Quantification of Endothelial Microparticles on Modified Cytometric Bead Assay and Prognosis in Chest Pain Patients

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**Background:** Endothelial microparticles (EMPs) are vehicles released from activated or apoptotic endothelium. The aim of this study was to establish a new cytometric bead assay for EMPs and investigate the prognostic value of EMPs in chest pain patients.

**Methods and Results:** We invented and verified the cytometric bead assay to quantify EMP level in vitro. A total of 80 healthy volunteers and 350 chest pain patients were recruited and the EMPs measured. The major adverse cardiovascular events (MACE) of documented coronary artery disease patients were recorded in the follow-up period. The level of EMPs statistically correlated with those of endothelin-1 (ET-1) and intercellular adhesion molecule-1 (ICAM-1) in vitro. The EMP level in healthy subjects was <300. The patients had a remarkably higher EMP level than healthy subjects. Diabetes mellitus, EMP, and ET-1 levels were significantly associated with future cardiovascular events in chest pain patients. There was a significantly higher event incidence in the top tertile EMP level than in the lower tertile in the acute coronary syndrome (ACS) patient group.

**Conclusions:** A novel EMP quantification assay has been successfully established. The EMPs in vitro and in patients were significantly correlated with ET-1 and ICAM-1 level. The patients with a higher EMP level had a higher risk of MACE. EMP level is a predictor for MACE in ACS patients. (*Circ J* 2014; 78: 206–214)

**Key Words:** Acute coronary syndrome; Chest pain; Endothelial microparticles; Prognosis; Risk factor

Endothelial dysfunction is an early event in the development of atherogenesis. Vascular endothelial cells play an essential role in regulating endothelial functions by maintaining normal vascular tone, modulating hemostasis, and preventing thrombogenesis. Many pathological triggers, such as dyslipidemia, hyperglycemia, pro-inflammatory cytokines, and turbulent blood flow, activate endothelial cells and induce endothelial dysfunction. By releasing several vasoactive substances, membrane microparticles (MPs), and chemotactic factors, the dysfunctional or denuded endothelium promotes local and systemic inflammatory response and atherogenic development.1–4

There are many invasive and non-invasive assays for evaluating endothelial dysfunction, such as acetylcholine stimulating test,5 high-resolution ultrasound assay,6 and serum soluble endothelial markers and nitric oxide synthase activity array.7–9 The disadvantages of these procedures (time-consumption, operational complexity, lack of consensus criteria, poor specificity, and potential harm to patients), however, limit their clinical application.

Endothelial microparticles (EMPs) shed from activated or apoptotic endothelial cells provide valuable information on the status of endothelial cells. There is growing evidence that EMPs are a circulatory marker of endothelial dysfunction.10,11 Their count and pathological activity reflect the degree of endothelial dysfunction.12,13 EMPs can function as important diffusible vectors of specific chemotactic molecules and cytokines, which promote pro-thrombogenesis and cellular interaction.14,15 EMP analysis is a new way to investigate pathology in various endothelium-related diseases, such as coronary artery disease (CAD), stroke, and Kawasaki disease.16–20 The quantitative relationship between EMP level and prognosis in chest pain patients, however, is still not clear.

To investigate this quantitative relationship, it is necessary to find a way to quantify MPs. Nevertheless, because of the small size and diversity, accurate and convenient quantification of MPs is a great challenge to clinical experts and pathologists. The main MP measuring methods currently in prac-
tice are flow cytometry and solid-phase capture assay. Flow cytometry enables the analysis of thousands of MPs in 1 sample. Meanwhile, it identifies subpopulations with multiple markers, but it is not sensitive enough for the extremely small MPs (<500 nm). Solid-phase capture assays, such as cytometric bead array (CBA), overcome this problem. In solid-phase assays, the MPs positive for solid binding markers are captured and analyzed regardless of size. The solid-based assays also reduce the challenges of analyzing a large number of samples.

