 column and separated at a flow rate of 400 ml/min using a mobile phase B gradient of 5% for 10 min, 5%-30% for 30 min, 30%-60% for 5 min, and 60%-80% for 3 min; maintained at 80% for 7 min; returned to 5% for 3 min; and maintained at 5% for 7 min. The eluted peptides were detected using Q-Exactive, and MS data were acquired using a data-dependent top 20 method by choosing the abundant precursor ions from the survey scan (350-20, 000 Da) using Higher Energy Collision Dissociation (HCD). Determination of the target value was based on Automatic Gain Control (AGC). Survey scans were acquired at a resolution of 70,000 and the resolution for HCD spectra was set to 17,500. The iTRAQ analysis was performed as two technical replicates to gather reliable quantitative information.

GO is an international standardization system of gene function classification. It has 3 ontologies that can describe molecular function, cellular component, and biological process, respectively. In this study, functional annotations of the proteins were conducted using Blast2GO program against the non-redundant protein database (NR; NCBI).

KEGG PATHWAY is a database resource and a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks between the identified differentially expressed proteins in the on-pump and off-pump groups [16,17].

Statistical analysis

All of the values are expressed as the mean ± SEM. Statistical analysis was performed with student's two-tailed unpaired t test or one-way ANOVA using Graph pad Prism 5. Statistical significance was accepted for all of the tests at $P < 0.05$.

Results

Clinical characteristics

In the two study groups, there was a steady baseline and no significant differences in the clinical characteristics (Table 1), such as age, gender, weight, ejection fraction, and other demographic variables. In all of the patients in the on-pump group, the roller-type rotary pump was used. The time required for the surgery was similar in the two groups, and it was 6 h ± 45.8 min.

The levels of Brain Natriuretic Peptide (BNP), C-Reactive Protein (CRP) and Myoglobin (Mb), had no statistical differences in two

Table 2: C-Reactive Protein (CRP), Brain Natriuretic Peptide (BNP) and Myoglobin (Mb) concentrations.

	BNP(ng/ml)		CRP(mg/dl)		Mb (ng/ml)	
	Pre-op	Post-op	Pre-op	Post-op	Pre-op	Post-op
on-pump(n=10)	114.0 ± 13.4	267.0 ± 52.1*	2.3 ± 1.0	16.3 ± 3.3*	59.8 ± 16.1	376.0 ± 41.4*
off-pump(n=10)	98.0 ± 42.3	139.0 ± 42.6*	1.7 ± 0.4	9.8 ± 0.5*	67.4 ± 13.4	195.4 ± 24.9*

Results are mean ± SEM; *P <0.05 post-op versus Pre-op, Pre-op: pre-operation; Post-op: post-operation; n: the number of sample.

groups before surgery. After surgery, these markers were obviously increased (Table 2, P<0.05), and compared with the off-pump group, the alterations were more significant in the on-pump CABG (Table 2, P<0.05).

Flow cytometry for different microvesicles

Flow cytometry revealed that microvesicles stained by markers usually present on platelets (CD41a), endothelial cells (CD144), and white blood cells (CD45), and Phosphatidylserine (PS) were already detectable in plasma samples at A0 (on-pump CABG before surgery) and B0 (off-pump CABG before surgery), and there were no statistical differences (Table 3, P >0.05). However, the number of microvesicles stained by these markers tended to increase at A1 (on-pump CABG after surgery) and this trend was significant only for CD41a, CD144, Phosphatidylserine (PS) (Table 4, P<0.05). Same results were also observed in the off-pump group at B0 (off-pump CABG before surgery) and B1 (off-pump CABG after surgery) (Table 5, P<0.05). All of the increased markers CD41a, CD144, Phosphatidylserine (PS) were detected more frequently in the on-pump CABG group than in the off-pump CABG group (Table 6, P <0.05). These results suggested that the process of surgery could stimulate origin-related cells (endothelial cells, platelets, etc.), releasing more microvesicles. CABG was associated with a rapid increase in plasma microvesicles.

