Sympathetic predominance is associated with impaired endothelial progenitor cells and tunneling nanotubes in controlled-hypertensive patients

Elena M. V. de Cavanagh,1,2 Sergio A. González,1,2 Felipe Inserra,1 Pedro Forcada,1,2 Carlos Castellaro,1,2 Jorge Chiabaut-Svane,1,2 Sebastián Obregón,1,2 María Jesús Casarini,2 Pablo Kempny,2 and Carol Kotliar1,2

1School of Biomedical Sciences, Austral University, Buenos Aires, Argentina; and 2Arterial Hypertension Center, Cardiology Department, Austral University Hospital, Buenos Aires, Argentina

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Sympathetic predominance is associated with impaired endothelial progenitor cells and tunneling nanotubes in controlled-hypertensive patients. Early endothelial progenitor cells (EPC) that emitted TNT were 41, 77, 50, and 88% lower than in normotensive subjects (15). Marrow-derived endothelial progenitor cells contribute to the repair and regeneration of the injured endothelium (15). Mononuclear cells were cultured on fibronectin- and collagen-coated dishes for early EPC and late EPC, respectively. Low (LF) and high (HF)-frequency components of short-term heart rate variability were analyzed during a 5-min rest, an expiration/inspiration maneuver, and a Stroop color-word test. Modulations of cardiac sympathetic and parasympathetic activities were evaluated by LF/HF (%) and HF power (ms²), respectively. In controlled-hypertensive patients, the number of EPC, early EPC that emitted TNT, late EPC, and late TNT were 41, 77, 50, and 88% lower than in normotensive subjects (114(107–119)/75(64–79) mmHg; 80% male) and 20 healthy normotensive subjects [137)/85(61–88) mmHg; 81.8% male] and 20 healthy normotensive subjects. In controlled-hypertensive patients, sympathetic overactivity/parasympathetic inhibition was negatively associated with EPC, parasympathetic underactivity were negatively associated with EPC, suggesting that reducing sympathetic/parasympathetic activation might favor endothelial repair.

Tissue ischemia and/or endothelial damage promote EPC mobilization from the bone marrow and EPC recruitment and incorporation at sites of vascular damage (15). Two distinct types of EPC have been identified in vitro cell culture of the blood mononuclear cell fraction, i.e., early EPC and late EPC, the latter also known as outgrowth endothelial cells. Early EPC, representing alternative activated M2 macrophages, promote vascular repair through the paracrine release of cytokines and late EPC by differentiating into endothelial cells and incorporating into blood vessels (20, 26). In addition, EPC can rescue damaged endothelial cells by transferring mitochondria and lysosomes through the recently discovered cell-to-cell communication channels referred to as tunneling nanotubes (TNT) (34, 35).

Studies in rodents showed that the sympathetic nervous system plays an essential role in EPC mobilization from the bone marrow (22) and that catecholamines are involved in the modulation of postischemic revascularization (31), suggesting that autonomic dysregulation can impair vascular endothelial repair. In humans, the relation between sympathetic activation and EPC egress from the bone marrow was indirectly suggested by reports showing a negative relation between the number of circulating EPC and both psychosocial stressors (13) and depression scores (8), which are known to be associated with autonomic derangements. In this context, it is noteworthy that in vivo endothelial repair capacity is reduced in prehypertension and hypertension patients (16) and that sympathetic overactivation has been calculated to be present in ~50% of essential hypertension patients (10, 30).

Considering all of the above, we postulated that autonomic imbalances characterized by sympathetic overactivation and/or parasympathetic inhibition would be associated with impaired endothelial repair capacity in treated hypertensive patients. To test this notion, in this study we assessed the relations between the number and function of EPCs and autonomic status in patients under treatment for hypertension that had achieved goal blood pressure and also investigated ex vivo EPC responses to added epinephrine and norepinephrine.