Here, we modified cytometric bead assay technology to detect circulatory EMPs. In reference to earlier studies of endothelium-specific markers, we detected circulatory EMPs based on their parent cellular markers, anti-CD146 (as the capture antibody) and anti-CD31 (as the detecting antibody). After testing for the stability, repeatability, specificity, and sensitivity of this new assay method, we analyzed whether EMPs correlated with outcome of chest pain.

Methods

The study protocol was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

Materials and Subjects

The carboxyl-polystyrene microsphere (diameter, 4.5 μm; catalog no., 73-CF-45-100) was purchased from Spherotech, USA. The Polylink Protein Coupling Kit (catalog no., PL01LN) was from Bangs Laboratories, USA. The CD146 MAb (Mouse IgG1; clone, 128018) and its isotype control Goat F(ab')2 Anti-mouse IgG-Fluorescein isothiocyanate (FITC) (catalog no., P0103B) were from R&D Systems (Minneapolis, MN, USA). FITC anti-human CD31 (Mouse IgG1; clone, WM-59, catalog no. 11-0319) and its isotype control (FITC-Mouse IgG1x; catalog no. 11-4714) were from eBioscience. The APC anti-human CD41 antibody (catalog no., 303710) and its isotype control were from Biolegend. The PE-Cy5.5 anti-human CD14 antibody (catalog no., ab25390) and its isotype control were from abcam. The PE-labeled CD235 (catalog no., A07792) and its isotype control were from Beckman Coulter (Fullerton, CA, USA). The FITC-antiCD54 (Mouse IgG1x; clone, 1A29) and its isotype control were from BD Pharrmingen™. Tumor necrosis factor-α (TNF-α; recombinant human TNF-α) and the ELISA kits to detect endothelin-1 (ET-1; catalog no., BEB5) and intercellular adhesion molecule-1 (ICAM-1; catalog no., DY720) were from R&D Systems. Human umbilical vein endothelial cells (HUVEC) were from ATCC (CRL-1730™).

Coupling Antibody to Microsphere

According to the Polylink Protein Coupling Kit manual, the antibody binds to carboxyl-polystyrene microsphere through 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide. The microsphere, Polylink Coupling Buffer, and Polylink Wash/Storage Buffer were warmed to room temperature. Then, the 12.5-mg microsphere was pelleted via centrifugation for 15 min at 1,500 g, and repeated twice. The antibody coated microspheres were stored at 2–8°C in Polylink Wash/Storage Buffer. Flow cytometry was used to detect binding efficiency.

Efficiency and Stability of Coupling

Antibody-coated microsphere (10 μl) was pipetted and mixed with 10 μl antibody-coated microsphere, and incubated for 30 min at room temperature with gentle shaking. The mixture was washed twice via centrifugation for 15 min at 1,000 g before being resuspended in 50 μl PBS. Then, 20 μl FITC anti-human CD31 or isotype control was added and incubated for 30 min at room temperature with gentle shaking. Three-color flow cytometry was performed on a FACS Calibur flow cytometer equipped with CellQuestPro software (Becton Dickinson, San Jose, CA, USA). The instrument setting and fluorescence compensation were adjusted using Calibrite 3 fluorescence beads (Becton Dickinson). After that, 10,000 microspheres were collected. The FL1 MFI of the CD31-positive gate microsphere was analyzed using FlowJo (version 5.7.2). The FL1 MFI of the sample represented EMP level.

Cell Culture and Procedures

HUVEC were cultured using a modification of the procedures described by Zhou et al. These primary cells were grown in DMEM medium supplement with 10% fetal calf serum, 0.5 mmol/L l-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded in 75-cm² flasks and maintained at 37°C in a 5% CO2 atmosphere. The cells were split 1:4 every 3 days. Experiments were performed on cells at passage 4 to passage 8 at 80–90% confluence.

TNF-α was dissolved in Hanks’ buffered saline solution (HBSS) with 0.5% bovine serum albumin (BSA) and stored in small aliquots at −80°C. Freshly thawed aliquots were used for each experiment.

