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Endothelial microparticles exert differential effects on functions of Th1 in patients with acute coronary syndrome☆

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ABSTRACT

Background: Endothelial microparticles (EMPs) can be involved in inflammatory process, blood coagulation, and regulation of vascular function. However, it remains unclear whether EMPs participate in the pathogenesis of ACS. The purpose of this study is to investigate the impact of EMPs on Th1/Th2 development and functions *in vitro*.

Methods: Eight-five patients were allocated into SAP group (n = 27), UAP group (n = 28), and AMI group (n = 30). Twenty hospitalized patients with normal coronary angiography were recruited as controls. The frequency of EMPs, IFN-γ, and IL-4 levels were measured, and the correlation between EMPs and Th1/Th2 cytokine was analyzed. PBMCs isolated from patients with ACS were treated *in vitro* with EMPs. This was followed by flow cytometry for Th1/Th2 counts, real-time PCR and western blotting for T-bet and GATA mRNA and protein expression, and ELISA for IFN-γ, TNF-α, IL-4, and IL-10.

Results: This study proved that the frequency of EMPs was significantly increased in ACS patients. There was a significant positive correlation between EMPs and IFN-γ. EMPs could significantly upregulate the differentiation and function of Th1 through increasing the expression of T-bet mRNA and protein. Furthermore, this study also indicated that EMP treatment *in vitro* could promote the expression of TNF-α, which exerts adverse effects on the pathogenesis and progression of atherosclerosis.

Conclusions: EMPs may be involved in the immune and inflammatory processes that take part in artery atherosclerosis and that they do so by regulating Th1/Th2 differentiation and function. They may play an important role in the pathogenesis of coronary atherosclerosis and plaque instability.

1. Introduction

Coronary atherosclerosis is a chronic immune disease mediated by CD4+ T lymphocytes and other immune cells [1]. A T-cell infiltrate is always present in atherosclerotic lesions and consists predominantly of CD4+ cells, which recognize protein antigens presented to them as fragments by MHC II molecules [2]. After appropriate activation, helper T cells (Th) differentiate into pro-inflammatory Th1 secreting interferon (IFN)-γ, interleukin (IL)-2 and TNF-α or anti-inflammatory Th2 cells secreting IL-4 and IL-10. Th1 cytokines, such as IFN-γ and IL-2, are present in the human plaque and contribute to inflammatory effects by activating macrophages, endothelial and smooth muscle cells. Therefore, Th1 cells play a major role in the pathogenesis of atherosclerosis. In contrast, IL-4, the prototypical anti-inflammatory cytokine of the Th2, is not frequently observed in human plaques. Experimental evidences involved in Th2 are contradictory, with some showing proatherosclerotic effects and others showing protective effects or no significant effect [3]. The current studies indicate that the outcome of the atherosclerotic process is partly determined by the balance between Th1 and Th2 driven responses. Furthermore, Th1/Th2 imbalance is not only involved in the occurrence and development of coronary atherosclerosis but also closely related to the occurrence of plaque instability and acute coronary syndrome (ACS) [4].

Recent studies have confirmed that the endothelial microparticles (EMPs), the tiny vesicles released from endothelial cell after activation or apoptosis, can indicate the status of endothelial function and dysfuncton in atherosclerosis and ACS [5]. Mallat et al. showed that EMPs are significantly elevated in patients with ACS compared with other coronary or non-coronary patients [6]. Bernal-Mizrachi et al. analyzed the relationships between EMP levels and the degree of morphological and found that EMPs are correlated positively with the extent and severity of coronary stenosis at angiography [7,8]. More importantly, because EMPs are effector substances, they can be involved in inflammatory process, blood coagulation, and regulation of vascular function [9]. EMPs may at least partly be responsible

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for the prothrombotic state associated with CAD. Heloire et al. proved that circulating EMPs could bind platelets, form aggregates, and be involved in thrombus formation in patients with acute myocardial infarction [10]. EMPs impaired ACh-induced vasorelaxation and nitric oxide production by aortic rings obtained from Sprague–Dawley rats in a concentration-dependent manner [11]. In addition, Angelot et al. [26] confirmed that EMPs specifically induce plasmacytoid dendritic cell maturation and production of inflammatory cytokines, which provided the evidence for the involvement of EMPs in immune modulations.

