Original Paper

Endothelial Cell-Derived Microparticles from Patients with Obstructive Sleep Apnea Hypoxia Syndrome and Coronary Artery Disease Increase Aortic Endothelial Cell Dysfunction

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Key Words

Obstructive sleep apnea hypoxia syndrome/OSAHS • Microparticle • Endothelial dysfunction • Coronary artery disease/CAD

Abstract

Background/Aims: Obstructive sleep apnea hypoxia syndrome (OSAHS) is an independent risk factor for coronary artery disease (CAD). Treatment of OSAHS improves clinical outcome in some CAD patients, but the relationship between OSAHS and CAD is complex. Microparticles (MPs) are shed by the plasma membrane by either physiologic or pathologic stimulation. In the current study, we investigated the role of MPs in the context of OSAHS. **Methods and Results:** 54 patients with both suspected coronary artery stenosis and OSAHS were recruited and underwent both coronary arteriography and polysomnography. Circulating MPs were isolated and analyzed by flow cytometry. CAD+OSAHS patients exhibited greater levels of total MPs (Annexin V⁺), erythrocyte-derived MPs (CD235⁺ Annexin V⁺), platelet-derived MPs (CD41⁺ Annexin V⁺), and leukocyte-derived MPs (CD45⁺ Annexin V⁺) compared to CAD alone patients or control. CAD+OSAHS patients expressed the greatest level of endothelial-derived MPs of all cellular origin types (CD144⁺ Annexin V⁺). Treatment of human aortic endothelial cells (HAECs) with MPs isolated from CAD+OSAHS patients markedly increased HAEC permeability (as detected by FITC-dextran), and significantly upregulated mRNA levels of ICAM-1, VCAM-1, and MCP-1. **Conclusion:** OSAHS+CAD patients harbor increased levels of MPs, particularly the endothelial cell-derived subtype. When administered to HAECs, OSAHS+CAD patients MPs increase endothelial cell permeability and dysfunction.

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Introduction

Estimated to affect 4% of men and 2% of women (over 18 million Americans) [1, 2], obstructive sleep apnea-hypopnea syndrome (OSAHS) is the result of intermittent upper airway collapse, leading to transient asphyxia and sleep fragmentation. As the population becomes more obese, OSAHS prevalence will continue to increase, as obesity worsens the pathophysiology of OSAHS. Shockingly, it is believed only 10% of people with sleep apnea are currently receiving treatment, and many more remain undiagnosed. Epidemiologic evidence has demonstrated the strong association between untreated OSAHS and the morbidity/mortality of various cardiovascular diseases [3-5]. OSAHS is an independent risk factor of coronary artery disease (CAD), particularly in men; up to 70% of CAD patients have undiagnosed OSAHS [6]. Long-term follow-up studies demonstrate that, although severe OSAHS significantly increases the risk of fatal and non-fatal cardiovascular events, continuous positive airway pressure (CPAP) therapy significantly improves cardiovascular disease clinical outcomes [7, 8] via incompletely understood pathophysiological mechanisms.

Microparticles (MPs, diameter 0.05-1 μ m) are shed by the plasma membrane by either physiologic or pathologic stimulation. Circulating MPs have origins from various cells types, including leukocytes, platelets, endothelial cells, erythrocytes, and progenitor cells. Circulating MPs levels correlate closely with the pathogenesis and severity of several cardiovascular diseases [9], like hypertension, atherosclerosis, and OSAHS. In patients with minimally symptomatic OSAHS, the levels of platelet and leukocyte derived MPs were elevated compared to control [10]. Moreover, withdrawn CPAP therapy for 2 weeks significantly increased endothelium-derived MPs [11]. However, the functional effects of MPs in the context of pathology such as CAD, with or without OSAHS, is unknown. In this study, we determined 1) whether OSAHS affected the circulatory MP level, and 2) the effect of administering MPs from OSAHS patients upon human aortic endothelial cells *in vitro*.

Materials and Methods

Patient selection

Patients (between ages 18 and 70) with CAD (defined as $\geq$ 50% stenosis in at least 1 epicardial coronary artery) or without CAD (or $<$ 50% stenosis in any epicardial coronary artery) by visual estimation in invasive coronary artery angiography were eligible for inclusion. The exclusion criteria were acute myocardial infarction (including ST-elevation myocardial infarction and non-ST-elevation myocardial infarction), stroke, cancer, chronic pulmonary disease, severe liver dysfunction, renal dysfunction, and acute/major pathology causing clinical decompensation. Then all patients were asked to complete the Berlin Questionnaire and Epworth Sleepiness Scale questionnaire to indicate a high risk of OSAHS. Overnight polysomnography (PSG) was performed by using two-channel (oximetry and nasal pressure) screening device (the ApneaLink, ResMed) as previously described. Hypopneas had to be associated with $\geq$ 4% oxygen desaturation. AHI $<$ 5 was considered as normal, while moderate and severe OSAHS means AHI $\geq$ 15. All enrolled patients signed the informed consent document approved by the ethical committee of Beijing Anzhen Hospital, Capital Medical University. Blood samples were collected the morning post overnight polysomnography.

