Effect of Green Tea Consumption on Endothelial Function and Circulating Endothelial Progenitor Cells in Chronic Smokers

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Background The present study was designed to investigate the effect and relationship of endothelial function and endothelial progenitor cells (EPCs) by green tea consumption in chronic smokers. The numbers of circulating EPCs have an inverse correlation with chronic smoking and endothelial dysfunction. Green tea catechin improved endothelial dysfunction in chronic smokers.

Method and Results In 20 young healthy smokers, endothelial functions, defined by flow-mediated endothelium dependent vasodilation (FMD) of the brachial artery via ultrasound as well as the number of EPCs isolated from peripheral blood, were determined at baseline and at 2 weeks after green tea consumption (8 g/day). Circulating EPCs were quantified by flow cytometry as CD45lowCD34+KDR2+ cells and by acyl-low-density lipoprotein and fluorescein isothiocyanate-lectin double positive cells after culture for 7 days. Clinical characteristics and laboratory findings were not significantly different between the baseline and at 2 weeks after green tea intake. EPC levels were inversely correlated with the number of cigarettes smoked. Circulating EPCs by flow cytometry (78.6±72.6 vs 156.1±135.8/ml, p<0.001) and cultured EPCs (118.2±35.7 vs 169.3±58.3/10 field, p<0.001) increased rapidly at 2 weeks after green tea consumption. FMD was significantly improved after 2 weeks (7.2±2.8 vs 9.3±2.4, p<0.001). The FMD correlated with EPC counts (r=0.67, p=0.003) before treatment and after 2 weeks (r=0.60, p=0.013).

Conclusions A short-term administration of green tea consumption induces a rapid improvement of EPC levels and FMD. Green tea consumption may be effective to prevent future cardiovascular events in chronic smokers. (Circ J 2006; 70: 1052–1057)

Key Words: Catechin; Cells; Endothelium; Smoking

A sahara et al in 1997 reported that in normal adults there are endothelial progenitor cells (EPCs), a kind of stem cell that makes vessels in peripheral blood. Later it was revealed that EPCs contributed to neo-angiogenesis, mobilized from bone marrow and peripheral blood through many kinds of ischemic stimulation. In addition, it has been revealed that EPCs play critical roles in maintaining the integrity of vessels and it also has a role as "repair cells" to remedy endothelial cell injury, an early step of atherosclerosis caused by a cardiovascular risk factor. In recent studies, the number of circulating EPCs was reduced when there were more atherosclerosis risk factors concerning the process of atherosclerosis. In the reduction of angiogenesis function, the function of EPCs was considerably suppressed too.

The assessment of flow-mediated, endothelium-depen-
The effects of green tea, in terms of circulating EPC numbers, has not been determined in smokers. Circulating EPC numbers and FMD are correlated closely and because catechin can activate eNOS, it can be hypothesized that green tea consumption by smokers increases the circulating EPC numbers and this is correlated with an improvement of endothelial cell function.

This present study was designed to investigate the endothelial dysfunction and the number of circulating EPCs, and to examine the effect and relationship of endothelial function and EPC numbers by green tea consumption in chronic smokers.

Methods

Subjects

This study was performed on 20 healthy chronic smokers. Heavy smokers (>20 cigarettes per day) were not included in the study because of cell culture problems.

Smokers who had underlying diseases such hypertension and diabetes were excluded from the present study. Smokers who consumed herbs or vitamins were also excluded. The Chonnam National University Hospital Ethics Committee approved this study. All volunteers provided written, informed consent.

Method and Study Design

FMD was measured before green tea consumption, and changes in vascular endothelial function and EPC numbers in peripheral blood were assessed in order to determine the effect of green tea consumption on vascular endothelial function and EPC numbers in smokers. Subjects consumed powdered green tea (8 g/day) (Amorepacific Co, Korea), which was dissolved in 1 L of warm water, for 2 weeks. The total catechin sum was 0.618% epicatechin gallate. The total catechin sum was 0.618% epicatechin, 3.20% epigallocatechin gallate (EGCG), and 1.339% caffeine, 4.659% epigallocatechin, 0.746% epicatechin, 3.20% epigallocatechin gallate (EGCG), and 0.618% epicatechin gallate. The initial seeding density was standardized at 6–10 × 10^6 cells per well. After appropriate gating with low cytoplasmic face antigens. In previous reports, circulating MNCs with CD34+VEGFR2+ cells were quantified and expressed as tentative PCs/EPCs. Four milliliters of PB was drawn from each subject and FMD was measured before green tea consumption, and flow cytometry assays and FMD measurements were blinded to subject identity and study sequence.