Collectively these data showed that EMPs may participate in the occurrence or development of coronary atherosclerosis or in the development of plaque instability by modulating immune responses. However, it remains unclear whether EMPs exert differential effects on functions of Th1 and Th2 in patients with acute coronary syndrome. This study was conducted to explore the relationship between the plasma EMPs and Th1, Th2 cytokine levels in patients with coronary heart disease and to investigate the role of EMPs in the regulation of peripheral blood Th1 and Th2 cells from patients with ACS. In this way, we attempted to determine the role of EMPs in the pathogenesis of ACS.

2. Methods

2.1. Reagents and instruments

Human umbilical vein endothelial cell line ECV-304 was obtained from ATCC, U.S.; improved RPMI1640 medium was obtained from HyClone Laboratories (U.S.); fetal bovine serum was obtained from Hangzhou Evergreen Biological Engineering Materials Co., Ltd., Hangzhou, China; human lymphocyte separation medium was obtained from Beijing Solai Bao Technology Co., Ltd., Beijing, China; TNF-α was obtained from PeProtech, U.K.; fluorescein isocyanate (FITC)-labeled CD42 monoclonal antibody (mAb) and phycoerythrin (PE)-labeled CD31 mAb were obtained from Proteintech Group, U.S.; standard microparticles of 0.8 μm or 3.0 μm in diameter were from Duke Co., U.S. The phycoerythrin green pigment dye 5 (PE-Cy5)-labeled mouse anti-human CD4 mAb, PE-labeled mouse anti-human IFN-γ mAb, and FITC-labeled mouse antihuman IL-4 mAb were obtained from Biolegend, U.S. TRNzol-A$^+$ total RNA extraction reagent was obtained from Beijing Tiangen Co., Ltd., Beijing, China. PCR primers were synthesized by Shanghai Biological Engineering Co., Ltd., Shanghai, China. A First-Strand cDNA Synthesis Kit and All-in-One™ qPCR Mix were obtained from GeneCopoeia, U.S. T-box expressed in T cells (T-bet), GATA binding protein 3 (GATA-3), and β-actin polyclonal antibody were obtained from Proteintech Group Company, U.S. An ECL chemiluminescence kit, horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG, and a DAB staining kit were obtained from DAKO, Denmark. Human IFN-γ, TNF-α, IL-4, and IL-10 enzyme-linked immunosorbent adsorption (ELISA) quantitation kits were obtained from (R&D, U.S.). An Epics XL flow cytometer was obtained from Beckman Coulter Inc., U.S. An ABI Prism 7500 fluorescence quantitative thermal cycler was obtained from ABI, U.S. A vertical electrophoresis unit, electrophoresis tank, protein membrane transfer device, and GelDoc2000 gel imaging analysis system were obtained from Bio-Rad, U.S. The medical X-ray film was obtained from Kodak, U.S.

2.2. Patients and control subjects

Twenty-seven patients with stable angina pectoris (SAP), twenty-eight patients with unstable angina pectoris (UAP), and thirty patients with acute myocardial infarction (AMI) were selected for this study. Twenty hospitalized patients with normal coronary angiography were recruited as the control group. All patients were admitted to the Second People's Hospital of Qinzhou City from January to December, 2011. A diagnosis of coronary heart disease was made according to WHO diagnostic criteria [12,13]: 1. Stable angina: the stress-caused sternum or precordial squeezing or tightening pain or discomfort, which could be alleviated after rest or sublingual nitroglycerin tablets within 5 min. 2. Unstable angina: New-onset angina, stress-deteriorated angina, and angina occurring at rest, at least one episode within 48 h before admission. 3. Acute myocardial infarction: No more than 24 h prior to enrollment, symptoms of myocardial ischemia persisting for more than 30 min, no relief after rest or taking nitrates, and serum markers of myocardial injury (CK-MB and troponin) exceeding 2-fold of the upper limit of normal. 4. Control group: chest pain but no ischemic electrocardiographic changes, with normal coronary angiography. Patients meeting the following criteria were excluded: infection, malignancy, severe liver and kidney insufficiency, connective tissue disease, endocrine diseases, autoimmune diseases, other heart diseases, such as myocarditis, endocarditis, rheumatic heart disease, or treatment with anti-inflammatory or immunosuppressant drugs in the past 3 weeks.