Endothelial cells were seeded at 1×10⁴ cells/well in 12-well plates. TNF-α (5 ng/ml, 10 ng/ml, or 20 ng/ml) or HBSS plus BSA were added and incubated for 4 h. CD54/ICAM-1 membrane expression was detected on flow cytometry. The supernatants were collected to detect the concentrations of ET-1 and ICAM-1 using the ELISA kits. EMP count was assayed using the present CBA. Scanning electronic microscopy was used to scan the surface of cultured endothelial cells.

Subjects, Follow-up, and Endpoints

All subjects (80 healthy volunteers and 350 patients) enrolled in the study signed an informed consent document approved by the ethics committee of the Experimental Research, School of Medicine, Shanghai Jiao Tong University. All of the chest pain patients underwent coronary angiography (CAG). The stenosis severity was judged by 2 independent coronary interventional experts. Patients with signs of chronic disease, hematological system disease, malignant tumor, acute inflammatory disease, or a history of cardiovascular events were excluded in the study. All the patients were followed for up to 1 year. The primary endpoint of the study was cardiovascular death, non-fatal myocardial infarction, unstable angina, ischemic stroke, or coronary revascularization to new lesions. All deaths were considered cardiovascular unless an unequivocal non-cardiovascular cause could be established. Hemorrhagic deaths were also considered
plasma was aliquotted (250-μl portions) and stored at −80°C until analysis. The extra plasma samples were collected for the detection of ET-1 and ICAM-1.

**Statistical Analysis**

Continuous data are expressed as mean±SD or median (interquartile range). Means between 2 categories were compared with 2-tailed, unpaired Student’s t-test. Frequencies were compared with chi-squared or Fisher’s test, as appropriate. Spearman rank correlation was used to test for the correlation of different markers of endothelial dysfunction. SigmaPlot (version 10.0) was used to design the figures. Kaplan-Meier survival curve was used to analyze endpoints in the quartile groups. Predictive value for cardiovascular events was assessed using Cox proportional hazards regression. The following variables were incorporated into the univariate model: age, sex, current smoking, hypertension, diabetes mellitus (DM), dyslipidemia, EMP, ET-1 and ICAM-1. Statistical analysis was performed using SPSS Statistics version 17.0.0 (SPSS, Chicago, IL, USA). P<0.05 was considered statistically significant. All experiments were performed at least 3 times.
EMPs Correlate With CV Events

Results

Efficiency and Stability of Coupling

The monolayer amount of protein bound to the microspheres was derived from the following equation:

\[ S = \frac{6}{\rho D} \times C, \]

where \( S \) is the amount of representative protein needed to achieve surface saturation (mg protein/g of microspheres), \( C \) is the capacity of microsphere surface for given protein, which for mouse IgG1 is 2.5 mg/m², \( \rho \) is the density of microspheres, which for polystyrene is 1.05 g/cm³, and \( D \) is the diameter of microspheres in microns (here it is 4.5 \( \mu \)m). In order to ensure the correct spatial orientation and decrease the likelihood of non-specific binding, we added proteins in a 3, 5, 8, and 10x excess of the calculated monolayer amount.

The amount of coupling antibody was raised from 40 \( \mu \)g to 320 \( \mu \)g, and the FL1 of antibody-coated microspheres is shown in Figure S1A. There was a rapid rise from 120 \( \mu \)g to 320 \( \mu \)g, followed by a plateau in FL1 MFI. Thus, we concluded that adding 320 \( \mu \)g antibody to 12.5-mg microsphere is the optimal situation to maintain high coupling efficiency. In the following 2 months, the FL1 of antibody-coated microsphere was evaluated and recorded every day. The results showed that the newborn antibody-coated microspheres maintained stability at 2–8°C in PolyLink Wash/Storage Buffer for at least 2 months (Figure S1B).

Confirming the Activation of HUVEC by TNF-\( \alpha \)

In order to confirm the activation of HUVEC after TNF-\( \alpha \) stimulation, we used flow cytometry to measure CD54 expression (Figure S1C). Cells treated with HBSS were defined as control. We found that TNF-\( \alpha \) increased CD54 expression in a dose-dependent manner (P<0.05; Table S1).