Isolation of EPC

We examined circulating EPCs in a cell culture assay system as reported previously. Briefly, peripheral blood (20 ml) was drawn by venipuncture using a heparin-coated syringe. Mononuclear cells (MNCs) were isolated using the density gradient method using histopaque-1077 (Sigma, USA), and resuspended in EBM medium supplemented with EGM-2 Bullet kit system (Clonetics; 10% fetal bovine serum, hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid, heparin). Cells were plated in 12-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) and coated with 2% gelatin (Sigma, St Louis, MO, USA). The initial seeding density was standardized at 6–10 × 10^6 cells per well. After 3 days of culture, non-adherent cells were removed and the media were changed. The culture was maintained through to day 7. At day 7, EPCs were characterized by a dual staining for Dil-acetylated low-density lipoprotein (Dil-AcLDL) incorporation (2.4 μg/ml; molecular probes) and fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin I (lectin, 10 μg/ml; Sigma). Attaching double-stained cells were counted manually in 10 random microscopic fields.

Quantification of EPCs by Flow Cytometry

(MNC^{CD34+*CD133+*})

We further examined circulating EPCs by using cell surface antigens. In previous reports, circulating MNCs with CD34+CD133+/CD34+ CD133+ VEGFR2+ were quantified as tentative PCs/EPCs. Four milliliters of PB was drawn and white blood cells were stained with allophycocyanin-conjugated anti-CD45 monoclonal antibody (mAb) (Caltag Laboratories, FITC-conjugated anti-CD34 mAb (Beckman Coulter, Inc), phycoerythrin-conjugated anti-VEGFR2 (VEGFR2) mAb (Sigma), and further incubated in the dark for 20 min. After appropriate gating with low cytoplasmic granularity and with low expression of CD45, the number of CD34+VEGFR2+ cells were quantified and expressed as the number of cells per 10^6 total events or number of cells per 200 μl blood. The numbers of CD45lowCD34+
VEGFR2+ cells were then counted. We further isolated CD45loCD34+VEGFR2+ cells by using a magnetic cell sorter device (Miltenyi Biotec) and by using a cell sorting system (fluorescence-activated cell sorter (FACS); Vantage; Becton-Dickinson), and examined whether these cells gave rise to EPCs.

Endothelial Function Assessment

Imaging studies of the right brachial artery were performed by using an ultrasound machine (SONOS 5500, Hewlett-Packard, USA) equipped with a 7.5 MHz linear-array transducer, based on a standard technique.15 Briefly, baseline data for the diameter and mean blood flow velocity of the brachial artery were quantified after 10 min of supine rest in an air-conditioned room at a position that was 1–2 cm above the elbow. Baseline measurement of the brachial artery diameter as well as a baseline measurement of arterial flow by pulsed Doppler with the range gate (1.5 mm) was made in the center of the artery. Endothelium-dependent vasodilation was assessed by measuring the change in the diameter of the brachial artery after 60 s of reactive hyperemia relative to baseline measurements after deflation of a cuff on the forearm that was inflated to 250 mmHg for 5 min. All images were coded and recorded on disk for subsequent blinded analysis. The arterial diameter was measured in millimeters as the distance between the anterior wall media-adventitial interface (“m” line) and the posterior wall intima-lumen interface at the end diastole, and in coincidence with the R wave on the ECG at 2 sites along the artery and for 3 cardiac cycles; these 6 measurements were then averaged. The FMD was expressed as the percentage change from baseline.