MPs isolation and analysis

Circulating MPs were determined as previously described [12]. In brief, 3cc venous whole blood was drawn into citrated tubes, centrifuged at 1500g for 15 minutes to obtain platelet-rich plasma. Further centrifugation at 13000g for 10 minutes obtained platelet-free plasma at room temperature. Total MPs were harvested after additional centrifugation at 20500g for 45 minutes. MPs was defined with anti-Annexin V (eBioscience, San Diego, CA), anti-CD235-FITC, anti-CD41-APC-H7, anti-CD45-PerCP-Cy5.5, and anti-CD144-Flour 647 (all BD bioscience, San Jose, CA).

For the *in vitro* assay, circulating MPs were isolated from venous blood [13]. Briefly, 4 ml of platelet-free plasma (PFP) was separated from whole blood. Additional centrifugation (20500g for 45 minutes) pelleted the MPs, which were washed with DMEM, and centrifuged again (20500g for 45 minutes). Pellets

were resuspended in the remaining 200 µl of supernatant, frozen, and stored at -80°C until use. All samples were frozen and thawed only once.

Human aortic endothelial cells (HAECs) culture and in vitro assay

Primary HAECs (ScienCell, Carlsbad, CA, USA) were cultured with endothelial cell medium (ECM, ScienCell) supplemented with 5% fetal bovine serum (FBS) and 1% endothelial cell growth factor. The endothelial cell permeability assay was performed in a transwell system (Corning, Corning, NY, core diameter 0.4 µm). Briefly, HAECs were cultured in the collagen (Sigma, St Louis, MO) coated upper well. 100 µL FITC-CM-dextran (1g/L) was added to the upper chamber after MP treatment for 24 hours. Medium (100 µL) was harvested from the bottom chamber after designated time pass from which FITC-CM-dextran was added, and analyzed by microplate reader. For THP-1 cells migration, HAECs was cultured on the collagen-coated upper well in a transwell system. For staining of actin, HAECs were incubated with Phalloidin conjugated with FITC (Phalloidin-FITC, Sigma) for 30 min.

Total mRNA isolation and real-time quantitative PCR

Total HAEC RNA was extracted by TRizol (Invitrogen, Carlsbad, CA) after three washings by PBS as described before [14]. 2µg of RNA was reversed transcribed with GoScript™ Reverse Transcription System (Promega) per manufacturer's instructions. Real-time quantitative PCR was performed via iQ5 system (Bio-Rad, Hercules, CA) with SYBR Green I (Takara, Shiga, Japan). Amplification was performed at 95°C for 5 minutes, then 95°C for 45 seconds, and 60°C for 1 minute for each step for 45 cycles, and. GAPDH served as housekeeping gene. We employed the below primers: ICAM-1: Forward 5'-TCTTCCTCGGCCTTCCCATA-3', Reverse 5'-AGGTACCATGGCCCAAATG-3'; VCAM-1: Forward 5'-GGACCACATCTACGCTGACA-3', Reverse 5'-TTGACTGTGATCGGCTTCCC-3'; MCP-1: Forward 5'-GACCATTGTGGCCAAGGAGA-3', Reverse 5'-TTGGGTTTGCTTGTCCAGGT-3'; IL-6: Forward 5'-TCAATATTAGAGTCTCAACCCCCA -3', Reverse 5'-TTCTCTTTCGTTCCCGGTGG-3'; GAPDH: Forward 5'-CTCTGCTCCTCTGTTCGAC -3', Reverse 5'-GCGCCCAATACGACCAAATC -3'.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation for continuous data and percentages for categorical variables (GraphPad Software, San Diego, CA). Data were compared using one-way ANOVA for continuous data and χ^2 test for categorical variables. P values <0.05 were considered statistically significant.

Results

Baseline characteristics of all study subjects

54 patients in this study underwent cardiac angiography and PSG, and were divided into 3 groups per cardiac vessel stenosis extent and apnea/hypopnea index (AHI): 1) Control group, characterized by stenosis $<50\%$ and AHI <5 ; 2) CAD group: stenosis $\geq 70\%$ and AHI <5 ; CAD+OSAHS group: stenosis $\geq 70\%$ and AHI ≥ 15 . There was no statistically significant demographic difference (age, sex, BMI, biological data, tobacco abuse, diabetes comorbidity) among all 3 groups (Table 1). The AHI and 4% oxygen desaturation index (ODI) in the CAD+OSAHS group were significantly greater compared to both the control and CAD groups. Moreover, the mean SaO_2 in the CAD+OSAHS group was decreased compared to the two other groups (Table 1).