Classification of differentially expressed proteins

Using the Proteome Discoverer software, we performed screening through mass spectrometry and a search *via* Mascot, and then, we performed quantitative analysis of the proteins with Uniprot-Human. Finally, we obtained a total of 201 regulated proteins from the on- and off-pump groups based on the databases. iTRAQ ratios >1.20 or <0.83 (P-value <0.05), respectively, were used to define proteins that were significantly up-regulated or down-regulated. In the on-pump CABG, 101 proteins showing changes were detected, and among them, 49 proteins were found to be up-regulated and 52 proteins were found to be down-regulated. In the off-pump CABG, 75 proteins showing changes in total, 35 proteins were found to be up-regulated and 40 proteins were down-regulated.

Table 3: The circulating microvesicles in plasma from on-pump and Off-pump CABG before surgery.

	A0 (n=10)	B0 (n=10)	P value
PS	3284.56 ± 1501.43	2887.23 ± 670.91	0.421
CD144	1364.63 ± 444.69	1092.35 ± 136.68	0.067
CD41a	872.38 ± 226.84	843.45 ± 171.65	0.74
CD45	32.87 ± 13.50	26.94 ± 8.73	0.235

Results are mean ± SEM; Phosphatidylserine, PS; *P <0.05 A0 versus B0; A0: On-pump CABG before surgery; B0: Off-pump CABG before surgery; n: the number of sample.

Table 4: The circulating microvesicles in plasma from on-pump CABG group.

	A0 (n=10)	A1 (n=10)	P value
PS	3284.56 ± 1501.43	11439.56 ± 7073.92	0.001*
CD144	1364.63 ± 444.69	2268.77 ± 1430.90	0.018*
CD41a	872.38 ± 226.84	1312.83 ± 413.52	0.002*
CD45	32.87 ± 13.50	42.45 ± 12.50	0.15

Results are mean ± SEM; Phosphatidylserine, PS; *P <0.05 A1 versus A0; A: on-pump CABG group; A0: Pre-operation; A1: Post-operation; n: the number of sample.

Table 5: The circulating microvesicles in plasma from off-pump CABG group.

	B0 (n=10)	B1 (n=10)	P value
PS	2887.23 ± 670.91	4661.23 ± 1092.85	0.0001*
CD144	1092.35 ± 136.68	1439.14 ± 143.9	0.0045*
CD41a	843.45 ± 171.65	1163.57 ± 205.29	0.0132*
CD45	26.94 ± 8.73	38.10 ± 9.71	0.13

Results are mean ± SEM; Phosphatidylserine, PS; *P <0.05 B1 versus B0; B: off-pump CABG group; B0: Pre-operation; B1: Post-operation; n: the number of sample.

Table 6: The circulating microvesicles in plasma from On-pump and Off-pump CABG after surgery.

	A1 (n=10)	B1 (n=10)	P value
PS	11439.56 ± 7073.92	4661.23 ± 1092.85	0.0075*
CD144	2268.77 ± 1430.90	1439.14 ± 143.9	0.0360*
CD41a	2312.83 ± 413.52	1163.57 ± 205.29	0.0260*
CD45	42.45 ± 12.50	38.10 ± 9.71	0.32

Results are mean ± SEM; Phosphatidylserine, PS; *P <0.05 A1 versus B1; A1: On-pump CABG after surgery; B1: Off-pump CABG after surgery; n: the number of sample.

Functional classification of the differentially expressed proteins

The screened proteins were functionally catalogued by GO in two different groups. All of the results showed that the related proteins were associated with the process of change during CABG with/without the use of CPB. Most of the proteins were responsed to inflammation and coagulation. In the off-pump CABG group, the proteins were involved in biological process including coagulation, Fc receptor-mediated stimulatory signaling pathway, regulation of response to stimulus, complement activation, response to stimulus, vesicle-mediated transport, response to stress, complement activation, Fc receptor signaling pathway, inflammatory response, cell communication, and regulation of blood coagulation, and so on. The identified proteins were separated according to the cellular component; include the cell part, the extracellular region part, the cellular component, and the membrane-bound organelle. The molecular function of the proteins was classified as molecular function, protein binding, and enzyme regulator activity etc. showed the changes in the on-pump CABG group, and similar to the off-pump CABG group, the proteins were involved in biological process including response to stimulus, Fc-gamma receptor signaling pathway, complement activation, alternative pathway, vesicle-mediated transport, acute inflammatory response, vesicle-mediated transport, platelet activation, and cell communication. The identified proteins according to the cellular component included the membrane, the membrane-bound organelle, the membrane-bound vesicle, and the cellular component. Molecular function of the proteins was classified as molecular function, binding, phospholipid binding, active transmembrane transporter activity, substrate-specific transporter activity etc. Enriched pathways participated *via* the different expressed proteins.