Figure 2. Specificity of the (A) newborn endothelial microparticle (EMP) assay and the (B–D) EMP/endothelin-1 (ET-1)/intercellular adhesion molecule-1 (ICAM-1) levels in enrolled subjects. (A) Expression of CD14, CD41, and CD235 on EMPs sorted on this newborn assay. (B) EMP FL1 level, examined with this novel assay. (C) EMP, ET-1, and ICAM-1 levels in healthy control, non-CAD, stable angina (SA), and acute coronary syndrome (ACS) patients. (D) Correlation between EMP and ET-1 (r=0.584, P<0.01), and EMP and ICAM-1 (r=0.308, P<0.01). (E) EMP vs. hs-CRP.
Correlation of EMPs With ET-1 or ICAM-1 In Vitro

TNF-α is a definite endothelial activator that upregulates the expressions of ET-1, ICAM-1, E-selectin, vascular cell adhesion molecule-1, and L-selectin. In the present study, we found that TNF-α also stimulated the production of EMPs as well (Figure 1A). This effect was in a concentration-dependent manner (Table S2).

The correlation between the levels of the new endothelial function marker (EMP) and the traditional ones (ET-1/ICAM-1) was then investigated. It was found that EMP level significantly correlated with the levels of ET-1 and ICAM-1 in HUVEC (Figure 1B). Thus, we concluded that the EMP-meas-

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### Table 1. Subject Characteristics

| Baseline clinical characteristics | Healthy controls (n=80) | Non-CAD (n=94) | SA (n=111) | ACS (n=145) |
|----------------------------------|------------------------|----------------|------------|-------------|
| Age (years)                      | 61±10                  | 63±12<sup>a</sup> | 62±11<sup>a,b</sup> | 61±14<sup>a,b,c</sup> |
| Male                             | 52 (65)                | 63 (67)<sup>a</sup> | 76 (68)<sup>a,b</sup> | 96 (66)<sup>a,b,c</sup> |
| SBP (mmHg)                       | 129±10                 | 131±14<sup>a</sup> | 133±15<sup>a,b</sup> | 131±16<sup>a,b,c</sup> |
| DBP (mmHg)                       | 73±5                   | 74±7<sup>a</sup>   | 75±13<sup>a,b</sup> | 74±16<sup>a,b,c</sup> |
| BMI (kg/m²)                      | 24±3                   | 24±4<sup>a</sup>   | 25±4<sup>a,b</sup>  | 24±5<sup>a,b,c</sup> |
| Creatinine (μmol/L)             | 81±15                  | 82±14<sup>a</sup>  | 85±17<sup>a,b</sup> | 87±27<sup>a,b,c</sup> |
| Smoking                          | 1 (1)                  | 27 (29)<sup>a</sup> | 29 (26)<sup>a,b</sup> | 46 (32)<sup>a,b,c</sup> |
| Hypertension                     | –                      | 27 (29)<sup>a</sup> | 35 (33)<sup>a,b</sup> | 52 (36)<sup>a,b,c</sup> |
| Dyslipidemia                     | –                      | 52 (55)<sup>a</sup> | 63 (57)<sup>a,b</sup> | 88 (60)<sup>a,b,c</sup> |
| Diabetes                         | –                      | 23 (24)<sup>a</sup> | 28 (25)<sup>a,b</sup> | 45 (31)<sup>a,b,c</sup> |
| Treatment at admission           |                        |                 |             |             |
| Aspirin                          | –                      | 94 (100)<sup>a</sup> | 111 (100)<sup>a,b</sup> | 145 (100)<sup>a,b,c</sup> |
| Thienopyridines                  | –                      | 94 (100)<sup>a</sup> | 111 (100)<sup>a,b</sup> | 145 (100)<sup>a,b,c</sup> |
| Statin                           | –                      | 94 (100)<sup>a</sup> | 111 (100)<sup>a,b</sup> | 145 (100)<sup>a,b,c</sup> |
| ACE inhibitor                    | –                      | 67 (71)<sup>a</sup> | 87 (78)<sup>a,b</sup> | 117 (81)<sup>a,b,c</sup> |
| β-blocker                        | –                      | 62 (66)<sup>a</sup> | 75 (68)<sup>a,b</sup> | 97 (67)<sup>a,b,c</sup> |
| CCB                              | –                      | 25 (19)<sup>a</sup> | 23 (21)<sup>a,b</sup> | 26 (18)<sup>a,b,c</sup> |