CAD increased MP formation, while OSAHS increased endothelial-derived MP formation in CAD patients

Circulating MP level is upregulated in CAD patients, particularly endothelial-derived MPs, which is correlated with clinical cardiovascular outcome [15]. Therefore, whether OSAHS affects circulatory MP level in CAD remains unknown. Flow cytometry analysis demonstrated increased circulating MP levels in the CAD group, both with and without OSAHS, compared to control (Fig. 1A). Further analysis was conducted upon endothelial

Table 1. Baseline characteristics of subjects included in this study. * $p < 0.05$ compared with control group

	Control	CAD	CAD+OSAHS	p Value
N	18	18	18	
Age (years)	42 ± 5.5	45 ± 6.3	46 ± 7.8	NS
Body mass index (kg/m ²)	25.8 ± 1.9	26.1 ± 2.5	26.9 ± 2.9	NS
Current smokers (n, %)	2 (11.1%)	2 (11.1%)	2 (11.1%)	NS
Diabetes mellitus (n, %)	3 (16.7%)	3 (16.7%)	3 (16.7%)	NS
Hypertension (n, %)	4 (22.2%)	6 (33.3%)	7 (38.9%)	NS
Dyslipidemia (n, %)	5 (27.8%)	9 (50%)	11 (61.1%)	NS
Apnea-Hypoapnea index (events/h)	2.27 ± 1.54	2.32 ± 1.45	26.35 ± 8.85*	<0.05
4% oxygen desaturation index (events/h)	2.15 ± 1.52	2.21 ± 1.41	25.32 ± 8.37*	<0.05
Mean SaO ₂ (%)	89.9 ± 1.68	89.9 ± 1.35	76.8 ± 6.31*	<0.05

Fig. 1. CAD increased MPs formation, while OSAHS increased endothelial-derived MP formation in CAD patients. Bar graph showed the MPs number of A) total MPs, B) endothelial MPs, C) leukocyte MPs, D) erythrocyte MPs and E) platelet MPs in control, CAD and CAD+OSAHS group detected by flow cytometry analysis. * $p < 0.05$ vs. control group, # $p < 0.01$ vs. control group, † $p < 0.05$ between CAD group and CAD + OSAHS group.

cell derived (CD144⁺Annexin V⁺), leukocyte derived (CD45⁺Annexin V⁺), erythrocyte derived (CD235⁺Annexin V⁺) and platelet derived (CD41⁺Annexin V⁺) MPs in all groups (Fig. 1B-E). All MP types, regardless of cellular origin, were increased in both the CAD and CAD+OSAHS groups compared to control. Additionally, there was no significant difference between MP subtype circulatory level between the CAD and CAD+OSAHS groups, except for the

Fig. 2. MPs from CAD patients with or without OSAHS increased endothelial cell dysfunction in vitro. A) HAECs were cultured in the upper well of transwell system and stimulated with MPs isolated from control, CAD and CAD+OSAHS patients. The curve showed fluorescence intensity of dextran-FITC in the lower chamber at the indicated time after being added to the upper well of transwell system; B) Representative images of FITC-phalloidin staining showed F actin arrangement in HAECs after being treated with MPs isolated from control, CAD and CAD+OSAHS patients. C) The bar graph showed migrated THP-1 cell number after being added to the upper well, with HAECs being cultured in the upper well and stimulated with MPs isolated from control, CAD and CAD+OSAHS patients. * $p < 0.05$ vs. control group, # $p < 0.01$ vs. control group, † $p < 0.05$ between CAD group and CAD + OSAHS group, ‡ $p < 0.01$ between CAD group and CAD + OSAHS group.

endothelial-cell derived MP subtype, which was markedly increased in the CAD+OSAHS group (Fig. 1B).

MPs from CAD patients with or without OSAHS increased endothelial cell dysfunction in vitro

To further investigate the effect of MPs upon endothelial cells, HAECs were cultured and treated with MPs purified from all three patient groups. Endothelial cell permeability was assessed via FITC-dextran. MPs harvested from CAD patients increased FITC-dextran permeation compared to control, and MPs from CAD+OSAHS patients increased FITC-dextran permeation to even greater degree (Fig. 2A). As actin stress fibers regulate cell permeability, we then performed FITC-phalloidin staining on HAECs subjected to MPs to reveal actin fiber arrangement. Compared to control, the actin stress fibers in HAECs treated with MPs from CAD patients were more pronounced (Fig. 2B). Utilizing a transwell system, we demonstrated significantly increased THP-1 cell migration in HAECs treated with MPs from CAD+OSAHS patients, compared to HAECs treated by MPs from the CAD group or control (Fig. 2C).

