Potential Link Between C3a, C3b and Endothelial Progenitor Cells in Resistant Hypertension

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Abstract: *Introduction:* Endothelial progenitor cells (EPC) and complement C3 are involved in the pathophysiology of arterial hypertension. C3a is the negative regulator of progenitor cells egress during their mobilization from bone marrow. Previously, higher plasma concentration of C3 was observed in resistant arterial hypertension (RAH) than in controlled arterial hypertension (CAH). Thus, we hypothesized that RAH would be associated with complement C3 activation and reduced number of circulating EPCs. *Objective:* To compare C3a, C3b and their correlation with circulating EPC in subjects with RAH and CAH. *Methods:* Blood pressure was measured by electronic sphygmomanometer. EPCs were identified as CD34+/CD133+/KDR+ cells by flow cytometry. C3a and C3b were determined using enzyme-linked immunosorbent assay (Quidel, CA). *Results:* RAH group (n = 20) and CAH group (n = 20) and 17 healthy individuals (control group) were recruited. In the RAH group, C3a (858.1 ± 70.6 μg/dL) was higher than in the CAH group (816.1 ± 123.3 μg/dL; $P < 0.001$), and in the control group (751.3 ± 98.8; $P < 0.001$), C3b (564.1 ± 54.7 μg/dL) was higher than in the CAH group (490.2 ± 58.5 μg/dL; $P < 0.001$). In control group (456.3 ± 98.8; $P < 0.001$), statistically significant negative correlation was observed between C3a and blood levels of EPC ($r = -0.523$, $P = 0.018$); statistically significant positive correlation was observed between systolic blood pressure and blood levels of C3a ($r = 0.52$, $P = 0.02$) and between systolic blood pressure and blood levels of C3b ($r = 0.57$, $P = 0.009$). *Conclusion:* RAH is characterized by higher levels of C3 component fragments and a negative correlation between circulating C3a and EPCs.

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Refractory or resistant arterial hypertension (RAH) is conventionally defined as systolic or diastolic blood pressure that remains uncontrolled above 140/90 mm Hg in the general population or ≤130/80 mm Hg in patients with diabetes or renal disease (serum creatinine >1.5 mg/dL in men or >1.3 mg/dL in women, or urinary protein excretion >300 mg over a 24-hour period) despite sustained therapy with at least 3 different classes (1 of them diuretic) of antihypertensive agents.[1]

In a recently published population-based longitudinal study, plasma C3 was found to be associated with a future blood pressure increase and the development of hypertension,[2] and higher plasma C3 levels were observed in hypertensive subjects in comparison with normotensive controls.[3–5] Several organs, including vascular endothelium, may regenerate by attracting stem cells that are mobilized from their niches in response to tissue or organ damage. Endothelial progenitor cells (EPC) are a heterogeneous group of endothelial cell precursors originating in the hematopoietic compartment of the bone marrow. They provide a circulating pool of cells that could form a cellular patch at the site of denuding injury or serve as a cellular reservoir to replace dysfunctional endothelium.[6] The mobilization of hematopoietic stem/progenitor cells (HSPC) from bone marrow into peripheral blood is still not fully understood, but mounting evidence suggests that their mobilization is part of the immune response and is mediated by innate immunity. Complement system cleavage fragments play a crucial role in both the retention and mobilization of CD34 (+) hematopoietic stem cells because they express the C3a receptor (C3aR), and C3a induces calcium flux in these cells.[7]

Fluid phase C3a and bone marrow–bound C3b/iC3b increase the responsiveness of HSPCs to a stromal-derived factor-1 (SDF-1) gradient.[8] Consequently, mobilization of HSPCs is envisioned as part of an immune response that requires complement activation by the classical Ig-dependent and/or Ig-independent pathways. Therefore, C3a is the first negative regulator of progenitor cells egress during their mobilization,[8,9] and the potentiating effect of C3a on SDF-1 is independent of the classical C3a receptor.[10] C3a may also directly modulate their homing by augmenting C3aR-mediated secretion of matrix metalloprotease-9 and cell adhesion.[11]

In the previous study, we observed higher blood levels of C3[12] in RAH than in controlled arterial hypertension (CAH). Thus, we hypothesized that RAH would be associated with complement activation and reduced number of circulating EPCs.

To check this hypothesis, a small pilot study has been conducted in which plasma C3a/C3b levels were measured and their correlation with circulating EPC were compared between the patients with RAH and CAH.

MATERIALS AND METHODS

Study Population

The local ethics committee approved the protocol, and the participants gave written informed consent according to Helsinki Guidelines. Data were collected from the Hypertension Unit and Medicine Departments of Barzilai Medical Center (Ashkelon, Israel) between January 2007 and December 2008. A total number of 500 individuals with a history of arterial hypertension of ≤3 years of duration from the city of Ashdod (Israel) were screened.

The hypertensive patients recruited in the RAH group had an uncontrolled or RAH based on conventional office blood pressure (BP) measurements [systolic BP (SBP) >140 mm Hg and/or diastolic BP (DBP) >90 mm Hg] and had been under treatment for >3 months with a stable scheme consisting of ≥3 antihypertensive drugs (including a diuretic), of adequate

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combination and dose. In patients with type 2 diabetes (13 individuals), the definition of RAH was BP >130/80 mm Hg. Moreover, all the patients in the RAH group underwent 24-hour ambulatory blood pressure monitoring at least once within last 12 months during their follow-up in the Hypertension Unit, and they really had resistant hypertension.

The hypertensive patients under treatment for >3 months consisting of ≤3 antihypertensive drugs with a stable scheme and with office SBP <140 mm Hg and DBP <90 mm Hg were considered to have CAH and were randomly recruited into the CAH group.

