Dietary intervention with Okinawan vegetables increased circulating endothelial progenitor cells in healthy young women

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ARTICLE INFO

Article history:
Received 31 January 2008
Received in revised form 15 July 2008
Accepted 24 September 2008
Available online 11 October 2008

Keywords:
Endothelial progenitor cells
Vegetable
Nutrition
Dietary intervention
Homocysteine
Okinawa

ABSTRACT

Objective: Circulating endothelial progenitor cells (EPCs) play a critical role in maintaining the integrity of vascular vessels. The number of EPCs inversely correlates with the number of atherosclerotic risk factors. Although nonpharmacological treatment represents the first approach to the primary prevention of atherosclerotic diseases, little is known about the effects of diet on EPCs. We investigated the effect of a dietary intervention with vegetables that are commonly eaten in Okinawa on the number of EPCs.

Methods and results: Forty-five healthy young women were employed and randomized to a dietary intervention group (n = 24) or a control group (n = 21). Subjects in the intervention group received typical Okinawan vegetables through home-parcel delivery for 2 weeks. After the dietary intervention, urinary potassium and magnesium excretion increased only in the intervention group and changes were greater than in the control group (p = 0.007, 0.010, respectively). The consumption of total vegetables correlated with changes in both urinary potassium and magnesium excretion. Serum folic acid increased and plasma homocysteine decreased in both groups but the change was significant only in the intervention group. The EPCs number significantly increased in the intervention group but did not in the control group. An inverse correlation was observed between EPC number and plasma homocysteine level (r = −0.272, p = 0.016). Changes in the EPC number inversely correlated with changes in both serum total cholesterol and low-density lipoprotein cholesterol level (r = −0.555, p = 0.0002; r = −0.626, p < 0.0001, respectively).

Conclusions: The consumption of vegetables increased the number of circulating EPCs; this change might be associated with a homocysteine-lowering effect.

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1. Introduction

Okinawa prefecture in Japan is well known as the leading area for longevity in the world and also has had the lowest rate of mortality due to cardiovascular disease, including coronary artery disease and stroke, until recently [1,2]. Epidemiological studies have revealed the importance of diet in preventing cardiovascular disease [3,4]. In fact, Okinawan elders have unique traditional eating habits characterized by low salt and fat intake and high consumption of antioxidant-rich vegetables [2,5]; this diet has been considered to be one of the major factors influencing longevity.

Circulating endothelial progenitor cells (EPCs) in the peripheral blood, mobilized from bone marrow, play important roles in maintaining the integrity of vascular vessels and endothelial function as well as in postnatal neovascularization [6]. Recent studies have shown an inverse relationship between the number of circulating EPCs and cardiovascular risk factors both in patients with cardiovascular disease and in healthy people [7,8]. Furthermore, a single measurement of EPCs is useful for predicting cardiovascular outcomes in patients with coronary artery disease [8,9], which suggests that EPCs can act as a prognostic biomarker. Certain drugs, such as statins, can increase the number of circulating EPCs [10]; however, few studies have been done to investigate the relationship between diet and EPC number. This study examined the effect of dietary intervention with typical Okinawan vegetables through home-parcel delivery on the number of circulating EPCs.

2. Methods

2.1. Subjects

This randomized controlled study employed 45 healthy free-living female volunteers living in Okinawa aged between 18 and 38 years. None of them were being treated for any disease at the.
time of the study. They were randomized to a dietary intervention group \((n = 24)\) or a control group \((n = 21)\). Six subjects were finally excluded from the statistical analysis due to incomplete samples collection \((n = 3)\) and smoking during the study period \((n = 3)\). As a result, 22 subjects in the intervention group and 17 in the control group were analyzed.