MATERIALS AND METHODS

Patients. The study adhered to the principles outlined in the Declaration of Helsinki and was approved by the School of Biomedical Sciences-Austral University Ethics Committee. Written informed consent was obtained from all subjects. A total of 90 individuals that consulted consecutively at the Center of Hypertension of the Austral University Hospital were screened for the following exclusion criteria: previous cardiovascular events, diabetes, smoking history, and cancer. Thirty treated essential hypertensive patients (Hypertensive group) that had

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achieved goal blood pressure were included. Patients were asked to withdraw their antihypertensive medications for 24 h in advance of the day when they underwent anthropometric, metabolic (fasting glycaemia and lipid profile), autonomic, and flow-mediated dilation evaluations. Twenty healthy normotensive volunteers (Normotensive group) that were not receiving any medication were studied to obtain reference values for EPC and TNT. Office blood pressure was measured with a calibrated and validated semiautomatic oscillometric device (Omron HEM-781CPINT; Omron Healthcare, Bannockburn, IL) according to JNC7 (9), with an appropriate cuff size and by averaging the second and third readings after the patients had been seated for 5 min.

Assessment of autonomic function. In Hypertensive patients, heart rate variability (HRV) was measured in the morning after 40 min of supine rest in a quiet room, at 22°C. Frequency domain analysis of short-term HRV was performed by using an autoregressive model (Kubios software) in three settings: basal (during a 5-min supine rest), during a deep breathing maneuver [expiration/inspiration (E/I) maneuver, with 6 metronomic breaths/min], and while performing a mental stress test (Stroop color-word test). Beat-to-beat R-R intervals (i.e., HRV) and systolic blood pressure (SBP) were continuously monitored throughout these maneuvers (29, 32a).

Spectral analysis of HRV data yielded the low (0.05–0.15 Hz, LF)- and high (≥0.15 Hz, HF)-frequency components of total power. For the assessment of autonomic balance, the cardiac parasympathetic reserve was evaluated by analyzing HF (ms²) during both the 5-min rest period and the E/I maneuver, whereas the cardiac sympathetic response was assessed by analyzing the LF-to-HF ratio (%) during the 5-min rest and the mental stress test.

EPC culture and characterization. A 40-ml sample of venous blood was used for the isolation of EPC by density gradient centrifugation (Histopaque 1077, catalog no. H8889; Sigma-Aldrich, St. Louis, MO). Samples were processed within 2 h after collection. The mononuclear peripheral blood cells were washed one time with phosphate-buffered saline and two times in growth medium (BioCoat Endothelial Cell Culture Environment, catalog no. 355054; Becton-Dickinson, Bedford, MA) supplemented with 20% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). The isolated cells were resuspended in growth medium and plated on human fibronectin-coated (catalog no. 354559) or rat collagen-coated (catalog no. 354557) six-well dishes (BioCoat cellware; Becton-Dickinson) for early EPC (5 × 10⁵ cells/well) and late EPC (2 × 10⁷ cells/well), respectively. After 48 h, the nonadherent cells were discarded, and the adherent cells were cultured during 9 days or 21 days for early EPC and late EPC, respectively (27). Growth medium was changed every other day, and digital photographs were obtained. To confirm the endothelial phenotype, at the end of the growth curve, cultured cells were characterized by exposure to Di-acetylated low-density lipoproteins (LDL; catalog no. L3484; Invitrogen, Carlsbad, CA), FITC-conjugated lectin from Ulex europaeus (catalog no. L9006; Sigma-Aldrich), and monoclonal antihuman PE-conjugated CD45 (catalog no. P7687; Sigma-Aldrich). CD14 and vascular endothelial growth factor receptor-2 (VEGFR-2) were revealed by indirect fluorescence immunohistochemistry, by using monoclonal anti-human CD14 (catalog no. C7673; Sigma-Aldrich) and VEGFR-2 (catalog no. V3003; Sigma-Aldrich) antibodies, followed by FITC-conjugated anti-mouse IgG (catalog no. F5687; Sigma-Aldrich). TNT were identified by microscopy and fluorescent phalloidin staining (Alexa Fluor 594 phalloidin, catalog no. A12379; Molecular Probes, Life Technologies, Carlsbad, CA) on days 9 and 21 for early EPC and late EPC, respectively. Digital images and CellS classic software (Texas A&M University of Technology) were used to count the cells in six randomly selected microscopic fields per well by one operator who was blinded to the corresponding subject’s group. TNT were quantified manually on digital images by one observer who was masked to the subject’s group. For EPC and TNT counting, intraserver variability, assessed by calculating the coefficient of variation (CV [%] = mean of SD × 100/data mean), were 5.0 and 5.3%, respectively. The interassay CV% were 7.5 and 7.1% for EPC and TNT counting, respectively.

