Platelet Aggregation Inhibitors in Hot Water Extract of Green Tea

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The effect of hot water extract of green tea on the collagen-induced aggregation of washed rabbit platelets was examined. The extract lowered submaximal aggregation and prolonged the lag time in a dose-dependent manner. After fractionation of the extract, it was revealed that the tea catechins (tannins) are active principles for inhibition and that ester-type catechins are more effective than free-type catechins. One of the ester type catechins, epigallocatechin gallate (EGCG), suppressed the collagen-induced platelet aggregation completely at the concentration of 0.2 mg/ml (= 0.45 mM). Comparing IC_{50} values of EGCG and aspirin it was found that the potency of EGCG is comparable to that of aspirin. Thrombin- and platelet activating factor (PAF)-induced aggregation was also inhibited by EGCG. The elevation of cyclic adenosine 3′,5′-monophosphate (cAMP) level was not observed in EGCG treated platelets.

Keywords tea; *Camellia sinensis*; tannin; catechin; (−)-epigallocatechin gallate; flavonoid; platelet aggregation; anti-platelet drug; anti-aggregant

Tea (*Camellia sinensis* L.) is used widely as a daily beverage and has several pharmacological effects, such as excitation, diuresis, pyretolysis and astringency. These actions are mostly caused by caffeine and tea catechins (tannins).[1] Recently it has been reported that tea inhibits platelet aggregation.[2-4]

Platelets play a central role in hemostasis after vascular injury and in formation of thrombus. They are also responsible for development of atherosclerosis. Therefore compounds which affect platelet functions could be useful for clinical purposes.

In the present study we tried to identify the active principles for inhibition of platelet aggregation in green tea extract and found that tea catechins, especially ester-type catechins are the main anti-aggregants.

Materials and Methods

Isolation of Effective Anti-aggregant from Green Tea Extract Commercially available green tea (sen-cha, dried leaves of *Camellia sinensis*) was used. Dry tea leaves (50 g) were extracted with 1 l of hot water (80°C) for 10 min. The aqueous extract was lyophilized and the green tea extract (fr. A, 10.7 g) was obtained.

As shown in Chart 1, fr. A was extracted with chloroform and ethyl acetate successively, and frs. B, C, D and E were obtained. Each fraction was lyophilized and tested for activity.

Fr. D, which showed the most potent activity, was further fractionated by column chromatography using Sephadex LH-20 (Pharmacia Fine Chemicals). The column was eluted successively with water and 15, 30 and 60% acetone, giving fr. 1 (water eluate), fr. 2 (15% acetone eluate) and fr. 3 (30% acetone eluate). The eluate with 60% acetone was separated into two fractions (frs. 4 and 5) based on the difference of color (the former was light yellow and the latter dark yellow).

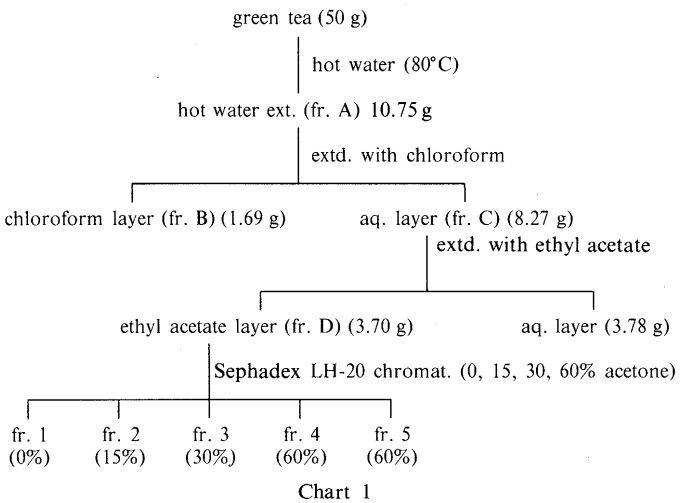

Chart 1

High Performance Liquid Chromatography (HPLC) Analysis Frs. 1—5 were analyzed by HPLC according to the method of Yayabe *et al.*[5] HPLC peaks corresponding to tea catechins were identified based on retention time.[5]

Preparation of Washed Platelets Blood was drawn from ear vein or aorta of male Japanese White rabbits. Washed platelets were prepared by centrifugation according to the method of Fukamachi *et al.*[6] Platelets were suspended in Tyrode-gelatin buffer (137 mM NaCl, 2.62 mM KCl, 1.05 mM $MgCl_2$, 0.012 M $NaHCO_3$, 0.25% gelatin (Merck)) containing 0.975 mM $CaCl_2$.