By using the computerized database, the prescriptions of antihypertensive agents were identified for each patient and were analyzed for 3 years. Adherence to treatment was evaluated in terms of persistence during the years and in terms of daily coverage. We identified the daily dose recommended for the maintenance therapy for each drug and then calculated the total number of each antihypertensive medication collected from the pharmacy by the patient during the year.

Exclusion criteria were (1) diabetes mellitus; (2) kidney disease (any type of renal failure with serum creatinine >1.5 mg/dL in men or >1.3 mg/dL in women, and/or urinary protein excretion >300 mg over a 24-hour period, and/or glomerular filtration rate <75 mL/min/1.73 m^2); (3) liver disease; (4) cancer; (5) acute myocardial infarction, or unstable angina within 6 months; (6) heart failure; (7) cerebrovascular disease in the previous months; (8) use of corticosteroids or other immunosuppressive therapy; (9) abnormal levels of plasma aldosterone, supine and standing plasma renin activity, 24-hour urinary catecholamines; (10) anomalies in the renal ultrasound scan or in renal arterial duplex, which was performed to exclude renal artery stenosis; (11) autoimmune disease; and (12) active infection.

Study Protocol

At the end of the run-in phase of 14 days the participants who met the inclusion criteria were included in the RAH and CAH groups. The normotensive controls were recruited from the subjects referred for several reasons to outpatient primary care clinics. Normotensive subjects were defined as those without cardiovascular disorders and with a office BP <140/85 mm Hg.

Fasting blood samples for biochemical and flow cytometric studies were drawn between 8 and 10 AM.

Blood Pressure Measurements

Measurements of blood pressure were performed at study entry, in the sitting position and after 10 minutes of rest, on the dominant arm by the automatic electronic sphygmomanometer (Omron M4; Omron Matsusaka Co., Japan). Three consecutive BP measurements were performed, and a mean of the measurements was used to define SBP and DBP. The clinic BP value was calculated as the average of the 3 readings taken on 3 different visits on days 0, 7, and 14.

To exclude the occurrence of a "false" RAH, due to a persistent white coat effect, all the patients in the RAH group also underwent home BP monitoring to assess the control of both office and out-of-office BP. All patients were taught to measure BP correctly with their own sphygmomanometer (Omron M4; Omron Matsusaka Co) at home. The home BP value was calculated as the average of 14 readings over 14 days. The "white-coat phenomenon" was considered to be present when the difference between the clinic BP and home BP was >20/10 mm Hg.

Laboratory Analyses

Blood hematology and chemistry analyses were performed at the Clinical Laboratory of Barzilai Medical Center (Ashkelon, Israel).

Flow Cytometry Analysis of EPCs

After overnight fast, blood samples were obtained through a 20-gauge needle from a forearm vein. All samples were processed within 2 hours. Peripheral blood progenitor cells were analyzed for the expression of cell surface antigens using fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) by flow cytometric analysis (FACScan, Becton Dickinson, Sunnyvale, CA) as previously reported.[13] In brief, 100 μL of peripheral blood was incubated for 15 minutes in the dark with 10 μL monoclonal antibodies against human KDR (Sigma) followed by PE-conjugated secondary antibody: with the FITC-labeled monoclonal antibodies against human CD45 (Santa Cruz), with the PE-conjugated monoclonal antibody against human CD133 (Santa Cruz), and with FITC- or PE-conjugated monoclonal antibodies against human CD34 (Santa Cruz). Isotype identical antibodies served as controls (Santa Cruz). Mononuclear cells were not separated from total peripheral blood. After incubation, cells were lysed, washed with phosphate-buffered saline, and fixed in 2% paraformaldehyde before analysis.

A morphological gate was used to exclude granulocytes. Then, we gated CD34+ or CD133+ peripheral blood cells in the mononuclear cell fraction and examined the resulting population for the dual expression of KDR. CD34+CD133+ cells were identified at the intersection of the CD34 and CD133 gates, whereas total KDR+ mononuclear cells were identified separately as cells with high KDR expression and low side scatter.

Triple-positive cells were identified by the dual expression of KDR and CD133 in the CD34 gate. Percentage of EPC is a cell count percentage of peripheral blood mononuclear cells in the lymphocyte gate.

The instrument setup was optimized daily by analyzing the expression of peripheral blood lymphocytes labeled with an anti-CD4 FITC/CD8 PE/CD3 PECy5/CD45 APC 4 color combination. The same operator (C.H.), who was blinded to the subjects' characteristics, performed all the tests throughout the study. Each analysis included 60,000 events.

C3a and C3b Analysis

Blood samples for measuring the concentrations of C3a and C3b were obtained through a 20-gauge needle from a forearm vein into tubes with EDTA. Immediately after collection, the blood samples were centrifuged, and the plasma was stored in individual tubes at −60°C for later analysis. The concentrations of plasma C3a and C3b were determined by sandwich enzyme-linked immunosorbent assay (Quidel, CA) according to the manufacturer's procedure. The limit of sensitivity of each assay was 1 ng/mL.

Data Analysis

Results are presented as mean ± SD. The nonparametric Mann-Whitney and χ^2 test were used to compare differences between means as appropriate. The Pearson test for correlations was used to analyze correlations between the variables. A P value <0.05 was considered significant. The statistical analysis was performed using the software Statistica 6 for Windows (Stat Soft, Chicago, IL).

RESULTS

The demographic, clinical, and laboratory data of the study are shown in Table 1. The group of the patients with RAH (n = 20) and the group of patients with CAH (n = 20) were similar with respect to their baseline demographic and laboratory characteristics, and 17 healthy individuals without