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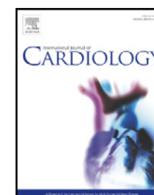

Reduced number and impaired function of circulating endothelial progenitor cells in patients with abdominal aortic aneurysm

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A B S T R A C T

Aim: Circulating endothelial progenitor cells (EPCs) are associated with coronary artery disease (CAD) and predict its outcome. Although the pathophysiology of abdominal aortic aneurysm (AAA) is different, it shares some risk factors with CAD. Therefore, the correlation between EPCs and AAA was investigated.

Methods and results: Seventy-eight subjects (age 77.2 ± 7.8 years) with suspected AAA were prospectively enrolled. Cut-off values (men, 3.5–5.5 cm; women, 3–5 cm) were used to define normal aorta, small AAA, and large AAA on thoraco-abdominal computer tomography. Endothelial function was measured by flow-mediated vasodilation (FMD). Flow cytometry and colony-forming units (CFUs) were used to evaluate circulating EPC numbers. Circulating EPCs were defined as mononuclear cells with low CD45 staining and double-positive staining for KDR, CD34, or CD133. Late out-growth EPCs were cultured from six patients with large AAAs and six age- and sex-matched controls to evaluate proliferation, adhesion, migration, tube formation, and senescence.

FMD was significantly lower with large (5.26% ± 3.11%) and small AAAs (6.31% ± 3.66%) than in controls (8.88% ± 4.83%, P = 0.008). Both CFUs (normal 38.39 ± 12.99, small AAA 21.22 ± 7.14, large AAA 6.98 ± 1.97; P = 0.026) and circulating EPCs ($CD34^+/KDR^+$ and $CD133^+/KDR^+$) were significantly fewer in AAA patients than in controls. On multivariate analysis, CFUs and circulating EPCs ($CD34^+/KDR^+$) were independently, inversely correlated to AAA diameter. Proliferation, adhesion, migration, tube formation, and senescence of late EPCs were significantly impaired in AAA patients.

Conclusion: The number and function of EPCs were impaired in AAA patients, suggesting their potential role in AAA.

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1. Introduction

Abdominal aortic aneurysm (AAA) accounts for more than 15,000 annual deaths in the US and is one of the leading causes of mortality in men aged more than 50 year-old [1]. Its prevalence increases with increasing age, and the risk of rupture increases with increasing aneurysm diameter. Once the aneurysm ruptures, only 18% of the patients survive. The presence of AAA shares similar risks, such as smoking, hypertension, and hypercholesterolemia, with atherosclerotic cardiovascular disease [2,3]. However, the managements of these risk factors failed to prevent the progression of AAA. AAA development and progression appear to be related to an imbalance between destructive and restorative vascular wall processes. Aneurysmal dilatation characteristically shows destruction of elastin and collagen in the media and adventitia, loss of medial smooth muscle cells, vessel wall thinning, and transmural lymphocyte and macrophage infiltration [4,5]. In addition, the loss of endothelial integrity was also noted in AAA from histological examination, which was also the fundamental cause of mural thrombosis [6]. Moreover, in a small series study of 30 patients with AAA, the endothelial function reflected by endothelium dependent vasodilation has a negative and linear correlation with the aneurysm diameter [7]. In an experimental AAA model, creation of high aortic flow status by creating AV fistula promoted re-endothelization of AAA and reduced its progression, when comparing with normal or low aortic flow status [8]. It

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seems that the endothelial injury was associated with the occurrence of AAA and the recovery of endothelial integrity correlated with the progression of AAA. Since the circulating endothelial progenitor cells (EPCs) are a cellular reservoir to repair or replace dysfunctional endothelium at vascular injury sites and restore endothelial function [9–11]. The number of circulating EPCs may predict adverse events and mortality in patients with different cardiovascular diseases [12,13]. It is believed that the circulating EPCs might be associated with the presence and progression of AAA. However, the only published data demonstrated an unexpected result. The circulating EPCs defined by positive surface markers of CD133 and/or CD34 increased in 25 subjects with AAA, comparing with 18 age-matched controls [14]. Therefore, we would like to investigate the associations of circulating EPCs as well as endothelial function with the progression of AAA in the present study.