Laboratory Assays

Blood samples for laboratory assays were obtained at approximately 07.00 h following an overnight fasting before and at the end of each treatment period for 2 weeks. C-reactive protein (CRP) was measured with the reagent N Latex CRP mono (Dade Behring Inc, Marrbug, Germany) and with the use of a Behring nephelometer analyzer (Dade Behring Inc, Marrbug, Germany). The normal reference value was below 0.5 mg/dl. The erythrocyte sedimentation rate (ESR) was tested by using the Starred ESR analyzer (Mechatronics R & R, Hoorn, Holland) and the normal reference value was 0–15 mm/h. Fibrinogen was measured with a Dade Thrombin reagent (Dade Behring Inc, Marbug, Germany) and by using a CA-6000 Sytem (TOA Medical Electronics Co, Japan). The reference value was 180–350 mg/dl. A standardized method was used to measure the leukocyte value and cholesterol level.

Statistical Analysis

Data are presented as mean±SD. Data were evaluated with a normality test, equal variance test, and a paired t-test. Data not normally distributed were analyzed by using non-parametric methods. In analysis of correlation, a non-parametric Spearman correlation test was used. It was performed with SPSS 11.0 for Windows and the value p<0.05 was considered to be statistically meaningful.

Results

Clinical Characteristics of Subjects

The clinical characteristics of subjects are shown in Table 1. The age of subjects was 27.6±3.6 years and all had a history of smoking (as 6 pack years). There was no significant change in terms of blood pressure, pulse rate, body mass index for laboratory analysis before and after green tea consumption.

Inflammatory Marker Analysis

Inflammatory markers of ESR, CRP and fibrinogen before and after green tea consumption were not changed significantly (Table 1).

Fig 1. Flow-mediated dilatation (FMD) was improved after green tea consumption (p<0.001).

Fig 2. Characteristics and quantification of endothelial progenitor cell (EPC). (a) Dil-acetylated low-density lipoprotein (DilAcLDL)/fluorescein isothiocyanate lectin double-positive attaching cells in 10 fields were counted. The number of attaching EPC was greater after green tea ingestion than baseline (p=0.0002). (b) Images of DilAcLDL uptake (A, B) and lectin binding of isolated EPC from subjects. Double-positive cells appear yellow in color (C, D).
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Changes in Endothelial Cell Function by FMD

The change in endothelial function was observed by measuring FMD before and after green tea consumption. FMD significantly ameliorated from 7.25±2.8 to 9.34±2.3% 2 weeks after consumption (p<0.001; Fig 1).

Changes in EPC Numbers

After 2 weeks of green tea consumption, EPC numbers in cultured cells were significantly increased from 118.2±35.7 at baseline to 169.1±53.3 (p<0.001; Fig 2).

The CD45<sup>low</sup>CD34<sup>+</sup>VEGFR2<sup>+</sup> cell numbers, which were defined as EPCs by FACS, was measured as 78.6±72.6/200µl, and 2 weeks later, it was increased to 156.1±135.8/200µl (p<0.001; Fig 3). CD45<sup>low</sup>CD34<sup>+</sup>VEGFR2<sup>+</sup> cells had an uptake of Dil-AcLDL and grew into EPCs, which were stained with FITC-lectin when they were cultured (Fig 3).

The number of EPCs that had a take up of Dil-AcLDL and which were stained with FITC-lectin through cell culture, as well as the number of CD45<sup>low</sup>CD34<sup>+</sup>VEGFR2<sup>+</sup> cells measured by FACS, was significantly correlated (r=0.57, p=0.02; Fig 4).

Relationship of FMD and EPC Changes

The quantitative analysis of CD45<sup>low</sup>CD34<sup>+</sup>VEGFR2<sup>+</sup> cells through baseline FACS was correlated with the value of FMD before green tea consumption (r=0.67, p=0.003; Fig 5a). After 2 weeks consumption, the increased value of cultured EPC numbers and FMD were also correlated with each other (r=0.60, p=0.013; Fig 5b).

Discussion

The present study demonstrated that reduced circulating EPC numbers and endothelial function in smokers were ameliorated significantly by consuming green tea. In addition, it was also shown that the number of circulating EPCs...
had a close relationship with endothelial function because an increase of cultured EPC numbers was significantly correlated with FMD.