Data given as mean±SD or n (%). *P>0.05, compared with healthy controls; †P>0.05, compared with non-CAD; ‡P>0.05, compared with SA; §P>0.01, compared with healthy controls. ACE, angiotensin-converting enzyme; ACS, acute coronary syndrome (includes unstable angina, non-ST segment elevation myocardial infarction, and ST segment elevation myocardial infarction); BMI, body mass index; CAD, coronary artery disease (defined as coronary stenosis ≥50%, with chest pain, with or without ST segment elevation); CCB, calcium channel blocker; DBP, diastolic blood pressure; non-CAD, no coronary stenosis, or stenosis <50%; SA, stable angina; SBP, systolic blood pressure.

### Table 2. Serum EMP, ET-1, and ICAM-1 Levels

| Group       | n  | EMP (pg/ml) | ET-1 (pg/ml) | ICAM-1 (ng/ml) |
|-------------|----|-------------|--------------|----------------|
| Healthy     | 80 | 240.81±30.10| 14.95±3.76   | 86.39±21.29    |
| Non-CAD     | 94 | 489.14±40.79| 15.11±4.23   | 132.47±27.56*  |
| SA          | 111| 701.06±41.24 | 50.28±9.43*  | 150.71±31.41*  |
| ACS         | 145| 901.10±55.21 | 123.16±13.47*| 225.10±38.29* |

Data given as mean±SD. *P<0.05 compared with healthy control; †P<0.05 compared with non-CAD. EMP, endothelial microparticle; ET-1, endothelin-1; ICAM-1, intercellular adhesion molecule-1. Other abbreviations as in Table 1.

### Table 3. Endothelial Dysfunction Markers vs. Risk Factors

| Group       | n  | EMP (pg/ml) | ET-1 (pg/ml) | ICAM-1 (ng/ml) |
|-------------|----|-------------|--------------|----------------|
| Normotension| 236| 515.12±46.42| 16.23±7.36   | 134.21±28.19   |
| Hypertension| 114| 789.67±53.14| 89.78±9.57*  | 178.63±30.61*  |
| Normolipidemia| 147| 479.46±38.11| 19.83±7.34   | 125.11±24.84   |
| Dyslipidemia| 203| 801.84±69.35| 78.54±10.96  | 187.71±31.41*  |
| Non-DM      | 254| 499.06±38.19| 20.11±9.48   | 130.53±28.99   |
| DM          | 96 | 699.91±53.75| 118.68±12.31*| 206.82±35.67*  |
| Non-smoker  | 258| 467.34±48.64| 14.50±6.91   | 141.65±32.47   |
| Smoker      | 102| 675.26±40.89| 100.35±9.38* | 174.73±36.82*  |

Data given as mean±SD. *P<0.05 compared with normotension group; †P<0.05 compared with normolipidemia group; ‡P<0.05 compared with non-DM group; §P<0.05 compared with non-smoker. DM, diabetes mellitus. Other abbreviations as in Table 2. 
Figure 3. Endothelial microparticle (EMP) level and endpoint incidence. (A) Major adverse cardiovascular events (MACE) in the stable angina (SA) group and acute coronary syndrome (ACS) group. (B) Cumulative non-events survival curve analysis (Left) and chart form (Right) of the quartile subjects according to circulatory EMP level. (C) Cumulative non-events survival curve analysis (Left) and chart form (Right) of the ACS tertiles according to circulatory EMP level. There was a significantly higher event incidence in the top tertile compared to the lower tertile. No significant difference of event incidence was observed between the middle tertile and lower tertile group. (D) Cumulative non-events survival curve analysis (Left) and chart form (Right) of the SA tertiles according to circulatory EMP level. No significant difference of event incidence was observed among the tertile groups in SA patients.
suring assay based on CBA is stable and credible in assessing endothelial dysfunction in vitro.

