

# (-)-Epigallocatechin gallate selectively inhibits adenosine diphosphate-stimulated human platelet activation: Suppression of heat shock protein 27 phosphorylation via p38 mitogen-activated protein kinase

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**Abstract.** (-)-Epigallocatechin gallate (EGCG) is a major component of green tea. It has been demonstrated that EGCG has an antithrombotic effect by inhibiting platelet aggregation. However, the detailed mechanisms underlying the effects of EGCG remain to be elucidated. The present study examined the effects of EGCG on human platelet activation by various stimulators and the exact underlying mechanisms. EGCG suppressed adenosine diphosphate (ADP)-stimulated platelet aggregation dose dependently between 30 and 70  $\mu$ M. By contrast, EGCG failed to affect platelet aggregation stimulated by collagen, U46619 (a TP agonist) or ristocetin (an activator of GPIIb/IX/V). EGCG attenuated the ADP-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase and heat shock protein 27 (HSP27). The ADP-stimulated release of platelet-derived growth factor (PDGF)-AB and the soluble CD40 (sCD40) ligand was inhibited by EGCG. These findings suggest that EGCG selectively inhibits ADP-stimulated human platelet activation and that EGCG reduces the release of PDGF-AB and the sCD40 ligand due to suppressing HSP27 phosphorylation via p38 MAP kinase.

## Introduction

Catechins are important components of green tea polyphenols with numerous favorable biological functions, including anti-inflammatory, anti-oxidative, anti-atherosclerotic, anticar-

cinogenic and anti-arthritis effects in humans (1-3). Green tea mainly contains four catechins, epicatechin, epigallocatechin, epicatechin gallate and (-)-epigallocatechin gallate (EGCG), and among them EGCG is the most abundant (4).

Platelets are important in primary hemostasis and the repair of vascular injury. Platelet adhesion and platelet aggregation are important steps in thrombus formation. Platelets initially adhere to the vessel wall at the sites of endothelial cell activation and develop into chronic atherosclerosis via adhesive receptors, including glycoprotein Ib/IX/V receptors, which mediate rolling and tethering of the platelets to von Willebrand factor at the sites of vascular injury and induce glycoprotein IIb/IIIa activation and the release of adenosine diphosphate (ADP), resulting in platelet aggregation (5-7). In addition, platelets engage collagen in the vessel wall through their adhesion receptors glycoprotein Ia/IIa (8). The interaction of von Willebrand factor and glycoprotein Ib/IX/V is known to be induced by ristocetin, an activator of glycoprotein Ib/IX/V (9). It has been reported that ristocetin-induced glycoprotein Ib/IX/V activation leads to the generation of thromboxane A<sub>2</sub> (TXA<sub>2</sub>) in platelets (7). ADP enhances platelet activation by engaging specific GTP-binding protein coupled receptors, P2Y<sub>1</sub>/P2Y<sub>12</sub> receptors, and activates glycoprotein IIb/IIIa and the cyclooxygenase (COX)-1 pathway, resulting in the stimulation of platelet aggregation (10,11). Then activated platelets release inflammatory agents, including soluble CD40 (sCD40) ligand and secrete mitogenic mediators, including platelet-derived growth factor (PDGF)-AB from granules into the local microenvironment. Previous studies (12,13) demonstrated that ADP induces heat shock protein 27 (HSP27) phosphorylation via p38 mitogen-activated protein (MAP) kinase and p44/p42 MAP kinase in human platelets, and is also associated with the secretion of PDGF-AB and the sCD40 ligand. In addition, it has been demonstrated that ristocetin stimulates the release of the sCD40 ligand from human platelets through TXA<sub>2</sub>-mediated activation of the TXA<sub>2</sub> receptor and that release of the sCD40 ligand via TXA<sub>2</sub> generation from platelets in atherosclerotic patients is elevated (14).

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Regarding the effect of EGCG on human platelets, it has been reported that EGCG exhibits a potent antithrombotic effect by the inhibition of platelet aggregation (15). However, its precise mechanism in human platelets has not yet been elucidated.

The present study examined the effects of EGCG on human platelet activation by various stimulators, including ADP, collagen, ristocetin and the TXA2 receptor agonist, and the exact mechanisms underlying the effect of EGCG. The present study also demonstrated that EGCG selectively inhibits ADP-stimulated human platelet activation and that EGCG reduces PDGF-AB secretion and sCD40 ligand release due to the suppression of HSP27 phosphorylation via p38 MAP kinase.

