Hypertension is a disease accompanied by severe complications such as stroke, ischemic heart disease, and nephrosclerosis. These complications, which are basically associated with arterial damage, are the target of therapy for patients with hypertension. Angiotensin II (Ang II) receptor blockers (ARBs) have been reported to have greater cardiovascular protective effects than other antihypertensive drugs. The protective effects of ARBs on vascular injury have been considered to be associated with inhibitory effects on Ang II–induced vascular growth, extracellular matrix formations, and oxidative stress.

Although several risks, such as hypertension, dyslipidemia, insulin resistance, and diabetes mellitus, primarily cause vascular injury, including endothelial damage, it is thought that abnormalities in the repairing of endothelial damage enhance vascular injury. Endothelial progenitor cells (EPCs) derived from the bone marrow that is present in peripheral blood usually seek out and repair vascular damage. Given that it has been shown that the life spans (cell cycle) of stem cells and progenitor cells, including EPCs, are shortened by oxidative stress, it would seem possible that oxidative stress induces endothelial damage by shortening the EPC life span and causing EPC dysfunction. Tissue Ang II is reportedly associated with cardiovascular complications in essential hypertension. The improvement of EPC function with the administration of angiotensin II receptor blockers is considered to be one of the cardiovascular protective effects.

**Keywords:** angiotensin II receptor blocker; blood pressure; colony formation; endothelial progenitor cell; essential hypertension; human; hypertension.

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We previously demonstrated that the formation and function of EPCs are impaired in salt-loaded stroke-prone spontaneously hypertensive rats (SHR-SPs) with increases in oxidative stress and that treatment with ARBs (losartan, candesartan, and valsartan) improved the functional impairment.\(^6\)\(^-\)\(^8\) In these studies, candesartan increased the number of cardiac stem cells in the heart,\(^7\) and valsartan increased the number of label-retaining cells as renal repairing cells in the kidneys.\(^5\) Thus, ARBs apparently improved the impaired function of the tissue stem cells and EPCs with oxidative stress in the hypertensive animal models. However, there have been no clinical studies to examine effects of ARBs on EPC functions in human essential hypertension.

In this study, we investigated basal EPC function in normotensive control subjects and patients with essential hypertension and evaluated the effects of the ARB losartan on EPC function in patients with hypertension.

**METHODS**

**Study design**

The study was approved by the Ethics Committee at the Nihon University School of Medicine. This was a prospective, randomized, and controlled open-label clinical trial at Nihon University Hospital (clinical trial authorization number: 20266/2004 Nihon University). We obtained written informed consent from all participants. Patients that fulfilled the criteria of essential hypertension were eligible and included in the study. Essential hypertension was defined by systolic blood pressure \(\geq 140\) mm Hg and/or diastolic blood pressure \(\geq 90\) mm Hg at outpatient or home measurement. From 2011 to 2013, eighteen normotensive control subjects and 36 patients with essential hypertension undergoing treatment at Nihon University Hospital were enrolled in this study. Table 1 shows profile of normotensive control subjects and patients with essential hypertension. Before this study, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, and fasting plasma glucose levels were analyzed in all subjects. Serum cholesterol and triglyceride levels were measured by enzymatic methods (Nescauto VL TC and Nescauto VL TG; Nippon Shoji, Osaka, Japan). Any antihypertensive drugs and statins were completely washed out for at least 2 weeks before study entry. Patients were randomly selected to receive 50 mg of losartan at first for 4 weeks and then 4 mg of trichlormethiazide (TCM) daily for 4 weeks or 4 mg of TCM at first and then 50 mg of losartan daily for 4 weeks as a crossover study.

**EPC colony-forming assay**

A modified EPC colony-forming assay was performed as described by Hill et al.\(^9\) In brief, 20 ml of heparinized peripheral blood was collected from normotensive control subjects once. For patients treated with losartan and TCM (on an outpatient basis), blood samples were taken before and after 4 weeks of treatment at Nihon University Hospital.

Mononuclear cells (MNCs) were separated by centrifugation with Histopaque-1083 density gradient medium (Sigma-Aldrich, St. Louis, MO). MNCs were suspended and mixed in 1 mL of EGM-2 medium (Clonetics, San Diego, CA) containing 10% fetal bovine serum, 4 mg/l human basic fibroblast growth factor (Sigma-Aldrich), 1 mg/l vascular endothelial growth factor (VEGF-A; R&D System, Minneapolis, MN), 1 mg/l recombinant insulin-like growth factor 1 (Sigma-Aldrich), 1 mg/l human epidermal growth factor (Sigma-Aldrich), 1 mg/l ascorbic acid, and 1 mg/l GA-1000 (BioWhittaker, Walkersville, MD). Twenty-four-well plates (Falcon, San Jose, CA) were precoated with rat vitronectin plates (Falcon, San Jose, CA) were precoated with rat vitronectin (0.1 µg/cm\(^2\)) plus 0.5% gelatin overnight at 37 °C. MNCs were inoculated into 6-well plates (10\(^6\) cells/well) and cultured in a carbon dioxide incubator at 37 °C for 7 days. The average number of colonies was calculated manually from a minimum of 4 wells under a light microscope by an observer who was unaware of the experimental design.