Effects of epinephrine and norepinephrine on cultured EPC. Early and late EPC were cultured in the presence of epinephrine hydrochloride (catalog no. E4642; Sigma-Aldrich) or norepinephrine bitartrate (catalog no. A9512; Sigma-Aldrich) that were dissolved in PBS and added to the culture medium at the final epinephrine concentrations reported for human plasma (11) at standing, during exercise, and in response to other types of stress (25, 100, and 400 ng/ml, respectively; n = 6 independent assays each) and at plasma norepinephrine levels reported at standing and during exercise (100 and 200 ng/l, respectively) (17). Control cells were added with PBS. To test the effects of these catecholamines on EPC adhesion to fibronectin and collagen, epinephrine or norepinephrine were added to the mononuclear cell culture medium at the time of seeding on the respective culture dishes, and adherent EPC were counted 48 h later; whereas, to test their effects on EPC growth, mononuclear cells were seeded on fibronectin or collagen in the absence of epinephrine or norepinephrine, and 48 h later, after having discarded nonadherent cells, the catecholamines were added to adherent EPC. The catecholamine-supplemented media were replaced every 48 h, and early and late EPC were counted on days 9 or 21, respectively, as was described in EPC culture and characterization.

Brachial artery flow-dependent endothelium-mediated dilation. To evaluate endothelial function status in treated hypertensive patients, brachial artery flow-dependent endothelium-mediated dilation was measured in the morning under fasting conditions, with patients supine in a quiet room kept at 22°C using a high-resolution device (Esaote Caris 7230, Genova, Italy). The international Brachial Arterial Reactivity Task Force guidelines were followed (7). In brief, brachial artery diameter measurements were obtained with a 10-MHz transducer positioned perpendicular to the vessel in the upper arm by using a stereotactic clamp to ensure that the measurements were made in the same arterial segment, and to avoid transducer displacement. Ultrasonic gel was used as the transmitting medium. Brachial artery blood flow velocity was obtained continuously by pulsed Doppler signal, in the arm opposite to that used for blood extraction. After positioning a blood pressure cuff in the upper arm, baseline vessel diameter and blood flow were acquired. The cuff was then inflated to ≥10 mmHg above SBP, to occlude arterial flow for 3 min. Continuous recordings of the longitudinal image of the artery were obtained starting 30 s before and up to 2 min after cuff deflation. To assess hyperemic flow, the Doppler signal was registered immediately after cuff release for a maximum of 15 s. The information obtained was processed with a Hemodyn 4M instrument. The flow-mediated dilator response, expressed as a percentage of the baseline brachial artery diameter, was used as an estimation of endothelium-dependent vasodilation. This method is routinely used in our laboratory.

Statistical analysis. Because the data were not normally distributed, differences between the Hypertensive and Normotensive groups in median values of EPC and TNT and of anthropometric variables were tested by the nonparametric Mann-Whitney U-test. Correlations between EPC parameters and autonomic evaluation parameters were assessed both by Spearman rank-order correlation test (for not normally distributed data) and by simple linear regression after natural logarithmic transformation of the variables. Multivariate linear regression analysis with either EPC or TNT as predicted variables and autonomic and evaluation parameters, and blood pressure as predictor factors was assessed according to the forward-stepwise method. Those parameters appearing clearly uncorrelated to EPC or TNT numbers by the Spearman test and simple linear regression were excluded from the multivariate analysis.