## Materials and methods

**Materials.** EGCG, ADP, U46619 and ristocetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagen was purchased from Nycomed Pharma GmbH (Munich, Germany). PDGF-AB enzyme-linked immunosorbent assay (ELISA) kits and sCD40 ligand ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Phospho-specific anti-p38 MAP kinase antibodies, p38 MAP kinase antibodies and phospho-HSP27 (Ser-78) antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-HSP27 antibodies and GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The other materials and chemicals were obtained from commercial sources.

**Preparation of platelets.** Human blood was donated from healthy volunteers into 1/10 volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was obtained from blood samples by centrifugation at 155 x g for 12 min at room temperature. Platelet-poor plasma (PPP) was prepared from the residual blood by centrifugation at 2,500 x g for 5 min. All participants signed an informed consent agreement following receiving a detailed explanation and the study was approved by the Committee of Ethics in Gifu University Graduate School of Medicine (Gifu, Japan).

**Platelet aggregation.** Platelet aggregation using citrated PRP was followed in a PA-200 aggregometer (Kowa Co. Ltd., Tokyo, Japan), which is able to determine the size of platelet aggregates based upon particle counting using laser scattering methods (small size, 9-25  $\mu\text{m}$ ; medium size, 25-50  $\mu\text{m}$ ; large size, 50-70  $\mu\text{m}$ ) (16), at 37°C with a stirring speed of 800 rpm. The platelets were pre-incubated for 1 min and then platelet aggregation was monitored for 4 min. The percentage of transmittance of the isolated platelets was recorded as 0% and that of the appropriate PPP (blank) was recorded as 100%. When indicated, PRP was pretreated with EGCG for 15 min.

**Protein preparation following stimulation.** Following stimulation with ADP, collagen, ristocetin or U46619, platelet aggregation was terminated by the addition of an ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000 x g at 4°C for 2 min. In order to measure PDGF-AB and the sCD40 ligand as described below, the supernatant

was isolated and stored at -30°C for subsequent ELISA. For the western blot analysis of p38 MAP kinase and HSP27, the pellet was washed twice with phosphate-buffered saline and then lysed and immediately boiled in lysis buffer containing 62.5 mM Tris/Cl, pH 6.8; 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol.

**Western blot analysis.** A western blot analysis was performed as described previously (17). Briefly, SDS-PAGE was performed by the method described by Laemmli (18) in a 10% polyacrylamide gel. The proteins in the gel were transferred onto polyvinylidene fluoride (PVDF) membranes, which were then inhibited with 5% fat-free dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST; 20 mM Tris; pH 7.6; 137 mM NaCl; 0.1% Tween-20) for 2 h prior to incubation with the indicated primary antibodies. The primary antibodies used in the present study were anti-phospho-specific p38 MAP kinase, p38 MAP kinase, phospho-HSP27 (Ser-78), HSP27 or GAPDH antibodies. Peroxidase-labeled anti-mouse IgG (Santa Cruz Biotechnology, Inc.) or anti-rabbit IgG antibodies (KPL, Gaithersburg, MD, USA) were used as secondary antibodies. The primary and secondary antibodies were diluted for optimum concentration, respectively, with 5% fat-free dry milk in TBST. The peroxidase activity on the PVDF membranes was visualized on X-ray film by means of an enhanced chemiluminescent western blotting detection system (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

**Measurement of PDGF-AB and the sCD40 ligand.** The PDGF-AB and sCD40 ligand levels in the samples were determined using each ELISA kit according to the manufacturer's instructions.

**Statistical analysis.** All figures are shown from representative results of five independent experiments. The data are presented as the mean  $\pm$  standard error of the mean. The data were analyzed by Student's t-test and values of  $P < 0.05$  were considered to indicate a statistically significant difference.

## Results

**Effects of EGCG on platelet aggregation induced by ADP, collagen, U46619 or ristocetin.** The effects of EGCG on platelet aggregation stimulated by ADP, collagen, U46619 (a TXA2 receptor agonist) or ristocetin (an activator of glycoprotein Ib/IX/V) were examined using a laser scattering system. ADP-stimulated platelet aggregation in percentage of transmittance was markedly reduced by EGCG in a dose-dependent manner in the range between 30 and 70  $\mu\text{M}$  (Fig. 1). EGCG dose dependently decreased the formation of large aggregates (50-70  $\mu\text{m}$ ) according to the analysis of the size of platelet aggregates whereas small aggregates (9-25  $\mu\text{m}$ ) and medium aggregates (25-50  $\mu\text{m}$ ) were markedly increased by EGCG (Fig. 1A).

