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Endothelial microparticles induce vascular endothelial cell injury in children with Kawasaki disease

J. TIAN[1], H.-T. LV[2], X.-J. AN[1], N. LING[1], F. XU[1]

[1]Department of Cardiology, Xuzhou Children's Hospital, Xuzhou, Jiangsu, P.R. China
[2]Department of Cardiology, Suzhou Children's Hospital, Suzhou, Jiangsu, P.R. China

Jing Tian is a Doctoral Candidate of Suzhou University

Abstract. – OBJECTIVE: To explore the role of microparticles produced by endothelial cells in the injury of vascular endothelial cells.

MATERIALS AND METHODS: We stimulated human umbilical vein endothelial cells (HUVEC) with TNF-α *in vitro*, analyzed the change of cellular morphology, and measured EMP level in the supernatant with a flow cytometer. Then, we evaluated the corresponding clinical indicators and the role of EMP in endothelial injury.

RESULTS: The endothelial cellular morphology underwent significant changes, and a large number of microparticles were secreted. In turn, these microparticles blocked cell cycle and induced apoptosis.

CONCLUSIONS: The microparticles produced by endothelial cells play an important role in the injury of vascular endothelial cells.

Key Words:
Kawasaki disease, Endothelial microparticle, Vascular endothelial cells, Cell cycle, Apoptosis.

Introduction

Kawasaki disease (KD) is one of the most common causes for acquired heart diseases in children. The pathogenesis of KD is due to systemic vasculitis, which predominantly involves the vessels of micro and medium diameter. Coronary artery disease is the most serious complication as it can induce a coronary aneurysm, leading to coronary artery stenosis, thrombosis and myocardial infarction. KD may be attributed to the injury of vascular endothelial cells due to large numbers of cytokines (e.g. TNF-α) and inflammatory mediators, which are the products of immune reactions to infection[1].

Endothelial microparticles (EMP) are secreted in the form of secretory vesicles from cell membrane when endothelial cells (EC) are activated or undergoing apoptosis. The diameter of EMP is approximately 0.05 to 1 μm. EMP comprises of membrane proteins and cytoplasm components of ECs, and it has procoagulant characteristics and proinflammatory roles and, thus, can change vascular function[2].

EMPs form when the transmembrane balance between the Ca^{2+}-dependent scramblase, lipid flippase and translocase on cell membrane is disrupted, with the resultant loss of cell membrane asymmetry, leading to the breakdown of the cytoskeletal fibers. Because of these changes in cellular morphology, EMPs form, detach and are released into the blood. The mechanism of EMP release is not clear. *In vitro* studies demonstrated that TNF-α, thrombin, C-reactive protein, bacterial lipopolysaccharide, uremic toxins, oxidative stress and low-density lipoproteins could induce the release of EMPs by EC in vitro[3-8].

It was reported that the amount and protein antigens of the EMPs produced in apoptosis pathway and activation pathway were different. In the case of EC apoptosis, the expression of intrinsic antigen (CD31>CD105) in EMPs was increased, while the expression of these components in EC was decreased. In the case of EC activation, expression of the induction markers of EMP (CD62E>CD54>CD106) was increased. The phenotype of EMP was useful in determining endothelial injury. The ratio of CD62E+ EMP to CD31+ EMP could be used as the discrimination criteria of activation and apoptosis. The ratio >10 indicated cellular activation, while the ratio <1.0 indicated apoptosis[9].

The role of EMP in vasculitis is gaining more attention[10]. Endothelial injury plays important role in vasculitis, as EMP can be involved in vasculitis through the injury and repair of EC[11]. Therefore, EMP could provide new target for the

Corresponding Author: Haitao Lv, MD; e-mail: tal0979@163.com

investigation and diagnosis of vasculitis. The relationship between KD and EMP level is not well understood. Clarke et al[12] reported the relationship between vasculitis and EMP in a small number of children (7 KD patients). This work showed increased serum level of CD144+ EMPs in vasculitis children. However, the importance of the relationship between CD31+EMP, CD62E+EMP in KD is not known.

In the present study, human umbilical vein endothelial cells (HUVEC) have been stimulated with TNF-α *in vitro*, and changes in cellular morphology, EMP level in the supernatant, and the corresponding clinical indicators have been measured, and the roles of EMP in endothelial injury have been explored.

Materials and Methods

Materials

Cell cultures of HUVECs are maintained in our laboratory. TNF-α was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-CD31 IgG, mouse anti-CD62 IgG, rabbit anti-mouse IgG-HRP, 4', 6-diamidino-2-phenylindole (DAPI), tetramethylbenzidine (TMB), propidium iodide (PI) and Annexin V-FITC were purchased from Zhongshan Jinqiao Biotechnology Co., Ltd, Beijing, China. Dulbecco's minimum essential medium (DMEM), fetal calf serum (FCS) and other common reagents were purchased from Invitrogen (Shanghai, China).