All experiments were performed in strict compliance with the 2008 Helsinki Declaration amendment on ethics requirements and in accordance with the Human Trial Guidelines issued by the Medical Ethics Committee of the Second People's Hospital of Qinzhou City [14]. The Medical Ethics Committee of the Second People's Hospital of Qinzhou City approved this study. The study was performed under the supervision of the ethics committee. Patients provided written informed consent before joining the study.

2.3. Assessment of plasma EMPs

Fresh peripheral vein blood samples (3 ml) were obtained using vacuum anticoagulant test tubes from patients with AMI or UAP immediately after admission. The fasting fresh peripheral venous blood samples were obtained from patients with SAP and from control group patients the morning after admission. Samples were centrifuged at 160 *g* for 10 min to produce platelet-rich plasma and then centrifuged at 1500 *g* for 6 min for platelet-poor plasma. Fifty microliters of platelet-poor plasma was mixed with 0.5 μg of fluorescent-labeled specific antibodies (anti-CD42-FITC and anti-CD31-PE, BD Biosciences, U.S.) or mixed with equal amounts of isotype-control antibodies (MICE IgG1/PE and MICE IgG1/FITC, BD Biosciences, U.S.) After 20 min of incubation at room temperature, 1 ml PBS was added and the mixture was subjected to flow cytometry (Beckman Coulter, U.S.), which was completed within 4 h of blood collection. EMPs were defined as CD31+/CD42− microparticles with diameters under 1.0 μm.

2.4. Isolation and in vitro culture of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from patients with ACS by Ficoll-Hypaque density gradient centrifugation. At 37 °C, 5% CO_2, the isolated PBMCs (2×10^6/ml) were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were stimulated using EMPs for 48 h at final concentrations of 0, 10^3, 10^4, 10^5, 10^6/ml.

2.5. In vitro culture of human umbilical vein endothelial cells (HUVECs)

Cells from human umbilical vein endothelial cell line ECV-304 were thawed and cultured at 37 °C, 5% CO_2, in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. When the cells reached sub-confluence, they were treated with 0.25% trypsin digestion and then passaged.

2.6. In vitro preparation and identification of EMPs

As described by Brown et al. [15], EMPs were prepared by culturing HUVECs (after three passages) for 48 h with TNF-α at a final concentration of 100 ng/ml. The supernatants were centrifuged at 4300 *g* for 5 min to remove cells and cell debris and then at 20,000 *g* for 2 h at 10 °C. The supernatant was discarded and the precipitate was washed 2 times with PBS to remove TNF-α residues. The EMPs were resuspended in PBS. Fifty microliters of EMPs were mixed with 10 μl of FITC-labeled anti-CD42 mAb and PE-labeled anti-CD31 mAb. The same amount of control antibody was added to another 50 μl of EMPs. After incubation at room temperature for 15 min, the labeled EMPs were washed 2 times and resuspended in 100 μl PBS for flow cytometry. The gating was set up using standard particles with diameters of 0.8 μm, and EMPs were quantified using the quantitative standard particles with diameters of 3 μm. Before flow cytometry analysis, EMP samples were mixed with 10^5 standard microparticles with diameters of 0.8 μm or with the same number of standard microparticles with diameters of 3 μm. Twenty thousand standard particles with diameters of 3 μm were counted and used to calculate the concentrations of EMPs. EMPs were defined as CD31+/CD42− microparticles with diameters under 1.0 μm.