By contrast, EGCG failed to affect the platelet aggregation stimulated by collagen, U46619 or ristocetin (Fig. 1B-D). In addition, EGCG had little effect on the ratio of the platelet aggregate size induced by collagen, U46619 or ristocetin (Fig. 1B-D).

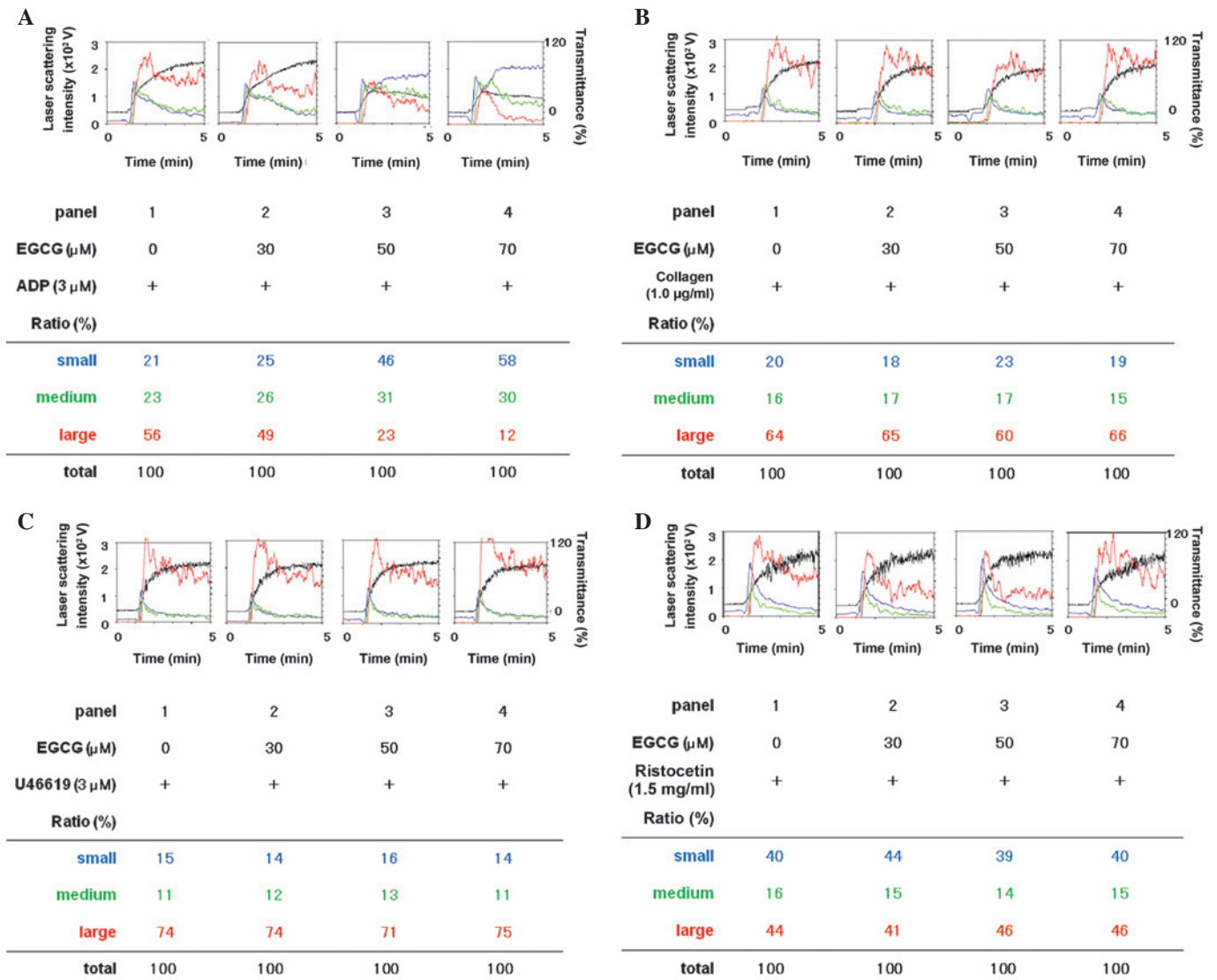


Figure 1. Effects of EGCG on platelet aggregation induced by ADP, collagen, U46619 or ristocetin. PRP was pretreated with various doses of EGCG at 37°C for 15 min and then stimulated by (A) 3  $\mu\text{M}$  ADP, (B) 1  $\mu\text{g/ml}$  collagen, (C) 3  $\mu\text{M}$  U46619 or (D) 1.5 mg/ml ristocetin for 4 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. The black line indicated the percentage of transmittance of each sample (the isolated platelets were recorded as 0% and platelet-free plasma was recorded as 100%). The blue line indicates small aggregates (9-25  $\mu\text{m}$ ); green line, medium aggregates (25-50  $\mu\text{m}$ ) and red line, large aggregates (50-70  $\mu\text{m}$ ). The distribution (%) of aggregated particle size was measured by the laser scattering methods. Representative results obtained from five healthy donors are indicated. EGCG, (-)-epigallocatechin gallate; PRP, platelet-rich plasma; ADP, adenosine diphosphate.

*Effects of EGCG on the ADP-induced phosphorylation of p38 MAP kinase or HSP27 in human platelets.* Previously, it has been demonstrated that ADP induces HSP27 phosphorylation via p38 MAP kinase activation in human platelets (12). Therefore, in order to examine how EGCG affects ADP-induced platelet aggregation, the effect of EGCG on the ADP-induced phosphorylation of p38 MAP kinase and HSP27 was examined. EGCG, which alone had little effect on p38 MAP kinase phosphorylation, markedly attenuated the ADP-induced phosphorylation of p38 MAP kinase (Fig. 2).

