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Circulating microparticles from obstructive sleep apnea syndrome patients induce endothelin-mediated angiogenesis

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ABSTRACT

Microparticles are deemed true biomarkers and vectors of biological information between cells. Depending on their origin, the composition of microparticles varies and the subsequent message transported by them, such as proteins, mRNA, or miRNA, can differ. In obstructive sleep apnea syndrome (OSAS), circulating microparticles are associated with endothelial dysfunction by reducing endothelial-derived nitric oxide production. Here, we have analyzed the potential role of circulating microparticles from OSAS patients on the regulation of angiogenesis and the involved pathway. VEGF content carried by circulating microparticles from OSAS patients was increased when compared with microparticles from non-OSAS patients. Circulating microparticles from OSAS patients induced an increase of angiogenesis that was abolished in the presence of the antagonist of endothelin-1 receptor type B. In addition, endothelin-1 secretion was increased in human endothelial cells treated by OSAS microparticles. We highlight that circulating microparticles from OSAS patients can modify the secretome of endothelial cells leading to angiogenesis.

1. Introduction

Obstructive sleep apnea syndrome (OSAS) is sleep-disordered breathing associated with increased cardiovascular morbidity that could result from intermittent hypoxia-related inflammation, oxidative stress and endothelial dysfunction. Recently, circulating microparticles (MPs), small vesicles of plasma membrane released during cell activation and apoptosis [1], have been proposed to contribute to the pathogenesis of vascular dysfunction in OSAS [2]. Thus, MPs from OSAS patients decrease nitric oxide production without affecting reactive oxygen species (ROS) generation in human endothelial cells [2]. Furthermore, injection of OSAS MPs into mice results in a reduced endothelium-dependent relaxation to acetylcholine [2] and an enhanced vascular contraction to serotonin in the aorta [3]. Interestingly, MPs expressing CD62L are positively correlated with the severity of OSAS according to the apnea–hypoapnea index [2,3].

Among the possible mechanisms responsible for the cardiovascular changes described in OSAS patients, activation of the endothelin-1 system has been proposed (for review see [4]). A very recent study shows single nucleotide polymorphisms in endothelin-1 gene, in which allelic frequencies are significantly altered in children with OSAS [5]. In OSAS patients, plasma endothelin-1 levels are increased [6,7] and positively correlated with the severity of nocturnal hypoxia, and decreased by positive airway pressure treatment [8]. However, another study shows that whereas positive airway pressure treatment improves circulating levels of inflammatory adhesion molecules such as ICAM-1 and plasminogen activator inhibitor-1, enhanced levels of plasmatic endothelin-1 are not corrected [9] suggesting that further treatments against OSAS need to be developed [10]. Furthermore, in an animal model of OSAS, rats exposed to chronic intermittent hypoxia display elevated levels of endothelin-1 as well as decreased endothelium-dependent vasodilation and increased vascular contraction to endothelin-1 which account for the increase of arterial pressure [11,12]. In addition, pharmacological treatment with bosentan abolishes deleterious consequences induced by chronic intermittent hypoxia [13].

Very recent data suggest that intermittent hypoxia in OSAS patients might be involved in the development of cancer [14]. Although the exact mechanism implicated remains to be determined, it is possible that the reoxygenation periods during intermittent hypoxia generate changes in gene expression which may regulate the activity of some transcription factors and signaling pathways involved in tumor growth-inducing angiogenesis [14]. Among the plausible mechanisms involved, an enhanced angiogenesis has been proposed in OSAS patients (for review see [15]). Indeed, an increased coronary collateralization has been described in OSAS patients [16]. In addition, upregulation of proangiogenic vascular endothelial growth factor (VEGF) [17] has

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been demonstrated in OSAS. One of the key features of MPs on cardiovascular system is their ability to modulate angiogenic program [1]. Therefore, the purpose of the present study was to determine whether MPs from OSAS patients can modulate angiogenesis in human aortic endothelial cells. To our knowledge, the study reported here is the first to provide experimental evidence that circulating MPs from OSAS patients are able to induce angiogenesis through the production of endothelin-1 by human endothelial cells via a mechanism sensitive to ETB receptor antagonist.

2. Materials and methods

2.1. Patients

Consecutive male patients (19 to 70 years old) investigated by polysomnography in the Sleep Unit of the Department of Respiratory Medicine of Angers University, for suspected OSAS were screened for the study. Exclusion criteria were previous treatment for OSAS, body mass index (calculated as weight in kilograms divided by height in meters squared) ≥ 35 kg/m^2, history of coronary artery disease, heart failure, stroke, hypertension, diabetes mellitus, dyslipidemia, and treatment with any drug known to affect endothelial function. Patients with an apnea–hypoapnea index of ≥ 5 events per hour were included in the OSAS group. Patients with an apnea–hypoapnea index <5 were included in the non-OSAS (N-OSAS) group. All the patients underwent evaluation of clinical profile and daytime sleepiness using the Epworth Sleepiness Scale [18]. Standard in-laboratory overnight polysomnography was performed as previously described [19] using a computerized recording system (CID 102; Cidelec, Angers, France) with the following channels: electroencephalogram, electrooculogram, chin electromyogram, arterial oxygen saturation (finger oximetry), nasal-oral airflow (pressure cannula), tracheal sound (suprasternal microphone), electrocardiogram, chest and abdominal wall motion (piezo electrodes), bilateral tibialis electromyogram, and body position. Respiratory events were scored manually using recommended criteria [20]. Hypopneas had to be associated with $\geq 4\%$ oxygen desaturation. The University of Angers ethics committee approved the study, and patients gave their informed consent.