Methods

HUVECs were maintained in DMEM + 10% FCS in a 6-well plate at a cell density of 10^6 cells/well. Monolayer of cells (80% confluence) formed after overnight incubation at 37°C. A serial concentration of TNF-α (5, 10, 50 and 100 ng/mL) was added in the treatment well. The cells were then incubated at 37°C overnight. The change in cellular morphology was observed with an inverted optical microscope. Alternatively, the cellular morphology was observed with a confocal fluorescence microscope after staining with 100 ng/mL DAPI (4' 6-diamidino-2-phenylindole).

The supernatant of cultured cells after the stimulation by TNF-α was analyzed by ELISA to detect EMPs. Mouse anti-CD31 IgG and mouse anti-CD62 IgG were used as primary antibody; rabbit anti-mouse IgG-HRP was used as secondary antibody. TMB was added as the substrate of HRP for color development. The results were measured at 450 nm.

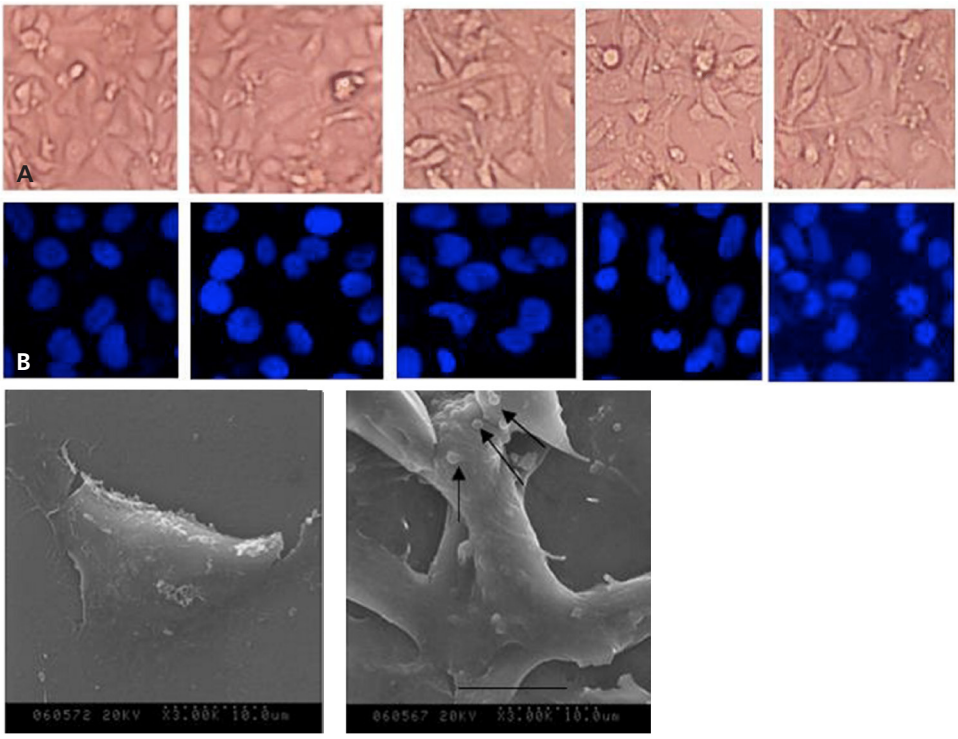
EMPs on the surface of HUVECs were observed with scanning electron microscopy (SEM). HUVECs were washed twice with phosphate buffered saline (PBS), incubated with 2.5% glutaraldehyde for 2 h to fix the cells. After washing with 0.1 M sulfate buffer (3 x 15 min), cells were incubated with the after-fixative 1 % osmic acid for 2 h. Following dehydration of the cells with gradient concentration of alcohol (50%, 70%, 80%, 90% and 95%), each for 15 min, changed with isoamyl acetate for dehydration for 15 min. After lyophilization, the cells were electroplated with gold film in a vacuum evaporator and mounted the on the stage for observation.

After stimulation with various concentration of TNF-α, the HUVECs were digested with trypsin and single cell suspension was prepared. Then Annexin V-FITC 2 μL and propidium iodide (PI) 5 μL were added, and incubated at room temperature (RT) for 20 min, and apoptosis was detected with flow cytometer. The results were analyzed with FlowJo software.

The detection of EMP with flow cytometer was as previously described[13]. Briefly, stimulated HUVECs with various concentration of TNF-α were collected by centrifugation, followed by washes with pre-cooled PBS, twice. Pre-cooled 70% alcohol was added for fixation at 4°C overnight. The cells were washed with PBS once, and PI 500 μg/mL, RNase A 100 μg/mL and 0.2% Triton X-100 were added. Then cells were incubated at 4°C in dark for 30 min. The cell cycle stages were detected with flow cytometer, and the results were analyzed with FlowJo software.

