Tea Flavonoids Induced Differentiation of Peripheral Blood-derived Mononuclear Cells into Peripheral Blood-derived Endothelial Progenitor Cells and Suppressed Intracellular Reactive Oxygen Species Level of Peripheral Blood-derived Endothelial Progenitor Cells

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Abstract – Endothelial dysfunction in atherosclerosis is associated with increasing oxidative stress that could be reversed by antioxidant. Therefore epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC) and catechin (C) of tea flavonoids were investigated for their roles in regenerating endothelial cell. Peripheral blood mononuclear cells (PB-MNCs) were isolated, plated and cultured in medium with/without treatment of EGCG, ECG, EGC and C. Results showed that among all EGCG, ECG, EGC and C concentrations tested, 12.5 μmol/L was not cytotoxic for peripheral blood-derived endothelial progenitor cells (PB-EPCs). Treatment of EGCG, ECG, EGC or C increased the percentages of CD34, CD133, VEGFR-2 expressions and suppressed hydrogen peroxide-induced percentages of reactive oxygen species (ROS) level in PB-EPCs. Taken together, our current results showed that EGCG, ECG, EGC or C of tea flavonoids could induce differentiation of PB-MNCs into PB-EPCs as well as protect PB-EPCs from oxidative damage by suppresing the intracellular ROS levels.
Keywords – Tea flavonoids, Antioxidant, Endothelial progenitor cell, Differentiation, ROS, Apoptosis

Introduction

Endothelial dysfunctions play a role in the pathogenesis of atherosclerosis and its complications.[1] Increasing oxidative stress and disorder antioxidant mechanism, especially inactivation of nitric oxide (NO) by reactive oxygen species (ROS) such as superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), and lipid peroxides, may contribute to the endothelial dysfunction, intact vessel wall, up to the rupture of a lipid-rich atherosclerotic plaque.[2] Endothelial dysfunction in atherosclerosis is associated with increasing oxidative stress that could be reversed by antioxidant therapy.[3] Antioxidants counter-balance the ROS production in cells and modify the regulatory pathways of cell growth.[4]

In several numbers of previous studies, tea consumption as a source of flavonoids was associated with low cardiovascular disease (CVD) and stroke-related mortality.[5] The mortality was significantly decreased along with the increase of tea flavonoids intake in a dose-dependent manner.[6] Some *in vitro* studies showed that flavonoids have strong ROS scavenging activity and prevent low density lipoprotein (LDL) to oxidize.[1] Atherosclerotic animal models study indicated that consuming dietary flavonoids can reduce atherosclerotic plaque development.[8] Flavonoids were reported to have anti-platelet, anti-inflammatory, and antioxidant activities.[9,10] In addition, an epidemiological study reported that consuming flavonoid-containing foods and beverages could reduce CVD risk,

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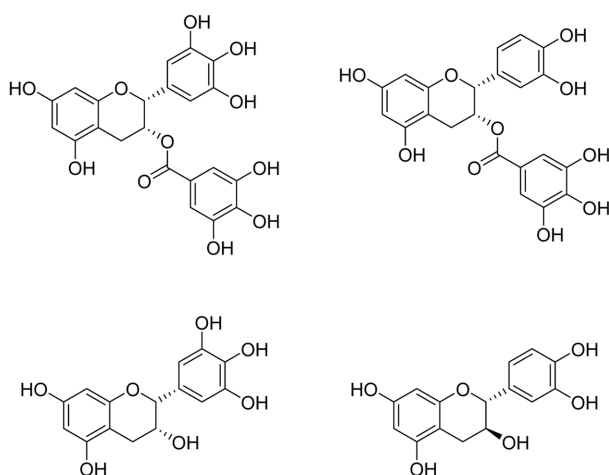
Fig. 1. Chemical Structures of catechins. Structures of (–)-epigallocatechin gallate (EGCG) (**a**), (–)-epicatechin gallate (ECG) (**b**), (–)-epigallocatechin (EGC) (**c**), and (+)-catechin (C) (**d**).

as well as increase endothelial function.[7]

The effects of green tea are mainly associated with polyphenols content, especially flavonoids with approximately 30% of dry weight of leaves.[11] Predominantly flavonoids consist of epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC) and catechin (C).[12,13] In our previous study, we found that EGCG enhanced proliferation of adipose tissue-derived mesenchymal stem cells (AD-MSCs) and differentiation of AD-MSCs into endothelial progenitor cells (EPCs). Tea flavonoids including EGCG, ECG, EGC and C could also have effects on peripheral blood-derived EPCs (PB-EPCs). Therefore we conducted a study in order to investigate proliferation and differentiation inducing properties of EGCG, ECG, EGC, and C (Fig. 1) on PB-EPCs.