In addition, EGCG, which alone did not affect HSP27 phosphorylation, markedly suppressed the ADP-induced phosphorylation of HSP27 (Ser-78; Fig. 3).

*Effects of EGCG on ADP-induced PDGF-AB secretion or sCD40 ligand release from human platelets.* In our previous

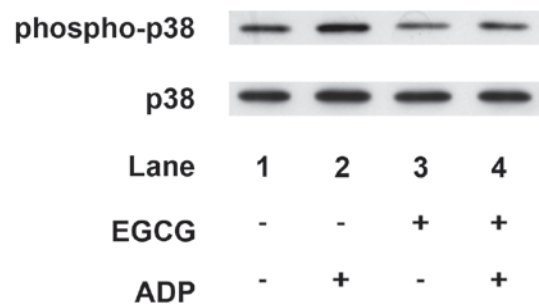


Figure 2. Effect of EGCG on ADP-induced phosphorylation of p38 MAP kinase in human platelets. PRP was pretreated with 70  $\mu\text{M}$  EGCG or the vehicle at 37°C for 15 min and then stimulated by 3  $\mu\text{M}$  ADP for 4 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. Lysed platelets were subjected to western blot analysis using antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. Representative results from five individuals are shown. EGCG, (-)-epigallocatechin gallate; PRP, platelet-rich plasma; ADP, adenosine diphosphate; MAP, mitogen-activated protein.

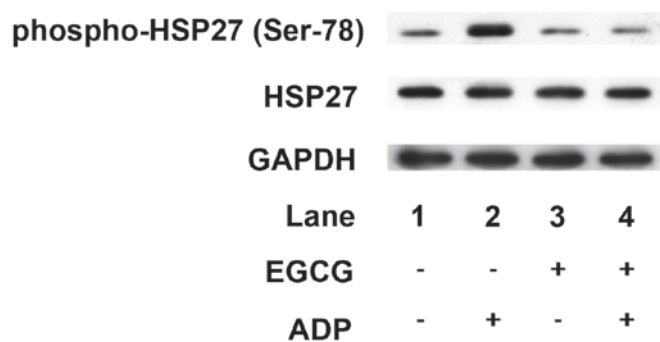


Figure 3. Effects of EGCG on ADP-induced phosphorylation of HSP27 (Ser-78). PRP was pretreated with 70  $\mu$ M EGCG or the vehicle at 37°C for 15 min and then stimulated by 3  $\mu$ M ADP for 4 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. Lysed platelets were subjected to western blot analysis using antibodies against phospho-HSP27 (Ser-78), HSP27 or GAPDH. Representative results from five individuals are shown. EGCG, (-)-epigallocatechin gallate; PRP, platelet-rich plasma; ADP, adenosine diphosphate; HSP27, heat shock protein 27.