To gain a deeper understanding of the proteins involved in the biological mechanisms of cMVs, a publicly available KEGG pathway database was used to provide biologists with excellent resources with respect to on- and off-pump. Approximately 50 KEGG pathways were

detected (data not shown). Among these proteins, VWF, SERPIND1, C1-8, F12, and F13 were found in the complement and coagulation cascade pathway; and VWF was found in the platelet activation pathway. The levels of these proteins were significantly increased after the surgery in the on-pump CABG group, but there was no difference in the off-pump CABG group.

Discussion

Our study demonstrated the simultaneous level changes of Brain Natriuretic Peptide (BNP), C-Reactive Protein (CRP), Myoglobin (Mb), and Circulation microvesicles in CABG patients. We showed that the changing of circulation microvesicles in postoperative patients was in line with the markers of inflammation, platelet activation and Myocardical dysfunction. Postoperatively all studied parameters, including microvesicles, BNP, Mb and CRP, increased statistically higher in both on-pump and off-pump. In contrast to on-pump surgery, off-pump approach less increased the levels of these markers. Simultaneously, the microvesicles was performed by flow cytometry, positively expressed Phosphatidylserine (PS), endothelial CD144 marker, platelet CD41a marker, and white blood cell CD45 marker.

Many factors are responsible for the inflammatory response, including surgical trauma, cardiopulmonary bypass, thrombin activation and Myocardical injury [18-23]. At present, there are no specific markers and no obvious difference to the inflammatory response in patients undergoing on-pump and off-pump. According to previous researches, CRP, IL-6 and IL-8 induced by the surgical trauma are associated with the level of inflammatory response in patients [1,11,23]. In addition, the complement system and fibrinolysis are also activated [24-25]. In our study, we demonstrated that the level of CRP significantly increased in postoperative patients, and was greater after on-pump group. Myocardical dysfunction could not avoid and directly affected the mortality of postoperative patients. Based on the previous studies, comparing to the on-pump, the incidence of Myocardical infarction was significantly less in off-pump patients [5,19,26]. In this study, BNP and Mb also have shown statistically increased in on-pump patients. Here, our result is consistent with the reported. Surgical trauma always causes a series of endothelial and soft tissue injury, which could lead to coagulopathy. However, it is difficult defined the extent of coagulation [27]. Microvesicles increasingly released under stress conditions, including surgery, inflammation and so on. They are considered as a potential means of intercellular communication, because they are capable of transferring various substances between cells and organs, such as proteins, mRNA, and miRNA. Earlier studies have suggested microvesicles in blood are derived from many kinds of cells, including platelets, erythrocytes, granulocytes, monocytes, lymphocytes, and endothelial cells. In physiology, platelet microvesicles are mostly detected [28-29]. In the process, the main change was the outward translocation of Phosphatidylserine (PS), which could be connected with Annexin-V [30]. This phenomenon also saw in this study, almost of microvesicles positively expressed PS. In inflammatory response, microvesicles was involved in many different mechanisms, for example, by stimulating the expression of pro-inflammatory genes in endothelial cells, leading to the production of cytokines and leukocyte-endothelial cell adhesion molecules in vitro, or by exposing complement components (C1q, C3, C4, and C5) [28]. Consequently, iTRAQ-based proteomic analysed further cMVs, we detected a total of 201 different expressed proteins in the two groups. These up-regulated proteins showed to participate in multiple biological processes (such as single-organism metabolic process, regulation of vesicle-mediated transport, and development process), and signaling pathways including cytokine-cytokine receptor interaction, the chemokine signaling pathway, ECM-receptor interaction, complement and coagulation cascades, and platelet activation. Based on the above data, we proposed the level of cMVs were associated with the injury degree in CABG. And circulating microvesicles participated and played a key role in the inflammatory response and coagulopathy in coronary artery bypass graft surgery.

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