2. Methods

2.1. Study population

Subjects referred for a thoraco-abdominal computer tomogram (CT) with suspected AAA were eligible. Patients with unstable angina, decompensated heart failure, inflammatory disease, ongoing infections, severe renal failure, and who were allergic to contrast medium were excluded. Written, informed consent, as approved by our Institutional Review Board, was obtained from each patient before enrolment. After overnight fasting, patients were studied in a quiet, temperature-controlled room. Blood pressures (BPs) were obtained by averaging three different measurements taken after a 15-minute rest. Serum and plasma samples were acquired for plasma creatinine (P_{Cr}), fasting glucose, lipid profiles, and high-sensitivity C-reactive protein (hsCRP). Height, weight, history, demographics, and prescribed medications were recorded. Estimated glomerular filtration rate (eGFR) was calculated from plasma creatinine (P_{Cr}) using a modified Modification of Diet in Renal Disease equation for the Chinese population: eGFR (mL/min/1.73 m^2) = $175 \times P_{Cr}^{-1.234} \times \text{age}^{-0.179} \times$ (0.79 if female) [15]. Serum hsCRP was determined by particle-enhanced immunoturbidimetry using latex agglutination (Toshiba, Tokyo, Japan).

2.2. AAA definitions

The maximal abdominal aorta diameter was measured by reviewing each coronal CT section. Cut-off values (men, 3.5–5.5 cm; women, 3–5 cm) were used to define normal aorta, small AAA, and large AAA.

2.3. Determination of endothelial function

Endothelial function was measured by endothelium-dependent flow-mediated vasodilatation (FMD) using a 7.5-MHz linear array transducer (Sonos 5500; Hewlett-Packard, Andover, MA, USA) to scan the brachial artery [16]. The procedure was performed in a quiet, air-conditioned room (22–25 °C) by an experienced technician blinded to the clinical data. The left arm was stabilized with a cushion, and a sphygmomanometric cuff was placed on the forearm. A baseline image was acquired, and blood flow was estimated by time-averaging the pulsed Doppler velocity signals from a mid-artery sample volume. Then, the cuff was inflated to ≥50 mm Hg above systolic pressure to occlude the arteries for 5 min and released abruptly. A mid-artery pulsed Doppler signal was obtained immediately upon cuff release, and brachial artery diameters were obtained at 30, 60, 90, 120, and 150 s after deflation. FMD was calculated as maximal post-occlusion brachial artery diameter relative to averaged baseline diameters.

2.4. Circulating EPC assay

The method for assessing the number of circulating EPCs has been previously described [17,18]. Briefly, a 10-mL volume of peripheral blood was incubated for 30 min in the dark with monoclonal antibodies against human kinase insert domain-conjugating receptor (KDR; R&D, Minneapolis, MN, USA) followed by phycoerythrin (PE)-conjugated secondary antibody, with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies against human CD45 (Becton Dickinson, Franklin Lakes, NJ, USA), with PE-conjugated monoclonal antibody against human CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany), and FITC-conjugated or PE-conjugated monoclonal antibodies against human CD34 (Serotec, Raleigh, NC, USA) and KDR (Sigma, St Louis, MI, USA). Isotype-identical antibodies served as controls (Becton Dickinson). After incubation, cells were lysed, washed with phosphate-buffered saline (PBS), and fixed in 2% paraformaldehyde before analysis. Each analysis included 100,000 events. The numbers of circulating EPCs were defined for $CD34^+KDR^+CD45^{low}$, $CD34^+CD133^+CD45^{low}$, and $KDR^+CD133^+CD45^{low}$, respectively. (Fig. 1).

2.5. EPC colony-forming assay

Isolated mononuclear cells (MNCs) were resuspended in growth medium (EndoCult; StemCell Technologies, Vancouver, Canada), and 5×10^6 MNCs were preplated onto a fibronectin-coated six-well plate in duplicate [18]. After 48 h, nonadherent cells were collected by pipetting the medium in each well up and down three times, and 1×10^6 cells were replated onto a fibronectin-coated 24-well plate. On day 5 of the assay, the colony-forming units (CFUs) per well for each sample were counted manually in a minimum of three wells by two independent observers.