Experimental

General Experimental Procedure – (–)-epigallocatechin gallate (EGCG), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), and (+)-catechin (C) with purity 95-99% (Fig. 1.) were purchased from Biopurify Phytochemical Ltd. (Chengdu, China). Human fibronectin was purchased from Roche (Basel, Switzerland). VascGrow[TM] medium was obtained from Stem Cell and Cancer Institute (Jakarta, Indonesia). Ficoll-Paque reagent was purchased from GE Healthcare (Uppsala, Sweden). Fluorescein isothiocyanate (FITC)-conjugated ulex europaeus agglutinin I (UEA-I), and 1,1-dioctadecyl-3,3,3,3-tetramethylindo carbocyanine-labeled acetylated LDL (Dil-acLDL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 4',6-diamidino-2-phenylindole (DAPI)

and 2'-7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Invitrogen (Carlsbad, CA, USA). FcR Blocking and phycoerythrin (PE)-conjugated anti-CD133 antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). FITC-conjugated anti-CD45/PE-conjugated anti-CD34 antibody was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). PE-conjugated anti-vascular endothelial growth factor (VEGFR)-2/Kinase Domain Receptor (KDR) antibody was purchased from R&D System (Minneapolis, MN, USA). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Fluorescence-activated cell sorting (FACS) was performed using a FACS Calibur Flow Cytometer Becton Dickinson (Franklin Lakes, NJ, USA). Cells were observed using inverted fluorescent microscope (Axiovert 40 CFL, Zeiss).

Isolation and Culture – Briefly, total PB-MNCs were isolated from peripheral blood of healthy young human volunteers with Ficoll density gradient centrifugation. PB-MNCs were plated in human fibronectin-coated culture dishes and cultured using VascGrow[TM] at 37 °C in a humidified, 5% CO_2 incubator. New media was applied after 4 days of culture and the culture was maintained for 7 days. Prior to blood collection, all volunteers signed informed consent. All procedures were approved by the ethics committee of Stem Cell and Cancer Institute, Jakarta and the joint ethics committee of Faculty of Medicine, Maranatha Christian University, Bandung, Indonesia and Immanuel Hospital, Bandung, Indonesia.

Fluorescent Staining – Fluorescent staining was performed to detect FITC-UEA-I and Dil-acLDL. To identify the uptake Dil-acLDL, adherent cells were incubated with 1 mg/mL Dil-acLDL at 37 °C for 4 hours. Cells were fixed with 3% paraformaldehyde for 10 minutes. Soon after cleansed, cells were incubated with 1 mg/mL FITC-UEA-I at 37 °C for 1 hour, followed by nucleus staining using DAPI. After staining, cells were observed using an Axiovert 40 CFL inverted fluorescent microscope from Carl Zeiss (Jena, Germany). Cells with double positive for both Dil-acLDL and FITC-UEA-I were defined as PB-EPCs.

MTS Assay – This assay was done to quantify viable PB-EPCs under treatment of EGCG, ECG, EGC and C. Briefly, 5×10^3 PB-EPCs were seeded in 96-well plates using serum-free medium for 24 hours. After EGCG, ECG, EGC or C treatment, PB-EPCs were added with 20 μL MTS and incubated at 37 °C in a humidified, 5% CO_2 incubator for 4 hours. Optical Density value was measured at 490 nm using a microplate reader from Biorad (Hercules, CA, USA).

Apoptosis Assay – PB-EPCs were cultured in 12-well plate and treated with EGCG, ECG, EGC or C for 24 hours. After that, PB-EPCs were collected, washed and stained with propidium iodide solution in phosphate buffer saline (PBS) for 15 minutes. The apoptotic cells were determined as SubG1 area using a fluorescence-activated cell sorting (FACS)Calibur flow cytometer from Becton Dickinson and presented as a percentage of total cells.

Immunophenotyping – PB-EPCs were detached using 2 mM ethylenediaminetetraacetate (EDTA) and pre-incubated for 15 minutes at room temperature with FcR Blocking. Then PB-EPCs were incubated at 4 °C with FITC-conjugated anti-CD45/PE-conjugated anti-CD34 and PE-conjugated anti-CD133 antibodies for 15 minutes, followed by PE-conjugated VEGFR-2/KDR antibody for another 40 minutes. Isotype-identical antibodies were also applied and used as controls to negative bindings. Each expression was analyzed using a FACSCalibur.