In a previous study, it was reported that circulating EPC numbers were decreased in smokers compared with non-smokers, which agrees with the results found in the study conducted by Vasa et al.10 At the cell culture stage, it was observed that EPCs from heavy smokers did not adhere and thus perished, which was assumed to be the result of the poison of smoking. It is still disputable about the EPC characterization. It is known that there are somewhat different types of EPC present depending on laboratory conditions, although there were no significant differences according to the cells used or experimental methods conducted. Hur et al. reported 2 types of EPCs, which have different morphological and molecular characteristics.30 In this study, EPCs were additionally measured by using a cell surface antigen marker. This method was not affected by cell culture condition and was helpful in the measurement of the direct effect of smoking on EPCs. The measurement value of EPCs with FACS was significantly related to that measured by cell culture. Therefore, the present study can present objective data. The reason to identify EPCs after gating CD45low cells in FACS is that in a previous pilot study, the CD45low part contained CD34+CD133+ cells and this gating increased which plays an important role in EPCs mobilization.21,25 In addition, smoking causes a dysfunction of vascular endothelial cells, which requires more EPCs to regenerate the injured vessels. This can be supported by the results of a previous study that reported the increased EPC numbers dramatically decreased after the commencement of smoking.

The vascular endothelial function by FMD was correlated with the number of EPCs. Green tea intake improves FMD. Therefore, it was assumed that green tea consumption could increase EPCs and improve FMD. Indeed, green tea consumption increased EPC levels and ameliorated FMD in smokers. These are well-known factors of green tea consumption; it improves FMD. EGCG in green tea mobilizes eNOS through phosphatidylinositol-3-kinase and the Akt-dependent pathway, and induces endothelial vasorelaxation. Green tea also has a strong antioxidant function.25,26 Murohara et al. reported that neovascularization was seriously repressed in lower limb ischemia in an eNOS knock-out mouse model. Aicher et al reported that eNOS played an important role in the mobilization of progenitor cells from bone marrow.28 Therefore, it can be thought that EGCG in green tea increased the circulating EPC numbers by the activation of eNOS and improved FMD.

The results of the present study indicate that short-term green tea consumption improved FMD and increased EPC numbers by the culturing and FACS methods. FMD was also correlated with the increased value of cultured EPC numbers after green tea consumption. This result is quite noticeable because the subjects continued smoking as they had done previously prior to the study.

The amount of green tea (8 g/day) in the present study was set at random. Normally, 1 g green tea is included in 1 cup of tea. Therefore, the amount of green tea used in the present study is a not significant. If a sample of green tea was put in 80°C water for 30 min, then it is known that the most water-soluble part of green tea has been extracted. However, it was noted that there was no difference in drinking and extract condition of green tea if it was in the powdered form.

There is a possibility that the clinical significance can be underestimated because all of the subjects in the present study were healthy adults who were disease free. However, in a recent autopsy study, a young smoker had a serious atherosclerosis lesion in the coronary artery.22 Also, green tea consumption was associated with a lower incidence of coronary artery disease in Japanese patients.31 Therefore, green tea consumption was recommended for young smokers.

Study Limitations

First, there was no additional experiment on the mechanism of growing EPCs through green tea consumption, although previous study mechanisms could be used.26,25,28 This limitation was so because the experiment was conducted on human beings. It is considered that the effect of EGCG should be studied on the circulating EPC numbers and function and on the change of eNOS in the future. Second, the plasma EGCG levels in patient blood was not measured after green tea consumption. Nagaya et al., however, had already reported that the intake of green tea only once could increase plasma EGCG concentration considerably.26 Finally, there was no response experiment to determine the relationship between green tea dosage, time-dependent FMD changes and EPC numbers.

Conclusion

The number of circulating EPCs and FMD was reduced in chronic smokers. A short-term therapy of green tea consumption induces a rapid improvement of EPC levels and FMD. Green tea consumption may be effective in preventing future cardiovascular events in chronic smokers.

References

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