### Table 1. Profile of normotensive control subjects and patients with essential hypertension

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normotensive control subjects</th>
<th>Hypertensive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>Male/Female</td>
<td>4/14</td>
<td>9/27</td>
</tr>
<tr>
<td>Age</td>
<td>53.9 ± 3.1</td>
<td>59.8 ± 2.0</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>118 ± 2.7</td>
<td>150 ± 4.0*</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>75 ± 1.2</td>
<td>92 ± 2.8*</td>
</tr>
<tr>
<td>MBP, mm Hg</td>
<td>90 ± 1.8</td>
<td>111 ± 2.8*</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>71.1 ± 4.0</td>
<td>63.6 ± 0.5</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>109.8 ± 6.8</td>
<td>129.3 ± 5.3</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>91.7 ± 15</td>
<td>126.4 ± 10.4</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>88.1 ± 2.1</td>
<td>114 ± 6.1</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. Abbreviations: DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MBP, mean blood pressure; SBP, systolic blood pressure.

\(^*P < 0.01\). vs. normotensive control subjects.
Thiobarbituric acid reactive substance assay

Thiobarbituric acid reactive substance (TBARS) in MNCs was measured with a commercial kit (Oxi-Tek TBARS Assay Kit; Zeptometrix, Buffalo, NY). In brief, MNCs (10⁶/100 µl) were mixed with 100 µl sodium dodecyl sulfonate solution. Thiobarbituric acid (TBA)/buffer reagent was prepared by mixing 0.5 g TBA with 50 ml acetic acid and 50 ml NaOH. TBA/buffer reagent (2.5 ml) was added to 200 µl sample/sodium dodecyl sulfonate mixture and incubated at 95 °C in capped tubes for 60 minutes. Samples were cooled to room temperature in an ice bath for 10 minutes and centrifuged at 300 g for 15 minutes. Supernatants were removed, and the fluorescence intensity was measured in semimicro cuvettes in a fluorometer (Bio-Rad, Hercules, CA). The concentration of TBARS was expressed in pmol/10⁶ cells by interpolation from the standard curve of malondialdehyde at concentrations of 0–200 pmol/l.

Statistical analysis

Data were analyzed by Student t test or Student unpaired t test where appropriate. Results are expressed as the mean ± SEM. Multiple regression analysis for systolic blood pressure, diastolic blood pressure, and EPC colony formation number was conducted using the stepwise method with other independent variables (StatView, version 5.0; SAS Institute, Cary, NC).

RESULTS

EPC colony formations

Typical EPC colony formation numbers are shown in Figure 1a. The number of EPC colonies was significantly lower (P < 0.01) in patients with essential hypertension (110 ± 18) than in normotensive control subjects (368 ± 42) (Figure 1b).

Correlation between EPC colony formation numbers and blood pressure

Figure 2 shows correlations between the number of EPC colony formations and systolic blood pressure, diastolic blood pressure, age, and plasma glucose in all subjects (n = 54), including normotensive control subjects. The number of EPC colony formations was significantly and inversely correlated with systolic blood pressure (R = −0.40; P = 0.002) and diastolic blood pressure (R = −0.35; P = 0.009). There was no correlation between the number of EPC colony formations and either age or fasting plasma glucose.

Correlation between EPC colony formation numbers and oxidative stress and serum lipids

Figure 3 shows correlations between the number of EPC colony formations and TBARS score and levels of triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol in all subjects. EPC colony number showed a weak inverse correlation that was not statistically significant with TBARS score (R = −0.10; P = 0.06), and there was a weak inverse correlation with serum triglyceride (R = −0.11; P = 0.046). There was no correlation between EPC colony number and high-density lipoprotein cholesterol or low-density lipoprotein cholesterol levels.

Effects of losartan on EPC colony formation numbers

Figure 4 shows mean blood pressure levels and EPC colony numbers in patients with essential hypertension before (baseline) and after treatment with TCM or losartan for 4 weeks. The level of significance for the decrease in mean blood pressure was the same after the administration of either treatment (P < 0.01). However, whereas the number of EPC colony formations was not significantly affected in the TCM-treated patients, there was significant increase (P < 0.01) in the number of EPC colony formations in patients with essential hypertension who were treated with losartan.