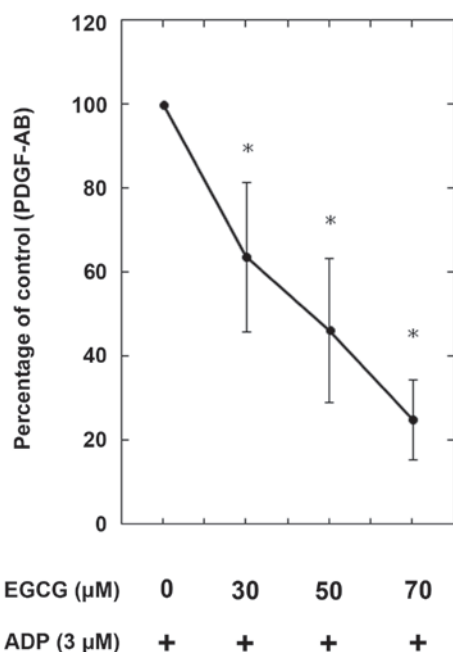


Figure 4. Effect of EGCG on ADP-induced PDGF-AB secretion in human platelets. PRP was pretreated with various doses of EGCG at 37°C for 15 min and then stimulated by 3  $\mu$ M ADP for 30 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000  $\times$  g at 4°C and the supernatants were then subjected to ELISA for PDGF-AB. The net increased levels of ADP alone were presented as 100%. The results from five individuals are shown. Each value represents the mean  $\pm$  standard error of the mean.  $P < 0.05$ , compared with the value of ADP alone. EGCG, (-)-epigallocatechin gallate; PRP, platelet-rich plasma; ADP, adenosine diphosphate; PDGF, platelet-derived growth factor; ELISA, enzyme-linked immunosorbent assay.

studies (12,13), ADP stimulated PDGF-AB secretion and sCD40 ligand release through HSP27 phosphorylation via p38 MAP kinase activation in human platelets. Thus, the effect of EGCG on the ADP-stimulated PDGF-AB secretion or sCD40 ligand release was examined. EGCG significantly reduced ADP-induced PDGF-AB secretion in a dose-dependent manner between 30 and 70  $\mu$ M (Fig. 4). Additionally,

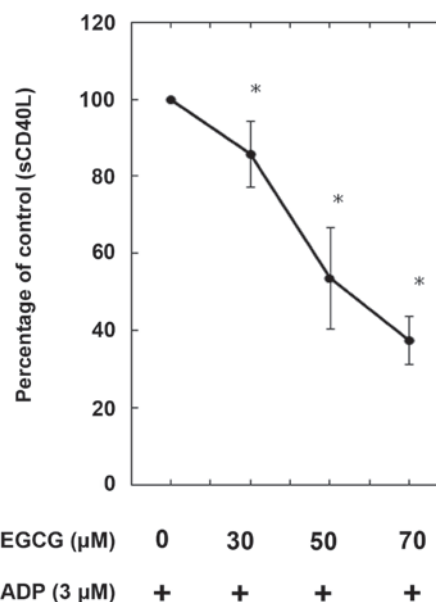


Figure 5. Effect of EGCG on ADP-induced sCD40 ligand release in human platelets. PRP was pretreated with various doses of EGCG at 37°C for 15 min and then stimulated by 3  $\mu$ M ADP for 30 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000  $\times$  g at 4°C and the supernatants were then subjected to ELISA for the sCD40 ligand. The net increased levels of ADP alone were presented as 100%. The results from five individuals are shown. Each value represents the mean  $\pm$  standard error of the mean.  $P < 0.05$ , compared with the value of ADP alone. EGCG, (-)-epigallocatechin gallate; PRP, platelet-rich plasma; ADP, adenosine diphosphate; ELISA, enzyme-linked immunosorbent assay; sCD40, soluble CD40.

the release of the sCD40 ligand stimulated by ADP was dose-dependently suppressed by EGCG (Fig. 5).