Results

We stimulated HUVECs with various concentration of TNF-α *in vitro*. As observed by inverted optical microscope and confocal fluorescence microscope, as the concentration of TNF-α increased, the cells gradually extended and cell gap increased. Some cells formed pseudopodia, with bean-shaped cell nucleus, and became long-columnar or irregular in shape (Figure 1A). SEM revealed secretion of small vesicles of a diameter of 1.0 μm from the membrane surface (Figure 1B). The levels of CD31+ EMPs and CD62E+ EMPs were significantly higher in the supernatant of HUVEC culture. This demonstrated increased release of EMPs from EC surface after the stimulation by TNF-α (Figure 1C).



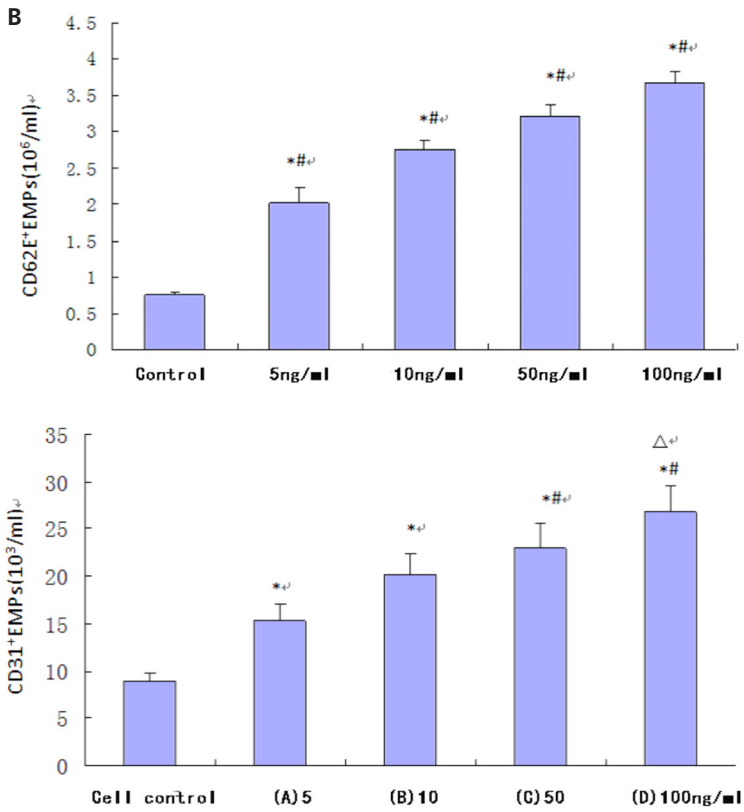


Figure 1. ***A,*** The morphological changes of ECs and cell nucleus after the stimulation by various concentration of TNF-α for 24 h. From left to right: normal control, 5, 10, 50, 100 ng/ml TNF-α. ***B,*** The secreted EMPs on EC surface (SEM). The left panel is normal control cells, the right panel is the cells stimulated by TNF-α, arrow indicates the secreted EMPs on cell membrane. ***C,*** Various concentrations of TNF-α promoted the release of CD31+ EMPs and CD62E+ EMPs. Group A, B, C and D were stimulated by 5, 10, 50 and 100 ng/mL TNF-α, respectively. *p<0.01 In comparison to control group,; #p<0.01 Inter-group comparison of Group A, B, C and D; Δ p<0.05 The difference between Group D and Group B. A: Normal control group; B: 104 EMPs/ml; C: 105 EMPs/ml; D, 106 EMPs/ml; E: 107 EMPs/ml.

Discussion

We measured the serum levels of CD62E+EMPs and CD31+EMPs in KD children. The level of EMPs was significantly increased in the acute phase and sub-acute phase of KD in children, and decreased in the recovery phase, but was still higher than normal