RESULTS

Subjects’ characteristics. The characteristics of the study subjects are shown in Table 1. The Hypertensive and Normotensive groups showed no differences in age and gender distribution, body mass index (BMI), LDL- and high-density lipoprotein (HDL)-cholesterol levels, and glycemia. In the
CD14 antibodies (red and green fluorescence, respectively) and steps:

1. We used an indirect approach consisting of two antibodies, because they both emitted light in the red region.
2. This was done to costain cells with CD45 fluorescent and Dil-acetylated LDL, which is used as a live cell marker.
3. Because we were interested in identifying early EPC, we stained these cells with a mixture of live and dead cells.
4. This allowed us to differentiate between early and late EPC.
5. Early EPC, but not late EPC, expressed the hematopoietic marker CD14.
6. The late EPC were characterized as thin, straight, actin-rich cytoplasmic projections, with a length equivalent to several cell diameters, and crossing from one cell to another (as shown in Fig. 2).

Ex vivo cultured early EPC, late EPC, and TNT are reduced in controlled-essential hypertensive patients. Table 2 shows the quantification of EPC and TNT (i.e., TNT projected by EPC). TNT counting refers to the total number of EPC-emitting TNT per microscopic field. For early EPC and late EPC, TNT started to be evident on days 5 and 10 of culture, respectively. In the Hypertensive group, early EPC, early TNT, late EPC, and late TNT were significantly less abundant than in the Normotensive group (43, 78, 51, and 89%, respectively).

The percentage of early EPC and late EPC that emitted TNT was significantly lower in Hypertensive patients than in Normotensive individuals (Table 2).

Relation between autonomic status and EPC numbers in controlled-essential hypertensive patients. Table 3 shows cardiac parasympathetic reserve and sympathetic response as evaluated by analyzing the HF and LF-to-HF ratio components of HRV in the Hypertensive group, during a 5-min rest, the E/I maneuver, and the mental stress test.

Considering that the data were not normally distributed, the Spearman correlation coefficient was used to assess the strength of the relation between variables. However, given that the robustness of Spearman correlation is lower than that of Pearson correlation coefficient, we also used the latter method after normalization by natural logarithmic transformation of the data.

In the Hypertensive group, late EPC number was positively related to the cardiac parasympathetic reserve during the E/I maneuver, both when assessed by statistical nonparametric analysis (Table 3) and after natural logarithmic transformation and linear regression analysis (Fig. 3).

In contrast, in the Hypertensive group, late TNT numbers were negatively associated with cardiac sympathetic response during the mental stress test (Table 3). During the rest period, no relations were found between either early EPC, early TNT, late EPC, or late TNT and cardiac parasympathetic reserve or sympathetic response.

To establish whether risk factors or hypertensive patients’ characteristics other than cardiac parasympathetic reserve and sympathetic response might have partly explained the reduced late EPC and late TNT counts, a multiple linear regression analysis was performed. No relations were found between any of early EPC, early EPC, late EPC, or late TNT and cardiac parasympathetic reserve or sympathetic response.

**Table 2. Subjects’ characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hypertensive (n = 30)</th>
<th>Normotensive (n = 20)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>49 (24–69)</td>
<td>50 (27–66)</td>
<td>0.780</td>
</tr>
<tr>
<td>Males, %</td>
<td>81.8</td>
<td>80.0</td>
<td></td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>130 (120–137)</td>
<td>114 (107–119)</td>
<td>0.008</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>85 (61–88)</td>
<td>75 (64–79)</td>
<td>0.039</td>
</tr>
<tr>
<td>Medications, %</td>
<td>63.5</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>ACEI/ARAII</td>
<td>41.7</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺ antagonist</td>
<td>33</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>17</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>18</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.0 (19.1–39.0)</td>
<td>25.0 (18.1–27.8)</td>
<td>0.1189</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>4.96 (3.93–6.44)</td>
<td>3.99 (3.41–5.17)</td>
<td>0.0518</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.34 (1.06–2.33)</td>
<td>1.86 (1.19–2.64)</td>
<td>0.1564</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>3.06 (1.81–4.19)</td>
<td>2.60 (2.12–3.15)</td>
<td>0.2120</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>1.05 (0.54–2.10)</td>
<td>0.85 (0.74–1.24)</td>
<td>0.0272</td>
</tr>
<tr>
<td>Glyceremia, mmol/l</td>
<td>5.05 (4.50–6.22)</td>
<td>4.91 (4.78–5.22)</td>
<td>0.4715</td>
</tr>
</tbody>
</table>