### 2.2. Study design

This study was approved by the Medical Ethics Committee of the University of the Ryukyus, and written informed consent was obtained from all participants before enrollment. The study design was described previously [11]. Briefly, the dietary intervention group received five kinds of vegetables that are commonly eaten in Okinawa (a total of 2.6 kg/week) at home through a home-parcel delivery service for a period of 14 days. The delivered vegetables included 800 g/week of Goya (Momordica charantia), 1200 g/week of green papaya (Carica papaya), 200 g/week of Handama (Gynura bicolor), 200 g/week of Karashina (Brassica juncea), and 200 g/week of one of three others: Njana (Crepidiastrum lanceolatum), Fuchiba (Artemisia vulgaris) or Pudanso (Beta vulgaris). The 2.6 kg/week supply of vegetables was based on the national recommendation for vegetable intake (350 g/day) from the Japan Ministry of Health, Labor and Welfare. Subjects in the control group were asked to avoid eating Okinawan vegetables. We asked the subjects to weigh and record all vegetables, fruits, and juices that they consumed during the intervention period. We employed young women for this study, because they usually cook at home by themselves. In addition, because the average vegetable intake of Japanese young women is lower than that of over 30’s women, we predicted that the effects of dietary intervention would be more obvious in this population. The following measurements of physical parameters and laboratory samplings were taken: fasting blood and 24h urine samples were taken before and after the dietary intervention; blood pressure was measured three times in the right arm in a sitting position after a short rest using an automatic digital sphygmomanometer (HEM-762, Omron Corp.); and reactive oxygen species (ROS) were assessed by TBARS (TBARS Assay Kit, ZeptoMetrix Corp.) and the d-ROM test (Free Radical Analytical System4, Wismerll) according to the manufacturer’s protocol. The laboratory personnel were blinded to the subjects and the coding system used to label the sample.

### 2.3. Isolation and characterization of EPC

We examined circulating EPCs as described previously [7]. In brief, immediately after collecting peripheral blood (25–30 ml), mononuclear cells were isolated by density-gradient using Histopaque-1077 (Sigma), seeded on culture dishes coated with fibronectin (Sigma) in endothelial growth medium-2 with Single-Quots (ECM-2 MV Bullet Kit, Cambrex Corp.) including 20% fetal calf serum, and incubated in a 5% CO\(_2\) incubator at 37 °C. After 4 days of culture, no adherent cells were discarded in a gentle wash with PBS, and fresh media were added. Adherent cells were then maintained in culture for 3 more days. After 7 days of culture, EPCs were characterized by dual staining for 11′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated LDL (2.5 μg/ml, Biogenesis) and FITC-conjugated Ulex europaeus agglutinin (UEA)-1 lectin (10 μg/ml, Sigma). We counted the number of EPCs in 10 random laser scanning confocal microscopic fields.

### 2.4. Statistical analysis

Data are presented as mean ± S.D., and continuous variables were evaluated using a non-paired \(t\)-test or paired \(t\)-test. Correlations between circulating EPCs and others were analyzed with Pearson’s correlation coefficient. Differences with \(p < 0.05\) were considered significant. Multiple regression analysis was used to exclude influence of the confounding factors. Statistical analysis was done with StatView version 5.0.

### 3. Results

#### 3.1. Characteristics of the subjects

The baseline characteristics of the subjects were similar between the two groups (Table 1). There were no differences in terms of body height, body weight, body mass index (BMI), and blood pressure. These parameters did not change after 2 weeks of dietary intervention in both groups (data not shown).

#### 3.2. Vegetable consumption

Subjects in the dietary intervention group consumed a higher amount of total vegetables than subjects in the control group: 356 g/day in the dietary intervention group and 200 g/day in the control group \((p = 0.0001)\). About 371 g/day of Okinawan vegetables were delivered to the intervention group, but the subjects consumed only 169 g/day of the Okinawan vegetables. In contrast, subjects in the control group, who were not supplied Okinawan vegetables, consumed only 40 g/day of Okinawan vegetables during the intervention period \((p < 0.0001)\). There were no differences in the mean amount of other vegetables consumed \((187 g/day versus 161 g/day, p = 0.468, in the intervention and control groups, respectively); therefore the total consumption of vegetables was higher in the intervention group. There was no significant difference in the mean amount of fruits consumed in both groups \((125 g/day versus 101 g/day, p = 0.356, in the intervention and control groups, respectively).

#### 3.3. Changes of urinary and blood laboratory data

After dietary intervention, 24h urinary potassium excretion significantly increased in the intervention group \((1.6 ± 0.4 g/day to 2.5 ± 1.4 g/day)\) compared with the control group \((1.7 ± 0.6 g/day to 1.6 ± 0.8 g/day, p = 0.007)\) (Table 2). Urinary magnesium excretion also increased in the dietary intervention group \((0.08 ± 0.02 g/day to 0.10 ± 0.05 g/day)\) compared with the control group \((0.09 ± 0.03 g/day to 0.07 ± 0.04 g/day, p = 0.010)\). Urinary sodium excretion decreased in the control group \((p = 0.021)\). Serum folate increased in both groups, but the change was significant only in the intervention group \((5.4 ± 2.1 ng/ml to 6.0 ± 2.6 ng/ml, p = 0.041)\). The level of plasma homocysteine decreased in both groups, but the decrease was significant only in the intervention group \((7.6 ± 2.9 nmol/ml to 7.2 ± 2.7 nmol/ml, p = 0.022)\). Changes of serum folate acid and plasma homocysteine were not different between two groups. An inverse correlation was observed between serum folate acid and plasma homocysteine in all subjects.

#### Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Intervention group ((n = 22))</th>
<th>Control group ((n = 17))</th>
<th>(p)-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>24.5 ± 4.1</td>
<td>26.2 ± 5.3</td>
<td>0.2428</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>156.4 ± 4.4</td>
<td>158.8 ± 5.1</td>
<td>0.1252</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>49.8 ± 5.3</td>
<td>51.3 ± 5.7</td>
<td>0.3911</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20.3 ± 1.6</td>
<td>20.4 ± 2.2</td>
<td>0.9415</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>109 ± 9</td>
<td>113 ± 10</td>
<td>0.1481</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>68 ± 7</td>
<td>69 ± 7</td>
<td>0.7399</td>
</tr>
</tbody>
</table>

Data are mean ± S.D. BMI: body mass index.
3.5. Effect of dietary intervention on EPCs number

The number of EPCs was not significantly different between the two groups at baseline (172 ± 83/field versus 138 ± 106/field, $p=0.268$ in the intervention and control groups, respectively). Dietary intervention significantly increased the number of EPCs only in the intervention group ($p=0.036$) but not in the control group ($p=0.641$). Changes of EPCs number did not have significant difference between two groups (Table 2).

3.6. Factors associated with EPC number

The number of EPCs was inversely correlated with plasma homocysteine level ($r=-0.272$, $p=0.016$) (Fig. 1A) in all subjects. When we included age, systolic blood pressure, homocysteine, LDL-C, urinary sodium, urinary potassium, urinary magnesium and ROS marker in a multivariate analysis, only plasma homocysteine level was negatively correlated with the numbers of EPCs ($\beta=-0.304$, $p=0.015$). To further investigate the effects of homocysteine on the EPC number, we isolated and cultured peripheral blood mononuclear cells in vitro from healthy volunteers with homocysteine for 3 days. Treatment with homocysteine decreased the attached and differentiated EPC number in a concentration-dependent manner (Fig. 1B).

There was no correlation between the number of EPCs and the consumption of vegetables, body weight, BMI, blood pressure, urinary potassium excretion, urinary magnesium excretion, serum HDL-C, TG and ROS. An inverse correlation was observed between the changes in the EPC number and the changes in total serum TC ($r=-0.535$, $p=0.0002$) and LDL-C concentrations ($r=-0.626$, $p<0.0001$) (Fig. 2). Then, by the use of multivariate analysis, changes in the EPC number were significantly correlated with only the changes in TC and LDL-C concentration ($\beta=-0.613$, $-0.686$; $p=0.0006$, $<0.0001$, respectively), when age, systolic blood pressure, homocysteine, LDL-C and dietary intervention were included in the model.
pressure, changes of homocysteine, ROS marker, urinary sodium, urinary potassium, urinary magnesium, and vegetable consumption were included.

4. Discussion

We demonstrated here that dietary intervention with typical Okinawan vegetables through home-parcel delivery increased the number of EPCs, urinary potassium and magnesium excretion in healthy young women. Consumptions of vegetables correlated with changes in both urinary potassium and magnesium excretion. The number of EPCs inversely correlated with homocysteine level. Changes in the EPC number inversely correlated with changes in both TC and LDL-C level.

Accumulating evidences has shown that increased consumption of vegetables and fruits is associated with reduced risk of cardiovascular diseases such as stroke and coronary artery disease [3,4]. The protective effects of vegetables and fruits against cardiovascular disease have been considered to be derived from nutrients such as potassium, folate, fiber, and antioxidant vitamins. Dietary folate is one of the determinants of plasma homocysteine levels. Although the effects of homocysteine-lowering therapy with folic acid and vitamins on cardiovascular disease endpoints are still conflicting [14], epidemiological data suggested that there was an association between plasma homocysteine and cardiovascular disease mortality [15,16], and Okinawans have plasma homocysteine levels among the lowest in the world [16]. We showed here that dietary intervention with Okinawan vegetables for 2 weeks increased serum folic acid and decreased plasma homocysteine level. Moreover, there was an inverse correlation between homocysteine levels and the number of EPCs in this study. We also confirmed that treatment with homocysteine dose-dependently inhibited the attached and differentiated EPC number in vitro. Zhu et al. reported that the number of EPCs decreased in patients with hyperhomocysteinemia compared with normohomocysteinemic subjects, and an inverse correlation was observed between total homocysteine level and EPC number [17]. Although the precise mechanism by which homocysteine reduced the EPC number remains to be determined, it was found that homocysteine decreased the EPC number by accelerating EPC senescence through Akt dephosphorylation [18]. These results suggest that greater consumption of vegetables increases the EPC number in part through decreasing the homocysteine level.