DISCUSSION

Bone marrow–derived EPCs in peripheral blood induce neovascularization and repair vascular damage as repairing...
cells. EPC colony-forming unit assay is a more suitable method for evaluating the vascular repair function than using a CD34 marker to count the number of EPCs in peripheral blood. Reduced EPC numbers have been reported in hypertensive patients with cardiovascular complications. Oliveras et al.\textsuperscript{11} reported that EPC number was reduced in patients with refractory hypertension. Lee et al.\textsuperscript{12} also reported that EPC number was reduced in hypertensive patients with left ventricular hypertrophy. Furthermore, a reduction in the number of EPCs, which was normalized after olmesartan treatment, has been reported in patients with type 2 diabetes mellitus.\textsuperscript{13} 

Regarding the vascular repair function of EPCs, Hill et al.\textsuperscript{9} demonstrated that the number of EPC colonies is inversely correlated with vascular injury and endothelial dysfunction, even in different risks such as hypertension, dyslipidemia, diabetes mellitus, and smoking, suggesting that the EPC dysfunction eventually determines the vascular injury in these risks. There are, however, no reports that evaluate EPC function in hypertensive patients. In this study, the number of EPC colony formations was obviously lower in patients with essential hypertension than in normotensive subjects. Moreover, EPC colony formation number was inversely correlated with blood pressure in all subjects. Endothelial function, which has been reported to be lower in patients with essential hypertension, determines blood pressure levels.\textsuperscript{14} It is possible that impaired EPC function leads to this reduced endothelial function. These results imply that a factor that impairs EPC function is also involved in the elevation of blood pressure. In this study, treatment with losartan significantly improved the reduction in the number of EPC colony formations in hypertensive patients, whereas treatments with TCM did not. Thus the common factor that reduces EPC function and elevates blood pressure might be the renin-angiotensin (RA) system, in particular the tissue Ang II.

We previously demonstrated the effects of ARBs on EPC function in hypertensive rats and made a number of findings: EPC colony formation number and EPC migration were markedly lower in SHR-SPs than in normotensive rats; TBARS scores were significantly higher in SHR-SPs than in Wistar-Kyoto (WKY) rats; and EPC colony formation number was markedly lower in SHR-SPs than in WKY rats. In SHR-SPs, the administration of ARBs markedly increased the reduced colony number with the inhibition of oxidative stress. This was accompanied by

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Correlations between endothelial progenitor cell (EPC) colony formation and (a) systolic blood pressure (SBP), (b) diastolic blood pressure (DBP), (c) age, and (d) plasma glucose (D) in normotensive control subjects (n = 18) and hypertensive patients (n = 36) (all subjects: n = 54).}
\end{figure}
Figure 3. Correlations between endothelial progenitor cell (EPC) colony formation and (a) thiobarbituric acid reactive substance (TBARS) score, (b) serum triglyceride, (c) high-density lipoprotein cholesterol, and (d) low-density lipoprotein cholesterol in all subjects. No correlation was found between EPC colony formation and any of these variables.

Figure 4. Effects of losartan and trichlormethiazide (TCM) on (a) mean blood pressure (MBP) and (b) endothelial progenitor cell (EPC) colony formation in patients with essential hypertension. Patients with essential hypertension were randomly selected to receive 50 mg of losartan or 4 mg of TCM daily for 4 weeks. Mononuclear cells isolated from peripheral blood were plated onto culture plates precoated with vitronectin. After 7 days of culture, nonadherent cells were removed, and attached cells formed distinct colonies. Data are shown as the means ± SEM. *P < 0.05, **P < 0.01.
a reduction in tissue expression of NADPH components gp91\textsuperscript{phox}, p22\textsuperscript{phox}, and p47\textsuperscript{phox}.\textsuperscript{6} Taken together, this indicates that EPC function was impaired in Ang II–dependent hypertension with oxidative stress and that the administration of ARBs improved the impaired EPC function in hypertensive rats by the reduction of oxidative stress. In human essential hypertension, tissue levels of Ang II are thought to be involved in cardiovascular injury associated with endothelial damage through the impairment of the EPC function.

It has been established that ARBs exert cardiovascular and renal protective effects in essential hypertension and diabetes mellitus beyond their blood pressure–lowering effects, which are thought to be associated with inhibition of tissue Ang II and oxidative stress.\textsuperscript{15} In the LIFE study, a large clinical trial, losartan was shown to prevent more cardiovascular diseases, including stroke and cardiovascular death, than atenolol, while achieving a similar reduction in blood pressure. This would indicate that losartan is of greater benefit because of its prevention of cardiovascular complications beyond blood pressure reduction.\textsuperscript{16} Thus the cardiovascular protective effects of ARBs have been established in the research and clinical fields, in which improved EPC function is a novel aspect of their cardiovascular protective effects.