Specificity of New Assay in Detecting EMPs
To confirm the specificity of this new assay, we investigated whether this new assay captures other MPs, such as platelet MP, red blood cell MP, and monocyte MP. We used FACS to detect the expression of platelet-specific protein CD41, red blood cell-specific protein CD235, and monocyte-specific protein CD14 in the sorted EMPs. As shown in Figure 2A, no protein CD14 in the sorted EMPs. As shown in Figure 2A, no

Baseline Subject Characteristics
A total of 80 healthy people and 350 chest pain patients were enrolled in the study. CAG was used to measure severity of coronary stenosis, and it was found that approximately three-fourths of the patients (73%) suffered from CAD, including 145 ACS patients. According to the angiographic results and symptoms, all chest pain patients were categorized as non-CAD, stable angina (SA), or ACS. Among these chest pain patients, there was a high prevalence of coronary risk factors, such as hypertension (33.5%), dyslipidemia (58.0%), DM (27.4%), and smoking (29.1%). The average patient age was 61.9±12.6 years. Detailed baseline characteristics are listed in Table 1.

EMPs, ET-1, and ICAM-1
The amount of circulatory EMPs was measured on newborn cytometric bead assay, and ELISA kits were used to analyze the levels of ET-1 and ICAM-1 (Figures 2B,C; Table 2). For EMPs, the level of EMPs in chest-pain patients was significantly higher than in the healthy control group. The CAD patients (SA and ACS) had a higher amount of EMPs than non-CAD patients (P<0.05). For ET-1, there was a statistically significant difference between the CAD (SA and ACS) patients and the healthy control/non-CAD group, but the difference between the healthy control and non-CAD group was not significant. For ICAM-1, the healthy control group had a significantly lower level than the chest pain groups (non-CAD, SA, and ACS). ACS patients had more ICAM-1 than the non-CAD group (P<0.05).

The correlation between the level of EMPs and the levels of ET-1 and ICAM-1 was analyzed. As shown in Figure 2D, there was a significant correlation between EMP level and that of ET-1 and ICAM-1. The correlation coefficients were 0.587 (P<0.01) and 0.308 (P<0.01), respectively.

Table 4. Cox Proportional Hazard Analysis for CV Events

| Predictor | HR (95% CI) | P-value |
|-----------|-------------|---------|
| Age       | 1.035 (1.008–1.093) | 0.01    |
| Sex (male) | 1.578 (0.825–2.643) | 0.17    |
| Current smoking | 0.899 (0.512–1.709) | 0.74    |
| Hypertension | 0.874 (0.461–1.385) | 0.47    |
| Diabetes mellitus | 1.986 (1.161–3.864) | 0.006   |
| BMI       | 0.957 (0.914–1.132) | 0.52    |
| dyslipidemia | 0.978 (0.962–1.013) | 0.19    |
| EMP       | 2.269 (1.603–4.842) | <0.001  |
| ET-1      | 1.212 (1.603–4.842) | 0.036   |
| ICAM-1    | 1.035 (0.947–1.168) | 0.13    |

Cl, confidence interval; CV, cardiovascular; HR, hazard ratio. Other abbreviations as in Tables 1, 2.

Cox Proportional Hazard Analysis for Cardiovascular Events
Twenty-nine cardiovascular events were recorded in documented chest pain patients during the follow-up period (Figure 3A). All these patients with major adverse cardiovascular events (MACE) were CAD patients. As shown in Table 4, univariate Cox proportional hazards analysis for cardiovascular events showed that age, DM, EMPs, and ET-1 were significantly associated with future cardiovascular events in chest pain patients (age: hazard ratio [HR], 1.035; 95% confidence interval [CI]: 1.008–1.093; P=0.01; DM: HR, 1.986; 95% CI: 1.161–3.864; P=0.006; EMP: HR, 2.269; 95% CI: 1.603–4.842; P<0.001; ET-1: HR, 1.212; 95% CI: 1.603–4.842; P=0.036). The proportional hazards assumptions were appropriate (P=0.64, Schoenfeld test).