Values are expressed as median (minimum-maximum value); n, no. of subjects. SBP, systolic blood pressure; DBP, diastolic blood pressure; ACEI, angiotensin-converting enzyme inhibitor; ARAII, angiotensin II receptor antagonist; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride.
than early EPC treated with PBS, but at 100 and 400 ng epinephrine/l, cell adhesion was progressively and significantly reduced by 34 and 63%, respectively (Fig. 4A1). A negative dose-response behavior was also observed when the cells were exposed to norepinephrine at 100 and 400 ng/l, which decreased EPC adhesion to fibronectin by 16% (not significant) and 50%, respectively, relative to PBS-treated cells (Fig. 4A1). However, when seeded over collagen, cell incubation with epinephrine at 100 and 400 ng/l or norepinephrine at 200 ng/l slightly but significantly reduced EPC adhesion (by 3.7, 7.7, and 3.6%, respectively) compared with cells incubated in the presence of PBS (Fig. 4A2).

In contrast, exposure to epinephrine showed a positive dose-response relationship on early EPC growth, i.e., early EPC count on day 9 of culture was 17 and 39% higher in cells incubated with epinephrine at 100 and 400 ng/l, respectively, relative to PBS-treated cells, whereas 25 ng epinephrine/l had no effect. Incubation of early EPC with norepinephrine at 100 and 200 ng/l stimulated cell growth by 43 and 41%, respectively, relative to incubation without norepinephrine (Fig. 4B1). When cultured over collagen, incubation with norepinephrine had no effect on late EPC growth, and only 400 ng epinephrine/l reduced cell growth by 20% vs. PBS-treated cells (Fig. 4B2).

The amount of cultured early EPCs is positively correlated with flow-dependent, endothelium-mediated dilation. In controlled-hypertensive patients, the median value for brachial artery flow-dependent, endothelium-mediated dilation (FMD) was 4.88%, with minimum and maximum values of 0 and 19.3%, respectively. Ex vivo cultured early EPC counts were positively correlated with brachial artery FMD [Spearman rho = 0.655; P = 0.049; and linear regression analysis (Fig. 5)].

No relation was found between the arterial blood flow response [median (maximum-minimum values) = 139 (68–]
181) ml/min] to reactive hyperemia and FMD (Spearman rho = 0.036; P = 0.8342), indicating that, although increases in blood flow-associated shear stress are known to trigger the vasodilatory response, other factors exerted a predominant influence on FMD modulation.

**DISCUSSION**

The present work shows that, in the treated essential hypertensive patients studied, although the blood pressure achieved was within the range recommended by the JNC7 guidelines, 1) the number and function of ex vivo cultured early and late EPC are reduced when compared with normotensive subjects, 2) an autonomic profile characterized by sympathetic overactivity/parasympathetic underactivity is negatively associated with EPC and TNT numbers, 3) epinephrine and norepinephrine negatively affect early and late EPC adhesion and to a lesser extent stimulate ex vivo early EPC growth, whereas epinephrine reduces late EPC growth, and 4) a positive correlation

Table 2. Quantification of cultured early EPC, early TNT, late EPC, and late TNT isolated from peripheral blood in treated essential hypertensive patients and normotensive subjects