Losartan treatment for 4 weeks significantly increased the number of EPC colonies from 110 per well to 170 per well in patients with essential hypertension. The level of increase in EPC colonies after treatment with losartan is lower in hypertensive patients than the 5 per well to 220 per well in the patients of this study than the 5 per well to 220 per well increase that we found with SHR-SPs;\textsuperscript{6–8} however, the basal number of EPC colonies is markedly higher in hypertensive patients than in SHR-SPs. In this study, the washout period for hypertensive patients who had received antioxidative medicines, including ARBs and statins, was 2 weeks. A possible explanation for the difference in basal number is that this 2-week washout period was insufficient. Moreover, patients with hypertension show rather different physical features and have life habits in the clinical studies than experimental studies using hypertensive models as SHR-SPs showing identical blood pressure and biological abnormalities including the increases in oxidative stress markers in blood. In addition, improvement of the impaired EPC function by losartan treatment without decreases in TBARS scores indicates that the tissue levels of Ang II impair the EPC function in hypertensive patients. Thus, the number of EPC colony formations might not be reversely correlated with TBARS scores in all subjects.

ARBs induce their antihypertensive effects by inhibiting the binding of Ang II to the AT1 receptor, which is present in several organs. A possible mechanism for the improvement of EPC function in hypertensive patients who receive losartan treatment is losartan acting on the RA system present in bone marrow. The presence of all major RA system components was confirmed in bone marrow cells, including stromal cells, hematopoietic stem cells, and mesenchymal stem cells. This finding leads to the concept of a potential autocrine/paracrine mechanism for local RA system–mediated regulation of the hematopoiesis.\textsuperscript{17} AT1 receptor signaling has been reported to regulate the hematopoietic proliferation by stimulation of a monocyte colony stimulating factor.\textsuperscript{18} Furthermore, Ang II has been recognized to induce apoptosis and senescence of EPCs through AT1 receptor signaling.\textsuperscript{19} Moreover, Ang-(1–7) formed from Ang II with angiotensin-converting enzyme 2 (ACE2) has been reported to have potent effects to hematopoietic cells in bone marrow.

Ang-(1–7) stimulates the proliferation of CD34-positive and mononuclear cells including EPCs.\textsuperscript{20} ARBs have been reported to increase Ang-(1–7) through the inhibition of the AT1 receptor, which suppresses ACE2-forming Ang-(1–7).\textsuperscript{21} Agata et al.\textsuperscript{22} demonstrated that ARB increases endogenous Ang-(1–7) through the overexpression of ACE2. Thus it is possible that losartan stimulates EPC colony formation through the generation of Ang- in bone marrow and/or suppression of the Ang II–mediated senescence of EPCs. Chen et al.\textsuperscript{23} demonstrated that the abilities of EPC migration and tube formation are impaired in human renin and angiotensinogen transgenic mice, which are improved with ACE2 overexpression. These effects were inhibited by ACE2 or eNOS inhibitor and further enhanced by Nox inhibitor, indicating that ACE2 improves EPC function by the regulation of eNOS and Nox pathways.

On the other hand, we investigated presence of RA system in EPCs and found that EPCs expressed renin, cathepsin D, chymase, and AT1 receptor and AT2 receptor mRNAs,\textsuperscript{7} indicating the presence of the RA system in EPCs. It is possible that losartan inhibits the binding of Ang II to the AT1 receptor in the EPCs themselves and/or in bone marrow. Thus ARBs may act on the local RA system present in bone marrow to potentiate hematopoiesis, which increases EPCs, and/or act on the RA system that is present in the EPCs to suppress their senescence.

In this study, there was a weak inverse correlation between EPC colony formation and serum triglyceride in all subjects. Bahlmann et al.\textsuperscript{13} reported that EPC number was decreased in patients with diabetes mellitus. There have also been reports of decreases in EPC number in peripheral blood in metabolic diseases such as dyslipidemia\textsuperscript{24} and nonalcoholic fatty liver disease.\textsuperscript{25} Thus the decreases in EPC number and function are observed in metabolic diseases and may be associated with the cardiovascular damage related to these diseases. In hypertensive patients, serum levels of triglyceride, cholesterol, and glucose are higher than those in normotensive control subjects. Thus hypertensive patients had more metabolic abnormalities that may be associated with the suppression of EPC function.

In summary, we demonstrated that basal EPC function was inversely correlated with blood pressure and impaired in essential hypertension. Such impairment may reduce the vascular regeneration potential and contribute to the pathogenesis of vascular complications in hypertension. Losartan significantly improved the impaired EPC function in hypertensive patients, which is considered to be one of the cardiovascular protective effects of ARBs. Thus, therapy with ARBs may provide a novel and effective therapeutic strategy for the repair of cardiovascular damage.
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DISCLOSURE
The authors declared no conflict of interest.

REFERENCES