EMP Level in Patients With Risk Factors
All patients were grouped into normotension or hypertension groups, normolipidemia or dyslipidemia groups, non-DM or DM groups, and non-smoker or smoker groups. The results, shown in Table 3, indicated that the plasma levels of EMP, ET-1, and ICAM-1 in patients with risk factors were significantly higher than in the patients without, indicating that the aforementioned risk factors may be attributed to the production of EMPs.

Discussion
In this study, we modified traditional assay technology to count circulatory CD146+/CD31+ EMPs. With this new technique, we investigated the quantitative relationship between EMP level and prognosis in chest pain patients. For the first time, we have shown that EMP level is associated with cardiovascular events in chest pain patients, acting as an independent predictor for MACE in ACS patients.

MPs are membrane vesicles shed from eukaryotic cells that express the surface antigens and functional markers of their parent cells. They have been shown to have a variety of activities, such as facilitating intercellular interactions, inducing EMP level with morpho-functional activities.
EMP Correlate With CV Events

Circulation Journal Vol.78, January 2014

To overcome this limitation of flow cytometry, Nomura invented an ELISA method to measure circulatory platelet MPs. The method is convenient and easy to carry out the clinical laboratory. Unfortunately, the program to detect other category MPs was not continued. Later on, Hugel et al designed a solid-phase capture assay for the determination of procoagulant activity of cellular MPs. They insolubilized biotinylated antibody onto streptavidin-coated microtitration plates, and the prothrombinase assay determined the amount of captured MPs according to phosphatidylserine presence on the surface of MPs. But because only 50% of all MPs express phosphatidylserine, this method is far from being sensitive.

How can we detect MPs with convenience, high throughput and accuracy? The cytometric bead assay provides a potential solution. Cytometric bead assay was developed to measure soluble analytes with a particle-based immunoassay. The advantages of cytometric bead assay are the broad dynamic range of fluorescence detection and the efficient capturing of analytes, which enables use of fewer sample dilutions to determine analyte concentration in substantially less time (compared to conventional ELISA). Now, many cytometric bead assays have been devised for detecting soluble trace protein, genetic polymorphism, and microRNA screening, but no cytometric bead assay for EMPs has yet been invented.

In the present study, we developed a cytometric bead assay to detect EMPs. We used the microsphere as the reaction carrier, which can provide a larger reaction surface than the conventional solid-phase-based method. In addition, the covariant binding between the capture antibody and microsphere ensures the stability and sensitivity of the antibody-coupled microsphere. The endothelial-specific membrane protein, CD146 (also known as melanoma cell adhesion molecule), was used as the capture target. In addition, the anti-CD31 antibody was used to detect antibody in this novel assay. Theoretically, this technique avoids the interference of other circulatory MPs, such as platelet MP, monocyte MP, and red blood cell MPs. To confirm this specificity, we examined the expression of CD41 (platelet-specific protein), CD235 (red blood cell-specific protein), and CD14 (monocyte-specific protein) in EMPs sorted by this new system. No expression of these proteins was observed, clearly demonstrating the specificity of this EMP assay. We tested the stability and repeatability of this new assay in a TNF-α-stimulating in vitro model. TNF-α is an endothelium activator. We found that after TNF-α stimulation, the HUVEC expressed a higher level of CD54/ICAM-1, shed more MPs, and released more ET-1 and ICAM-1. This indicates that in this in vitro model, the HUVEC was activated and the endothelial function was damaged. After 2 months monitoring of FL1 MFI in the in vitro model, the coefficient of variation of FL1 MFI was <2%. This satisfactorily confirmed the stability and repeatability.