<table>
<thead>
<tr>
<th>Type of EPC or TNT (No./microscopic field)</th>
<th>Hypertensive (n = 30)</th>
<th>Normotensive (n = 20)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early EPC</td>
<td>223.5 (87.8–551)</td>
<td>392 (248–681)</td>
<td>0.0015</td>
</tr>
<tr>
<td>Early TNT</td>
<td>13.2 (0.83–54.5)</td>
<td>59.7 (24.8–99.8)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Late EPC</td>
<td>157.6 (52.4–409)</td>
<td>324 (215–634)</td>
<td>0.005</td>
</tr>
<tr>
<td>Late -TNT</td>
<td>2.60 (0–29.3)</td>
<td>24.2 (19.2–44.6)</td>
<td>0.008</td>
</tr>
<tr>
<td>(Early TNT/early EPC) × 100, %</td>
<td>5.30 (0–17.6)</td>
<td>12.3 (5.89–28.2)</td>
<td>0.012</td>
</tr>
<tr>
<td>(Late TNT/late EPC) × 100, %</td>
<td>2.30 (0–13.1)</td>
<td>7.51 (3.0–15.6)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Values are expressed as median (maximum–minimum value); n, no. of subjects. Early TNT, no. of late EPC that project tunneling nanotubes (TNT); late TNT, no. of late EPC that project tunneling nanotubes. The numbers of cultured early (day 9) and late (day 21) EPC/microscopic field that were isolated from peripheral blood in controlled-essential hypertensive patients (Hypertensive) and normotensive individuals (Normotensive) and seeded on fibronectin (early EPC) or collagen (late EPC) are shown. Also shown is the percentage of cultured early EPC (day 9) and late EPC (day 21) that emitted TNT in cells isolated from treated essential hypertensive and normotensive subjects.
Table 3. Cardiac parasympathetic reserve and sympathetic response and the relation between autonomic status and EPC or TNT numbers in treated essential hypertensive patients

<table>
<thead>
<tr>
<th>Settings at the Time of Measurement</th>
<th>HF, ms²</th>
<th>LF/HF, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>During a 5-min rest</td>
<td>177 (22–934)</td>
<td>2.15 (0.40–10.5)</td>
</tr>
<tr>
<td>During an E/I maneuver</td>
<td>431 (37–2791)</td>
<td>10.3 (1.30–50)</td>
</tr>
<tr>
<td>During a mental stress test</td>
<td>120 (6–570)</td>
<td>4.10 (0.40–14.1)</td>
</tr>
</tbody>
</table>

Parameters Related

<table>
<thead>
<tr>
<th>Correlation (Spearman rho)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late EPC no. and cardiac parasympathetic reserve*</td>
<td>0.450</td>
</tr>
<tr>
<td>Late TNT and cardiac sympathetic response†</td>
<td>-0.426</td>
</tr>
</tbody>
</table>

Values are expressed as median (maximum-minimum value). In hypertensive patients (n = 30), autonomic status was assessed by frequency domain analysis of short-term heart rate variability (HRV). Low (LF)– and high (HF)-frequency components of short-term HRV were analyzed during a 5-min rest, an expiration/inspiration (E/I) maneuver, and a Stroop color-word test. In hypertensive patients, late EPC no. was positively related to the cardiac parasympathetic reserve during the E/I maneuver, and late TNT numbers were negatively associated with sympathetic response during the mental stress test.

exists between cultured early EPC counts and the extent of brachial flow-mediated dilation, indirectly suggesting that EPC alterations are associated with, and can have detrimental effects on, vascular function.

Given that no relations were found between any of the early EPC, late EPC, early TNT, late TNT, and plasma TGs, total cholesterol, or SBP/BDP it follows that the lower number and function of ex vivo cultured early and late EPC observed in hypertensive patients relative to normotensive subjects was not dependent on the differences observed in these particular parameters between both groups.

To the best of our knowledge, this is the first report to show that both cultured late EPC counts and the number of early and late EPC that emit TNT are lower in cultured patients relative to normotensive subjects. The relevance of TNT formation by EPC is evidenced by puzzling data showing that both cultured late EPC counts and the number of early and late EPC that emit TNT are lower in cultured hypertensive patients relative to normotensive subjects. The relation between cardiac sympathetic response and the number of late-EPC no. was positively related to the cardiac parasympathetic reserve during the E/I maneuver, and late TNT numbers were inversely related to cardiac sympathetic response during the mental stress test.