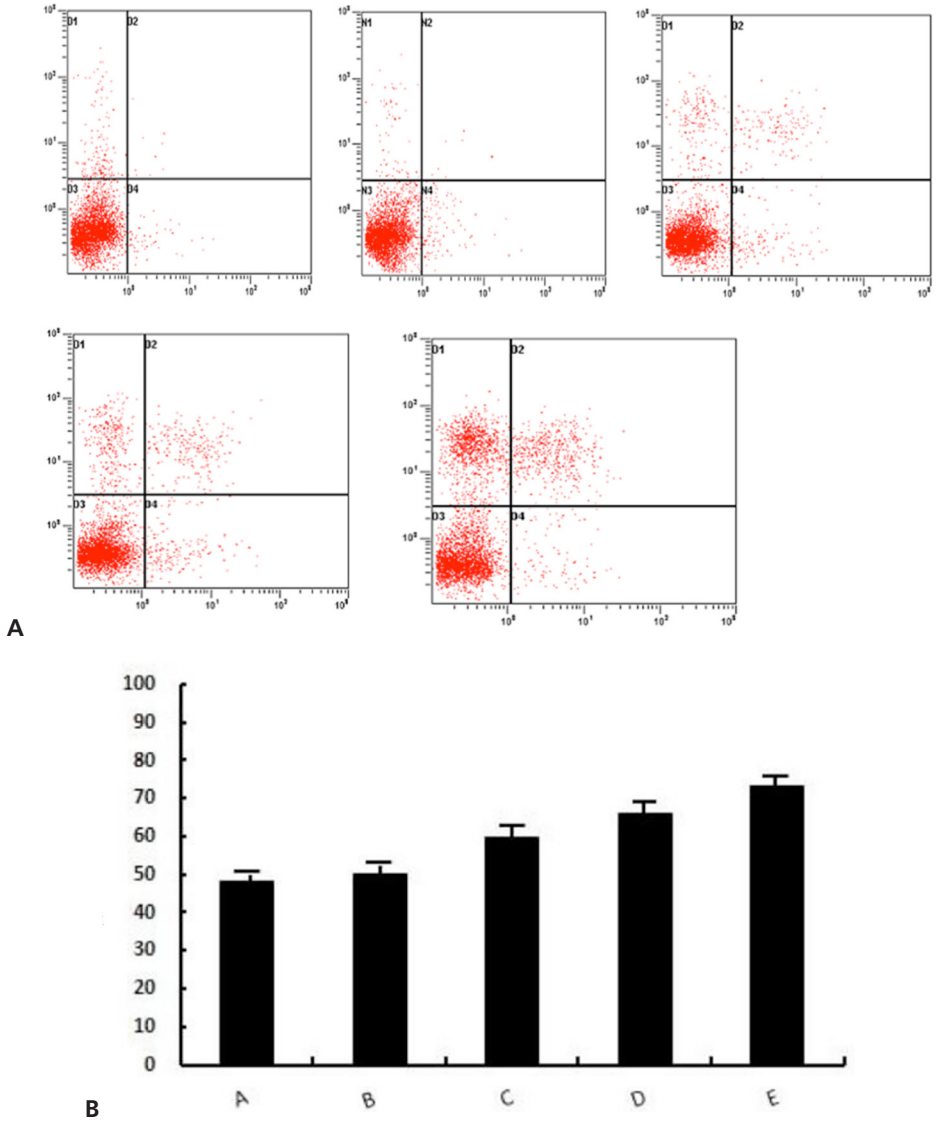


Figure 2. ***A,*** Various concentrations of CD62E+ EMPs induced apoptosis in HUVECs. ***B,*** Various concentrations of CD62E+ EMPs arrested the cell cycle in G0/G1 phase. One-way ANOVA was used to analyze the ratio of G0/G1 in these 5 groups, $p<0.001$; q-test showed significant differences between these groups, except for the difference between Group B and normal control group ($p<0.05$).

level. This indicated that high level of EMPs might be related to the persistence of endothelial dysfunction. Current researches focused on the significant increase of EMPs in KD children and suggested this may be related to endothelial dysfunction. The mechanism by which EMPs could affect the EC function in the peripheral blood of KD children requires further investigation. Some reports found high level of microRNA in the microparticles in the patients with hematologic diseases and non-hematologic solid tumors[14]. Other investigations also found high level of specific microRNA in the EMPs of the patients with cardiovascular diseases, such as Coronary Heart Disease, in which the core function of EMPs was to deliver microRNA in the blood circulation[15]. This study also found high level of miR-199b-5p in the EMPs in KD children. As EMPs, the precursor of miR-199b-5p could damage vascular endothelial cells (data not shown). Therefore, the microRNA in EMPs may play important roles in the vascular injury by EMPs. Other studies suggested Kawasaki disease in children as the primary systemic non-granulomatous vasculitides of medium-sized vessels[16].

Conclusions

In vitro stimulation of HUVECs with TNF-α leads to changes in morphology of cells, with a large number of EMPs – predominantly CD31+ EMPs and CD62E+ EMPs, being secreted by the cells. The addition of CD62E+ EMPs in HUVECs could directly induce apoptosis and arrested cell cycle in G0/G1 phase. As the concentration of CD62E+ EMPs increased, the apoptosis rate gradually increased. In conclusion, EMPs play important role in the endothelial injury in KD patients, but the precise mechanism requires further investigation.

Conflicts of interest

The authors declare no conflicts of interest.

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