## Discussion

The present study demonstrated that EGCG, a predominant polyphenolic compound in green tea (4), markedly suppressed the human platelet aggregation induced by ADP, however, not by collagen, ristocetin or U46619 (a TXA<sub>2</sub> receptor agonist). It is generally recognized that platelets initially interact with subendothelium. Under high shear stress conditions, von Willebrand factor binds to the platelet membrane glycoprotein Ib/IX/V, which mediates initial tethering of platelets and initiates signals leading to platelet adhesion. This interaction is able to be induced by ristocetin, an activator of glycoprotein Ib/IX/V (9). It has been demonstrated that glycoprotein Ib/IX/V activation first generates TXA<sub>2</sub> by COX-1, leading to ADP secretion (19). In addition, at the injured vascular sites, collagen in the vessel wall induces platelet activation through their adhesion receptor glycoprotein Ia/IIa, a major collagen receptor (8). TXA<sub>2</sub> potentially activates glycoprotein IIb/IIIa through signal transduction from the TXA<sub>2</sub> receptor, TP (20). Subsequently, platelet aggregation develops by the induction of glycoprotein IIb/IIIa activation, resulting in thrombus formation. Platelet aggregation is important in the development of thrombus formation. Based on our findings that demonstrated that EGCG selectively suppressed ADP-induced platelet aggregation, it appears unlikely that EGCG inhibits human platelet adhesion, the first step of platelet activation. ADP is recognized to be a

weak stimulator in comparison with other platelet activating agonists, including collagen (10). However, ADP is a necessary cofactor for the normal activation of human platelets by other stimulators. Low concentrations of ADP enhance or potentiate the effects of agonists for platelet activation (10). Therefore, it is most likely that EGCG suppresses human platelet aggregation, which is amplified by ADP, the second step of platelet activation.

Thrombus formation is associated with the release of granule contents, including PDGF-AB and serotonin, and the release of inflammatory substances, including sCD40 ligand from platelets. It has previously been reported that ADP stimulates the phosphorylation of HSP27 via p38 MAP kinase activation in human platelets and that the ADP-induced HSP27 phosphorylation via p38 MAP kinase correlates with PDGF-AB secretion and sCD40 ligand release from human platelets (12,13). Therefore, the present study examined the effect of EGCG on the phosphorylation of p38 MAP kinase and HSP27 induced by ADP in human platelets. It was demonstrated that EGCG markedly attenuated the ADP-induced phosphorylation levels of p38 MAP kinase and HSP27. Based on these findings, it is possible that the suppression of ADP-stimulated platelet aggregation by EGCG is at least in part mediated by the attenuation of HSP27 phosphorylation through the p38 MAP kinase signaling pathway in human platelets. It has previously been reported that EGCG inhibits phospholipase C activity in human platelets (15); however, the exact mechanism remains unclear. Further investigation is required to clarify the details regarding the effects of EGCG on human platelets.

It is firmly established that the materials stored in the specific granules, including  $\alpha$ -granules are secreted from activated platelets. Large adhesive and healing proteins, including PDGF-AB, are stored in  $\alpha$ -granules (21). PDGF-AB released from platelet  $\alpha$ -granules is a potent mitogenic growth factor, which mainly acts on connective tissue, including vascular smooth muscle cells and promotes arteriosclerosis (22). In addition, activated platelets release inflammatory mediators of atherosclerosis, including the sCD40 ligand. The CD40 ligand is stored in the cytoplasm of unstimulated platelets and rapidly translocated on the surface following platelet activation by agonists, including collagen (23,24). The CD40 ligand expressed on the activated platelet surface undergoes a cleavage that generates a functional soluble fragment termed the sCD40 ligand. It is recognized that the sCD40 ligand released from platelets induces inflammatory responses via CD40, which is expressed on vascular endothelial cells and neutrophils (25). It has been demonstrated that the elevation of plasma sCD40 ligand is associated with an increased risk of cardiovascular events in patients with unstable coronary artery disease (26). The present study demonstrated that EGCG significantly inhibited the ADP-induced secretion of PDGF-AB and release of the sCD40 ligand from human platelets. Taking these findings into account, it is most likely that EGCG is important as an agent of anti-atherosclerosis and anti-inflammation through diminishing the levels of PDGF-AB and the release of the sCD40 ligand. The present study was able to provide a possible mechanism of the anti-inflammatory and anti-atherogenic effects of EGCG, the most abundant catechin in green tea.

In conclusion, the present findings suggest that EGCG selectively inhibits ADP-stimulated human platelet activation and that EGCG reduces the release of PDGF-AB and the sCD40

ligand by suppressing HSP27 phosphorylation via p38 MAP kinase.

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