2.6. EPC culture

Peripheral blood samples (20 mL) were obtained in heparin-coated tubes from six patients with large AAAs and six age- and sex-matched patients with normal aortas to study late EPCs in culture. MNCs were isolated by density gradient centrifugation with Histopaque-1077 (Sigma), and the serum was preserved [17]. Briefly, MNCs (5×10^6) were plated in 2-mL of endothelial growth medium (EGM-2 MV Cambrex, East Rutherford, NJ, USA), with 15% individual serum on fibronectin-coated, 6-well plates. After 4 days of culturing, the medium was changed, and nonadherent cells were removed; attached early EPCs appeared elongated with spindle shapes. Some MNCs were allowed to grow into colonies of late (out-growth) EPCs, which emerged 2–3 weeks after the start of MNC culture. The late EPCs exhibited a 'cobblestone' morphology and monolayer growth pattern typical of mature endothelial cells at confluence. Late EPCs were collected for the functional assays.

2.7. EPC characterization

Early EPCs were characterized as adherent cells, double-positive for acetylated LDL uptake and lectin binding by direct fluorescent staining, as previously described [17]. Briefly, the adherent cells were first incubated with 2.4 mg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated LDL (DiI-acLDL; Molecular Probes, Eugene, OR, USA) for 1 h, then fixed in 2% paraformaldehyde and counterstained with 10 mg/mL FITC-labeled lectin from *Ulex europaeus* (UEA-1; Sigma). The late EPC-derived outgrowth endothelial cell population was characterized by immunofluorescence staining for expressions of vascular endothelial (VE)-cadherin, platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD-31), and CD34 (Santa Cruz). Fluorescent images were recorded under a laser scanning confocal microscope.

2.8. EPC functional assays

2.8.1. EPC adhesion assay

EPCs' ability to adhere to the injured site to initiate the repair process was evaluated by plating 1×10^4 late EPCs onto a fibronectin-coated, 6-well plate and incubating for 30 minutes. Gentle washing with PBS three times was performed after a 30 min adhesion, and adherent cells in six random, high-power (×100) microscopic fields (HPF) of each well were counted by independent, blinded investigators.

2.8.2. EPC proliferation assay

The proliferation of EPCs was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide (MTT) assay [17]. After being cultured with 1×10^4 late EPCs in fibronectin-coated 24-well plates for 3 days, the EPCs were supplemented with MTT (0.5 mg/mL; Sigma) and incubated for 4 h for the proliferation assay. The blue formazen was dissolved with dimethyl sulfoxide and measured at 550/650 nm. In addition, simultaneous culturing with human aortic endothelial cells (HAECs) served as the internal control [19]. The EPC proliferation activity was presented as the ratio to HAECs.

2.8.3. EPC migration assay

EPC migration was evaluated by a modified Boyden chamber assay (Transwell, Coster; Corning Inc., Acton, MA, USA) [17]. Isolated EPCs were detached as described above with trypsin/EDTA, and 3×10^4 EPCs were placed in the upper chambers of 24-well transwell plates with polycarbonate membranes (8-mm pores) that contained serum-free EGM; vascular endothelial growth factor (VEGF, 50 ng/mL) was added to the medium in the lower chambers. After incubation for 24 h, the membrane was washed briefly with PBS and fixed with 4% paraformaldehyde. The upper side of the membrane was wiped gently with a cotton ball. The membrane was stained using hematoxylin solution and carefully removed. The number of migratory late EPCs was evaluated by counting the migrated cells in six random HPFs.

2.8.4. EPC tube-formation assay

In vitro tube formation was assayed using the In Vitro Angiogenesis Assay Kit (Chemicon) [17]. ECMatrix gel solution was thawed at 4 °C overnight, mixed with ECMatrix diluent buffer, and placed in a 96-well plate at 37 °C for 1 h to allow the matrix solution to solidify. EPCs were harvested as described above with trypsin/EDTA, then 1×10^4 EPCs were placed on matrix solution with EGM-2 MV medium with glucose or mannitol, and incubated at 37 °C for 16 h. Tubule formation was inspected under an inverted light microscope (×100). Four representative fields were taken, and the average total area of complete tubes formed by cells was compared using Image-Pro Plus software (Media Cybernetics, Inc., MD, USA).

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