Intracellular ROS Assay – Treated PB-EPCs were incubated in 37 °C with 10 μmol/L DCF-DA for 30 minutes and washed with PBS containing KCl solution. Level of intracellular ROS were measured using a FACSCalibur.

Statistical Analysis – Data are presented as mean and standard deviation. Statistical analysis was perfomed using IBM SPSS Statistics for Windows software version 20.0. (Armonk, NY, USA). One-way analysis of variance test was performed, followed by Duncan's post-hoc tests.

Result and Discussion

Lectin binding and Dil-acLDL uptake of the CD34^{+}, CD133^{+} and VEGFR-2^{+} cells – Cultured cells were adherent with spindle-shaped morphology. Fig. 2B showed lectin binding property, while Fig. 2C showed Dil-acLDL uptake of the cells, marked by fluorescent cells in both Fig. 2B and 2C. These 2 characteristics
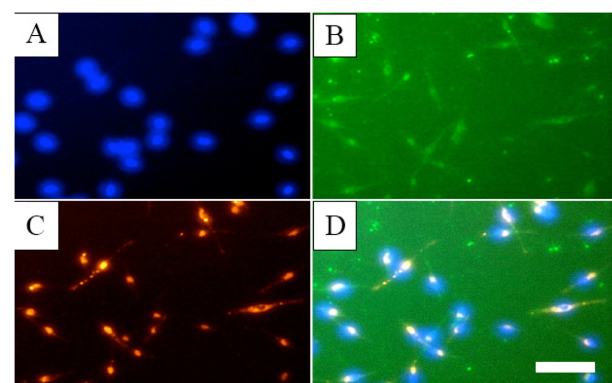
Fig. 2. PB-EPCs lectin binding and LDL uptake. PB-EPCs were seeded in 96-well plate and subjected to Fluorescent Staining as described in Experimental. A: DAPI, B: FITC-UEA-I, C: Dil-acLDL, D: Merge of A, B and C. White bar: 50 μm.
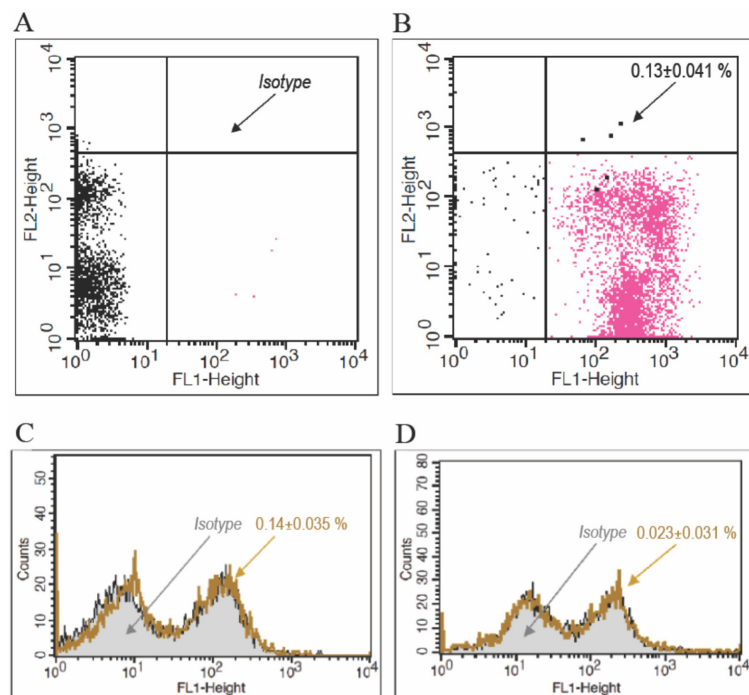
Fig. 3. PB-EPCs immunophenotypes. PB-EPCs were detached and subjected to Immunophenotyping as described in Experimental. A: isotype, B: CD34/45, C: CD133 and its isotype, D: VEGF-R2 and its isotype.