The number of TNT reported here represents the basal level of TNT expression by cells under normal tissue culture conditions. The lower early and late TNT numbers observed in cultured cells from controlled hypertensive patients relative to normotensive subjects cannot be extrapolated to the in vivo situation; however, considering that TNT contribute to cell survival, the present ex vivo finding is consistent with the well-documented reduction of in vivo endothelial repair capacity that occurs in prehypertension and hypertension patients. At present, the signals that guide TNT formation are not completely understood. It should be mentioned that, apart from EPC, TNT were described in other cell types. Also, TNT can facilitate not only the transfer of lysosomes but also that of mitochondria, plasma membrane components, vesicles, 
Ca²⁺, pathogens, and electrical signals. This novel type of intercellular communication participates in such vital processes as tissue regeneration, signal transmission, development, and immunity (1). According to our present results, it can be suggested that, in treated hypertensive patients, the alterations in TNT formation may negatively affect EPC function despite having achieved adequate blood pressure values through pharmacological treatment.

Here we observed that, in treated hypertensive patients, the cardiac parasympathetic reserve was positively related to ex vivo cultured late EPC levels, whereas cardiac sympathetic response was negatively associated with TNT numbers in late EPC cultures. These results suggest that normalization of the sympathetic/parasympathetic balance may increase EPC levels and improve their function, thereby enhancing the capacity for endothelial repair in essential hypertensive patients. The latter concept is supported by both the observed negative dose-response relationship on ex vivo EPC adhesion to fibronectin and collagen, as well as epinephrine’s inhibitory effect on late EPC growth. Although both epinephrine and norepinephrine
stimated early EPC growth, the magnitude of the effect was smaller than the detrimental influence on EPC adhesion. The observed stimulation of early EPC growth by catecholamines is a puzzling finding for which we can only provide a speculative reasoning. Because early EPC represent alternative activated M2 macrophages, we have drawn a potential interpretation from known macrophage responses to catecholamines. The sympathetic nervous system has a dual role, i.e., either anti-inflammatory or proinflammatory, on local inflammatory responses mediated by the actions of norepinephrine and epinephrine on immune cell adrenoreceptors (12). In the case of macrophages, the effects of catecholamines on cell functions seem to be complex and subject to inconsistencies. It has been proposed that the discordance between stimulatory and inhibitory actions of catecholamines is dependent upon the activation state of macrophage populations and the associated changes in the expression levels of different adrenoreceptor types. In this context, to clarify the significance of our unexpected observation that catecholamines stimulated the in vitro growth of early EPC, other studies are needed to assess the expression of adrenoreceptors under the present experimental conditions. Finally, considering that early EPC are supportive cells that, by releasing cytokines can modulate the vasculogenic activity of late EPC, it is feasible that, in the setting of autonomic dysregulation, the stimulation of early EPC growth by epinephrine and norepinephrine might represent a counter-balancing mechanism aimed at buffering the negative effects of both catecholamines on early and late EPC and minimizing any deleterious consequences on vascular maintenance.