Although all the subjects of our study had normocholesterol level and no significant change was observed in lipid levels due to dietary intervention, an inverse correlation was observed between the changes in EPC number and changes in both TC and LDL-C levels. These data are consistent with the results reported by Croce et al. [19], who showed that dietary intervention for 4 weeks increased the number of EPCs, assessed as in the present study by Dil-actLDL/UEA-1 dual positive cells, in patients with hypercholesterolemia (LDL-C; between 158.7 mg/dl and 189.6 mg/dl) without other cardiovascular risk factors. They also showed an inverse correlation between changes in LDL-C levels and changes in EPC number. Chen et al. showed that the number of EPCs was lower in subjects with elevated serum cholesterol levels compared with age-matched middle-aged control subjects, and they also showed an inverse correlation between the number of EPCs and TC and LDL-C level, including control subjects [20]. The number of EPCs, assessed by a colony-forming-units assay, was reduced not only in coronary artery disease patients, but also in relatively healthy subjects with elevated serum cholesterol [8]. Taken together, these results suggest that TC and LDL-C even in the normal range may affect the number of EPCs.

The mechanisms by which hypercholesterolemia decreased the number of EPCs still remains to be determined. Oxidized LDL has been proposed to play an important role in the development of atherosclerosis [21,22], and the possibility has been reported that oxidized LDL accelerated EPC senescence by telomerase inactivation [23]. It is also reported that elevations in ROS formation subsequently decrease EPC number [24]. Vegetables and fruits are rich sources of potassium, folate, fiber and antioxidants such as vitamin C and vitamin E. Several reports have shown that the consumption of vegetables and fruits showed a significant inverse association with lowering ROS [25], and antioxidant vitamins and folic acid reduced LDL oxidation in vitro [26]. Potassium supplementation also has inhibitory effects on free radical formation [27]. According to the national health and nutrition survey in Japan, the average vegetable intake of Japanese women aged 20–29 years was 235.4 g/day. Subjects in the intervention group in this study consumed many more vegetables during the study period than the national average, and as a result urinary potassium and magnesium excretion increased after the dietary intervention. However, we could not find any change of ROS as assessed by the d-ROM test. This may be an inappropriate method for detecting small changes in ROS level in our study. Small dense LDL is easily oxidized and is an atherogenic lipoprotein [28]. The ratio of TG/HDL-C is considered to be an estimator of small dense LDL-C and an inverse correlation was observed between LDL particle size and TG/HDL-C ratio in healthy subjects [12]. We showed here that both urinary potassium and magnesium excretion were negatively correlated with TG/HDL-C ratio, which may suggest that potassium or magnesium may
increase the size of LDL particles. Thus, high vegetable consumption may increase the number of EPCs through improving characteristics of cholesterol such as LDL particle size and oxidizability.

There were several limitations of our study. The sample size is small, and we did not evaluate other lifestyles such as physical exercise volume, which may affect EPC numbers. Okinawan vegetables such as Fuchiba and Njana were known to have relatively much of β-carotene and strong antioxidant activities [29], but we did not evaluate such variables of these respective vegetables in the present study. Since the differences in such contents are not large and we could not find any change of ROS marker in this study, vegetables other than Okinawan vegetables may also affect the numbers of EPCs. In addition, none of the subjects in this study had risk factors for atherosclerotic disease. Therefore, it may be difficult to detect an association between changes in risk factors and EPC number.

In conclusion, the present study demonstrated that dietary intervention with typical Okinawan vegetables significantly increased urinary potassium and magnesium excretion, and also the number of circulating EPCs. It was found that a vegetable-rich diet contributes to anti-atherosclerosis, and the level of EPCs may be useful as a biologic marker of a healthy diet.

Acknowledgements

We would like to thank Dr. Kui-Cheng Zheng and Dr. Mallet K.-N. Tuekke of the Department of Environmental and Preventive Medicine, Dr. Minoru Nakamoto of the Department of Cardiovascular Medicine, Nephrology and Neurology, Faculty of Medicine, University of the Ryukyus, for their support.

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