Next, we investigated whether this new EMP assay was sensitive and specific compared with traditional endothelial function biomarkers. Although there is a lack of evidence at present that those traditional circulatory markers, such as ICAM-1, and ET-1, can specifically predict endothelial dysfunction in vivo, they have been widely used in detecting endothelial function in vitro. In the present study, a significant correlation between EMP and ET-1/ICAM-1 was observed in vitro. In the in vivo study, there was a significant, but obviously weaker, correlation. Why was there a difference of correlation coefficient between the in vitro and in vivo testing? EMPs are MPs that are produced only by endothelium, regardless of being in vitro or in vivo. But endothelium is not the only source of ICAM-1 and ET-1. In vivo, other cells (such as leukocytes, platelets, and activated lymphocytes) also produce ICAM-1 and ET-1. This could explain the difference of correlation coefficient, and why ET-1/ICAM-1 is a good biomarker of endothelial function in vitro, but not in vivo. Because of the significant correlation, we showed that CD146/CD31 EMPs are sufficiently sensitive and specific to represent endothelial function.

DM, smoking, hypertension and dyslipidemia are risk factors of CAD. They all have deleterious effects on endothelial function. Considering the role of EMPs in reflecting endothelial function, we explored the association between EMP level and those risk factors. It was found that the EMP level in patients with risk factors was dramatically higher than in the patients without, which further demonstrated the biomarker role of CD146/CD31 EMPs in endothelial dysfunction.

Given that endothelial dysfunction is the early event in the development of atherogenesis, researchers have explored the association of EMPs with cardiovascular disease. Nozaki et al found that Cd146-EMP can independently predict future cardiovascular events in patients at high risk for CAD. Sinning et al showed that circulating CD31+/annexin V+ EMPs correlate with cardiovascular outcome among SA patients. Whether EMPs are also a predictive factor for MACE in chest pain patients, however, especially in ACS patients, is still not clear. Thus, we investigated the quantitative relationship between the level of Cd146+/CD31+ EMPs and prognosis in chest pain patients. In the present study, we analyzed the levels of serum EMPs, ET-1 and ICAM-1 in 80 healthy volunteers and in 350 chest pain patients. It was found that CAD patients had higher serum levels of EMPs and ET-1 than healthy controls and non-CAD subjects. After 1 year’s follow-up in chest pain patients, we analyzed the primary endpoints. During that time, a total of 29 events were observed. The survival curve showed a significant difference in the incidence of the endpoint event between the lowest quartile patients and the top quartile patients. Cox proportional hazard model was then used. The Cox regression results showed that EMP level is associated with prognosis in chest patients. We then further analyzed subgroups to demonstrate whether EMP level is an independent predictor for all chest pain patients. We found that ACS patients comprised 41% of the total chest pain patients. A total of 79% of the cardiovascular events occurred in the ACS group (21% in the SA group and 0% in non-CAD chest pain patients). In addition, no significant difference of event incidence was observed among the tertile groups according to circulatory EMP level in the SA group (Figure 3D). In contrast, there was a significantly higher event incidence in the top tertile than in the lower tertile in ACS patients (Figure 3C).

Therefore, according to the Cox proportional hazard analysis for cardiovascular events (Table 4) and the cumulative non-events survival curve analysis according to circulatory EMP level in total chest pain patients, we concluded that EMP level is a prognostic marker in ACS patients, but not in non-CAD patients or SA patients. EMPs may be a useful
tool for risk stratification in ACS patients.

Conclusions
We have introduced a modified cytometric bead assay to measure EMPs. With this newborn EMP-quantifying CBA assay, we have proved that EMPs correlate with outcome in chest pain patients. Quantification of EMPs may be useful for further risk stratification in ACS patients.

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Supplementary Files
Supplementary File 1
Figure S1. Examining the efficiency and stability of coupling to (A,B) microspheres and confirming the activation of (C) HUVEC.
Table S1. Expression of CD54 on HUVEC membrane
Table S2. EMP, ET-1, and ICAM-1 levels in HUVEC supernatants
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