Finally, we investigated the mRNA levels of inflammatory cytokines (ICAM-1, VCAM-1, MCP-1, and IL-6) in HAECs subjected to MP treatment. ICAM-1 and MCP-1 mRNA levels were increased in HAECs treated by CAD or CAD+OSAHS MPs compared to control (Fig. 3A, C).

Fig. 3. MPs from CAD patients with or without OSAHS increased endothelial inflammation *in vitro*. The real-time PCR analysis showed mRNA levels of A) ICAM-1, B) VCAM-1, C) MCP-1 and D) IL-6 in HAECs after being stimulated with MPs isolated from control, CAD and CAD+OSAHS patients. * $p < 0.05$ vs. control group.

VCAM-1 mRNA level was upregulated only in HAECs treated by CAD+OSAHS MPs (Fig. 3B). There was no difference in IL-6 expression in HAECs treated by any MP group (Fig. 3D).

Discussion

OSAHS affects up to 7% of all middle-aged individuals, becoming more prevalent with age [16]. CAD patients with OSAHS experience twice the morbidity compared to CAD patients without OSAHS [3]. Both basic and clinical research investigations have demonstrated OSAHS contributes to CAD initiation and progression [17-19]. OSAHS has been linked to cardiovascular disease via endothelial dysfunction, sympathetic activation, inflammation, and oxidative stress, but the precise responsible molecular mechanisms remain unclear [3, 20]. We report in the present study that OSAHS increased endothelial cells-derived MPs formation in CAD patients, which exacerbated endothelial dysfunction by increasing endothelial permeability and inflammatory cytokine expression.

Small membranous vesicles shed from cells in response to various stimuli [21], MPs have various roles in the pathogenesis of CAD [22, 23]. In acute coronary syndrome (ACS) patients, procoagulant endothelial MPs are found in greater circulatory concentration compared to control patients, and may contribute to intracoronary thrombus generation [22]. MPs deliver biological messages to target cells, and regulate endothelial cell dysfunction, inflammation [24], oxidative stress [25], angiogenesis [26], and permeability [27], processes intricately implicated in the pathogenesis of CAD and its sequelae. Moreover, MPs isolated from human atherosclerotic lesions stimulated endothelial cell proliferation and angiogenesis in CD40/CD40L dependent fashion [23]. In CAD patients with type 2 diabetes, plasma levels of CD144⁺ MPs were increased compared to CAD patients without diabetes, and predictive of endothelial dysfunction [28]. Our results [MPs from CAD+OSAHS patients promoted *in vitro* endothelial dysfunction (Fig. 2 and 3)] support such reports, and suggest MPs may predict endothelial injury *in vivo*.

OSAHS affect the production and cellular origin of MPs. Childhood obstructive sleep apnea is associated with greater circulating MP levels that may promote cardiovascular risk [29]. Platelet-derived MPs are significantly associated with vascular dysfunction associated with pediatric OSA, and have been implicated in altering endothelial function [29]. Total, platelet-derived, and leukocyte-derived MPs are all increased in patients with even minimally symptomatic OSAHS compared to control, with potentially increased cardiovascular risk [10]. MPs from OSA patients experiencing marked nocturnal hypoxemia exhibit reduced endothelial-NO release and increased endothelial adhesion molecule expression [30].

In summary, CAD patients exhibited increased circulating MP levels compared to control. MPs from CAD patients (with or without OSAHS) increased endothelial cell dysfunction *in vivo* when administered to HAECs. OSAHS increased endothelial derived MP formation, and HAECs subjected to MPs from CAD+OSAHS patients manifested the most severe endothelial dysfunction *in vitro* of any tested group.

Two limitations must be explored further. Firstly, it is necessary that implement a study that incorporates treatment of OSAHS (for example, continuous positive airway pressure administration) to determine the potential endothelial effects of MPs derived from such patients. Secondly, a long-term follow-up study assessing the roles of MPs upon endothelial function in OSAHS patients is warranted.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (81470567, 81570423), Beijing Nova program (Z171100001117019), the International Science & Technology Cooperation Program of China (2015DFA30160), the Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (ZYLX201605) and Incubating Program (PX2016048), the Beijing Municipal Administration of Hospitals Ascent Plan (DFL20150602), and the Beijing Municipal Science & Technology Commission (Z141107002514014).

Disclosure Statement

The authors have no conflicts of interest.

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