2.2. MP isolation and characterization

MP characterization was performed in the morning after sleep recording, at approximately 7 or 8 am, before breakfast. Routine laboratory tests, including glucose, glycated hemoglobin, triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and blood cell count, were also performed using a morning blood sample. For MP isolation, blood samples were collected in EDTA tubes (Vacutainer; Becton Dickinson, Le Pont de Claix, France) from a peripheral vein using a 21-gage needle to minimize platelet activation and were processed for assay within 2 h. Samples were centrifuged for 20 min at 270 *g*, and plasma was then harvested and centrifuged for 20 min at 1500 *g* to obtain platelet-free plasma (PFP). Two hundred microliters of PFP was frozen and stored at −80 °C until use. As previously described [2,3], the remaining PFP was subjected to two series of centrifugation at 21,000 *g* for 45 min to eliminate plasma and to pellet MPs for studies, and supernatant was replaced by 0.9% NaCl saline solution. Finally, MP pellets were suspended in 150 μL of 0.9% saline salt solution and were stored at 4 °C until subsequent use.

MP subpopulations were discriminated into PFP according to the expression of membrane-specific antigens by flow cytometry. MPs derived from platelets, lymphocytes, and endothelial cells were identified using anti-CD41, anti-CD45, and anti-CD146 antibodies, respectively. Anti-CD62L antibody was used to identify MPs derived from activated L-selectin$^+$ leukocytes. Irrelevant human IgG was used as an isotype-matched negative control for each sample. Five microliters of PFP was incubated with 5 μL of specific antibody (Beckman Coulter, Villepinte, France), and after 45 min of incubation, samples were diluted in 300 μL of 0.9% NaCl. Annexin V-FITC (BioVision Research Products, Mountain View, CA) binding was used to count phosphatidylserine-expressing MPs. To determine the MP concentration, equal volumes of sample and FlowCount beads were then added to calculate the MP concentration, and samples were analyzed using a 500 MPL system flow cytometer (Beckman Coulter). Regions corresponding to MPs were identified in forward and side-angle light scatter intensity dot plot representation set at logarithmic gain, depending on their diameter (0.1 to 1.0 μm). Sample analysis was stopped after counting 10,000 events.

2.3. Cell culture

Human aortic endothelial cells (HAoECs) (Promocell, Heidelberg, Germany) were cultured (37C, 5% CO_2) in Endothelial Cell Growth Medium MV2 (Promocell) complemented with the supplements according to the manufacturer's instructions. All ECs were used at <10 passages. Cells were treated for 24 h in the absence or presence of N-OSAS or OSAS MPs at the circulating levels of MPs detected in the plasma of each patients (OSAS group; range, 3885 to 69,480 MPs per microliter of plasma; N-OSAS group; range, 2,887 to 8,5725 MPs per microliter of plasma), as previously described for other pathologies [21,22].

2.4. Plasmatic VEGF

Measurement of plasmatic VEGF was performed on PFP using a commercially available ELISA assay (Pierce Biotechnology, Rockford, IL).

2.5. VEGF expression by Western Blotting

MPs (30 μg of proteins) were separated on a 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA). Blots were probed with anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA). Tubulin (Santa Cruz Biotechnology) was used to visualize protein gel loading. The membranes were then washed at least three times in Tris buffer solution containing 0.05% Tween and were incubated for 1 h at room temperature with the appropriate horseradish peroxidise-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ). The protein–antibody complexes were detected by enhanced chemiluminescence plus reagent (Amersham Biosciences) according to the manufacturer's instructions.

2.6. Apoptosis measurement by flow cytometry

HAoECs were exposed to MPs or actinomycinD (1 μM as positive control; Sigma-Aldrich, St. Louis, MO) for 24 h and then fixed in 70 % ethanol at 4 °C for at least 4 h. After a centrifugation at 15,000 *g* for 5 min, cells were re-suspended in PBS containing 0.05 mg/mL RNase (Sigma-Aldrich) and 10 μg/mL propidium iodide (Sigma-Aldrich). Cellular DNA content was analyzed on a Cytomics FC 500 MPL flow cytometer (Beckman Coulter). In all cases at least 10,000 events were collected for analysis.

2.7. Cell adhesion assay

Evaluation of adherent cells was performed using crystal violet staining. Then, 5×10^4 cells per well were seeded into 96-well plates and were treated for 24 h. After incubation, the plate was shacked for 15 s. The supernatant with non-adherent cells was removed by three washes with washing buffer (0.1% BSA in medium without serum). Attached cells were fixed with 4 % of paraformaldehyde for 15 min at room temperature. Cells were rinsed two times with washing buffer, stained with crystal violet (Sigma-Aldrich) (1 mg/mL in 2 % of ethanol) for 10 min at room temperature and extensively washed with distilled water. Then, sodium dodecyl sulfate 2% was added and incubated for 30 min at room temperature. Absorbance was then evaluated using a microplate reader at 550 nm (Synergy HT, Biotek).