Table 1. Effect of EGCG, ECG, EGC and C on PB-EPCs viability

Treatment	Percentage of Viable PB-EPCs (Mean±SD)			
	EGCG	ECG	EGC	C
Untreated	100.00 ± 8.27 [cA]	100.00 ± 8.27 [cA]	100.00 ± 8.27 [cA]	100.00 ± 8.27 [cA]
0.5% DMSO	98.68 ± 5.04 [cA]	98.68 ± 5.04 [cA]	98.68 ± 5.04 [cA]	98.68 ± 5.04 [cA]
12.5 μmol/L	93.20 ± 5.29 [cA]	94.00 ± 2.27 [cA]	93.73 ± 1.04 [cA]	93.39 ± 0.33 [bcA]
25 μmol/L	78.28 ± 4.02 [bA]	68.45 ± 4.96 [bA]	69.23 ± 4.32 [cA]	93.96 ± 4.84 [bcB]
50 μmol/L	71.29 ± 4.18 [bA]	62.18 ± 2.27 [bA]	63.47 ± 3.55 [bA]	86.21 ± 5.81 [bB]
100 μmol/L	30.59 ± 4.96 [aA]	32.43 ± 4.96 [aA]	54.67 ± 3.06 [aB]	61.76 ± 5.94 [aB]

PB-EPCs were treated with 12.5, 25, 50 or 100 μmol/L of EGCG, ECG, EGC or C for 24 hours. MTS Assay was carried out as described in Experimental. Each treatment was done in triplicate. [a,b,c]Means in the same column containing the same superscript are not significant ($p \geq 0.05$), while means in the same column containing different superscript in small letter indicate significant differences ($p < 0.05$). [A,B]Means in the same row containing the same superscript are not significant ($p \geq 0.05$), while means in the same row containing different superscript in capital letter indicate significant differences ($p < 0.05$). Statistical analysis was performed based on Duncan's post-hoc test. SD: standard deviation.

Table 2. Effect of EGCG, ECG, EGC and C on apoptosis of PB-EPCs

Treatment	Percentage of SubG1
Untreated	11.68 ± 1.20 [a]
0.5% DMSO	14.93 ± 1.60 [a]
EGCG	14.48 ± 6.83 [a]
ECG	14.57 ± 7.67 [a]
EGC	12.31 ± 3.00 [a]
C	15.97 ± 3.62 [a]

Ten thousands PB-EPCs were seeded in each 12-well plate and treated with 12.5 μmol/L EGCG, ECG, EGC or C for 24 hours. After 24 hours, Apoptosis Assay was carried out as described in Experimental. Each treatment was done in triplicate. The apoptotic cells were determined on the basis of the SubG1 area. The data are presented as mean ± standard deviation. [a]Means in the same column containing the same superscript are not significant ($p \geq 0.05$). Statistical analysis was performed based on Duncan's post-hoc test.

Table 3. Effect of EGCG, ECG, EGC and C on PB-EPCs immunophenotypes

Treatment	CD34 (%)	CD133 (%)	VEGFR-2 (%)
Untreated	52.86 ± 8.44 [b]	0.63 ± 0.02 [ab]	0.22 ± 0.02 [a]
0.5% DMSO	28.96 ± 9.68 [a]	0.36 ± 0.14 [a]	1.78 ± 0.15 [c]
EGCG	51.09 ± 2.77 [b]	0.83 ± 0.41 [ab]	1.22 ± 0.19 [b]
ECG	60.48 ± 2.60 [b]	0.61 ± 0.08 [ab]	2.42 ± 0.13 [c]
EGC	54.70 ± 3.74 [b]	1.13 ± 0.71 [b]	1.21 ± 0.22 [b]
C	62.24 ± 9.22 [b]	1.01 ± 0.13 [ab]	1.47 ± 0.33 [bc]

Isolated-PB-MNCs were cultured with addition of 12.5 μmol/L EGCG, ECG, EGC or C. PB-EPCs Culture was then carried out as described in Experimental. PB-EPCs were then detached for Immunophenotyping as described in Experimental. Each treatment was done in triplicate. The data are presented as mean ± standard deviation. [a,b,c]Means in the same column containing the same superscript are not significant ($p \geq 0.05$), while means in the same column containing different superscript in small letter indicate significant differences ($p < 0.05$). Statistical analysis was performed based on Duncan's post-hoc test.

implied the cultured cells as PB-EPCs. The cells were further immunophenotyped for CD34, CD133 and VEGFR-2. Flow cytometric results showed that the cells expressed CD34, CD133 and VEGFR-2 (Fig. 3). Hence, our cultured cells were confirmed as EPCs.