In recent years, the sympathetic nervous system was identified as a decisive determinant in progenitor cell mobilization from the bone marrow (22). In relation to this, EPC were shown to express functional β₂-adrenergic receptors that upon stimulation induce EPC migration, proliferation, and differentiation, resulting in improved neoangiogenesis in experimental hind limb ischemia (14). Interestingly, in chronic obstructive pulmonary disease patients, β₂-adrenergic receptor expression in early EPCs was higher than in healthy controls, and treatment of EPC with a β₂-adrenergic receptor antagonist (ICI-118551) increased EPC migration and proliferation compared with treatment with the agonist norepinephrine (24). Concerning the link between the parasympathetic nervous system and
EPC, approximately a decade ago, a previously unknown cholinergic angiogenic pathway mediated by nicotinic acetylcholine receptors (nAChRs) on endothelial cells was described (19). Later, nAChR activation by both local and systemic administration of nicotine was shown to increase the mobilization and/or recruitment of mouse EPC to the site of angiogenesis in ischemic tissues (18); also, nAChRs were identified on fibronectin–cultured human EPCs, and nicotine, via nAChRs, was found to improve human EPC functional activity (36). The archetypal endogenous nAChR agonist is acetylcholine (ACH). The broad hydrolyzing activity of acetylcholinesterase and butyrylcholinesterases in neuronal and nonneuronal tissues, including vascular cells (23), reduces ACh actions to local paracrine or autocrine effects. Of note, choline, which is released from ACh by acetylcholinesterase, can activate at least one of the nAChR subtypes, i.e., the homomeric α6- nAChRs (3); therefore, after ACh hydrolysis, a chance for ACh signaling still remains. Considering the above evidence, it is feasible that ACh (or other longer-lasting nAChR agonists) released from nerve endings may be involved in vascular growth or maintenance. Circumstantial evidence supports the latter concept; thus, as pointed out by Cooke et al. (5), the reduced foot-healing capacity associated with diabetes is preceded by a severe neuropathy, raising the question of whether, in diabetic patients, the loss of nerve-produced trophic agents, including ACh, might participate in the deterioration of angiogenesis. Finally, although parasympathetic innervation to the bone marrow had not been previously reported, parasympathetic activity was recently detected in the skeletal bone marrow, where ACh functioned to decrease bone resorption (4).

Our observations that peripheral blood mononuclear cells from controlled-hypertensive patients gave rise to a lower number of cultured early EPC than those from normotensive subjects, and that early EPC numbers are positively related to flow-mediated dilation, are in line with previous findings by others (16).

Although the antihypertensive treatments were discontinued for 24 h previous to blood sampling, some of the drugs used by the study population [renin-angiotensin-aldosterone system (RAAS) blockers, dihydropyridine Ca2+ antagonists] may have influenced autonomic balances. However, it should be considered that the cardiac sympathetic response during the mental stress test was negatively associated with late TNT numbers, but both kinds of compounds have been described to lower sympathetic activity. Therefore, if an autonomic effect did occur, a confounding influence by RAAS blockers or dihydropyridine Ca2+ antagonists would have weakened the above association instead of strengthening it. Also, although statins have been shown to increase the level of circulating EPC (32), no differences were observed here between hypertensive patients receiving and not receiving statins.

Injured tissues release a variety of mediators that attract EPC to the site and ensure their engraftment (25, 28). In this setting, in coronary artery disease patients, hypertension was identified as a major independent predictor of early EPC blunted migratory capacity (33). Also, compared with healthy individuals, in prehypertension and hypertension subjects in vivo endothelial repair capacity by early EPC is considerably reduced, and this is related to accelerated early EPC senescence and impaired endothelial function. Of note, early EPC telomere length is negatively related to SBP (16). In addition, both in the rat and in humans, hypertension is associated with increased numbers of senescent circulating EPC (38). EPC mobilization from the bone marrow is dependent on NO-mediated activation of matrix metalloproteinase (MMP)-9. Recent experimental evidence supports the concept that, in hypertension, the impairment of EPC mobilization results from insufficient bone marrow NO synthase activity, and the consequent deficiency in NO/MMP-9 signaling (2). Interestingly, in experimental renovascular hypertension, improvements of in vivo repair capacity, in vitro EPC proliferation, and tube formation and homing signals (VEGF and homing receptor expression) are present in the early phase of the disease; however, this putative early compensatory vascular response is lost with longer-lasting hypertension (39). In the present paper, we report additional factors that can interfere with adequate EPC function in hypertension.

Our findings may help to explain why hypertensive patients that have achieved target blood pressure levels through pharmacological treatment still display a high residual cardiovascular risk. Of note, a recent review (11, 30) emphasized the concept that sympathetic nervous system activation is frequently responsible for the origin and maintenance of elevated blood pressure in essential hypertension and that, at present, sympathetic antagonists are underused as treatments for hypertensive patients. If further studies replicate these findings, the next step would be to test whether, by antagonizing the sympathetic nervous system, it is possible to increase EPC numbers in association with the improvement of endothelial function.

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AUTHOR CONTRIBUTIONS


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