EGCG, ECG, EGC and C reduced percentages of viable PB-EPCs – Based on the results of MTS assay (Table 1), PB-EPCs treated with higher concentration of EGCG, ECG, EGC, and C showed significant lower percentages of viable PB-EPCs. Among all concentrations of EGCG, ECG, EGC and C tested, 12.5 μmol/L had the lowest potential in reducing viable PB-EPCs percentages. In accordance, under treatment of 12.5 μmol/L EGCG, ECG, EGC or C, percentages of apoptosis resulted were almost similar to the untreated (Table 2). These results were similar to our previous research showing that >25

μg/mL green tea extract induced cytotoxic in PB-EPCs[14], while 12.5 μg/mL was not cytotoxic for EPCs.

EGCG, ECG, EGC and C increased expressions of CD34, CD133 and VEGFR-2 – EPCs are defined by their cell surface expressions, including CD34, CD133 and VEGFR-2[15,16]. CD34 and CD133 indicate the plasticity of the cells bearing the stem cell characteristic, whereas VEGFR-2 indicates endothelial characteristic. Based on our current flow cytometric results, EGCG, ECG, EGC or C increased the percentages of CD34, CD133 and VEGFR-2 expressions (Table 3). The highest percentage of CD34 expression was induced by C, the highest percentage of CD133 expression was induced by EGC, meanwhile the highest percentage of VEGFR-2 expression was induced by ECG. These results suggested that EGCG, ECG, EGC

Table 4. Effect of EGCG, ECG, EGC and C on ROS of H_2O_2-induced PB-EPCs

Treatment	ROS Level (%)	Ratio of All to Negative Control (%)	Ratio of All to Positive Control (%)
Untreated (negative control)	7.20 ± 1.65 [a]	99.95 ± 22.97 [a]	22.81 ± 5.24 [a]
H_2O_2 (positive control)	31.55 ± 1.10 [e]	438.15 ± 15.22 [e]	99.99 ± 3.47 [e]
EGCG + H_2O_2	12.92 ± 0.70 [c]	179.49 ± 9.67 [c]	40.96 ± 2.21 [c]
ECG + H_2O_2	10.83 ± 2.35 [bc]	150.46 ± 32.62 [bc]	34.34 ± 7.44 [bc]
EGC + H_2O_2	18.66 ± 2.81 [d]	259.21 ± 39.09 [d]	59.15 ± 8.92 [d]
C + H_2O_2	8.85 ± 1.13 [ab]	122.96 ± 15.73 [ab]	28.06 ± 3.59 [ab]

PB-EPCs were treated with 12.5 μmol/L of EGCG, ECG, EGC or C for 30 minutes. PB-EPCs were then treated with H_2O_2 with final concentration of 200 μmol/L for 1 hour. Treated-PB-EPCs were subjected to Intracellular ROS Assay as described in Experimental. Each treatment was done in triplicate. The data are presented as mean±standard deviation. [a,b,c,d]Means in the same column containing the same superscript are not significant (p ≥ 0.05), while means in the same column containing different superscript in small letter indicate significant differences (p < 0.05). Statistical analysis was performed based on Duncan's post-hoc test.

and C induced differentiation of peripheral blood mononuclear cells (PB-MNCs) into PB-EPCs. Our previous results also showed that ECGC induced differentiation of AD-MSCs into EPCs[17]. In addition, resulted PB-EPCs showed that VEGFR-2 was the most elevated marker, indicating high endothelial characteristic.

EGCG, ECG, EGC and C suppressed H_2O_2-induced ROS level in PB-EPCs – DCF-DA has been used for several studies dealing with the effect of ROS in cell culture[18,19]. DCF trapped within the cells was measured to represent the intracellular ROS level[20,21]. Compared with the untreated, induction of H_2O_2 increased percentages of ROS level in PB-EPCs significantly (Table 4). By pretreatment of EGCG, ECG, EGC or C, H_2O_2-induced percentages of ROS level was suppressed. These results were consistent with our previous results. Green tea extract that was supposed to contain flavonoids including EGCG, ECG, EGC and C, suppressed H_2O_2-induced ROS level in PB-EPCs[14]. Among all investigated flavonoids, C had the highest ROS suppressing capacity in PB-EPCs, since C was reported to have high 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging and high superoxide dismutase activities[22].

Taken together, our current results showed that EGCG, ECG, EGC or C of tea flavonoids, could induce differentiation of PB-MNCs into PB-EPCs as well as protect PB-EPCs from oxidative damage by suppresing the intracellular ROS levels. In addition, concentration of 12.5 μmol/L was not cytotoxic for EPCs. This study suggests that tea flavonoids might be related to PB-EPCs production and protection.

Acknowledgments

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