Aggregation Studies Platelet aggregation was measured by turbidimetry using a dual channel aggregometer (Niko Bioscience Inc. Tokyo). Each fraction of green tea extract was dissolved in saline (0.9% NaCl), and aspirin (Wako Chemical) was dissolved in methanol at various concentrations. Two microliters of these samples or their solvents were added to 400 μl of platelet suspension (2×10^8 cell/ml) and incubated at 37 °C for 1 min with stirring in the aggregometer cuvette. Then the stimulants were added and aggregation was observed as an increase in light transmission. The stimulants used were bovine tendon collagen (Niko Bioscience Inc., 25 μg/ml; final concentration), thrombin (Sigma, 0.5 U/ml) and platelet activating factor (PAF) (Bachem Feinchemikalien, 0.1 nM).

To calculate degree of inhibition (%), the light transmittance values at 3 and 5 min after the addition of the stimulant were measured for thrombin- and collagen-induced aggregations, respectively and for PAF-induced aggregation the maximal light transmittance was measured.

Platelet Cyclic Adenosine 3′,5′-Monophosphate (cAMP) Assay Aliquots (0.4 ml) of platelet suspension (5×10^8 cell/ml) were incubated with epigallocatechin gallate (EGCG) in saline (0.05—5 mM, final concentration) or saline for 1 min at 37 °C. The reaction was stopped by adding cold trichloroacetic acid to a final concentration of 6%. Extraction and determination of cAMP were performed according to the instruction manual of the cAMP [^{125}I]RIA kit (New England Nuclear).

Other Quantitative Analysis The concentration of total tea catechins (tannins) was determined according to the official method of analysis of tea.[7] Caffeine concentration was determined by HPLC based on peak area.[8]

Other Materials EGCG (mol. wt = 502) was donated by Mr. Yayabe (Ito-en Central Research Institute). All other chemicals were of analytical grade.

Statistical Methods The significance of the change in platelet cAMP level owing to EGCG treatment was evaluated by use of Student's *t*-test.

Results and Discussion

Anti-aggregant Component of Green Tea Extract As shown in Fig. 1, green tea extract lowered the extent of submaximal aggregation and prolonged the lag time in a

dose-dependent manner. At the concentration of 1 mg/ml the aggregation was completely suppressed.

The green tea extract was fractionated and the effect of each fraction was examined (Fig. 2). At the first step both the caffeine-rich fraction, fr. B (caffeine content: 54%) and fr. C (0.44%) showed inhibitory activity. Caffeine is known to inhibit adenosine diphosphate (ADP)-induced platelet aggregation at high concentrations[9] and in our experiment it also inhibited collagen-induced aggregation by 33% at the concentration of 2 mM (0.39 mg/ml). However, the caffeine content of the green tea extract (6.3%) was not enough to cause significant inhibition and its contribution to the inhibitory activity of the extract seemed to be small. At the next step the inhibitory activity of fr. C shifted to the catechin fraction, fr. D, and the remaining aqueous fraction (fr. E) had no activity. The contents of tea catechins in frs. A, B, D and E were 34.6%, 10.4%, 96.4% and 0.1%, respectively. Thus, we concluded that the anti-platelet principles in the green tea extract are tea catechins.

Fr. D was further separated into five fractions by Sephadex LH-20 chromatography. Frs. 1 and 2, which did not contain tea catechins, had no inhibitory activity. On the other hand frs. 3, 4 and 5 effectively inhibited the aggregation and the potencies of frs. 4 and 5 were greater than that of fr. 3 (Fig. 2b). As shown in Fig. 3, fr. 3 consisted of free-type catechins, namely (−)-epigallocatechin (EGC) and (−)-epicatechin (EC), and fr. 5 consisted of EGCG and (−)-epicatechin gallate (ECG), which are ester-type catechins. Fr. 4 contained both types of catechins. Therefore it appeared that ester-type catechins were more effective than free-type catechins.

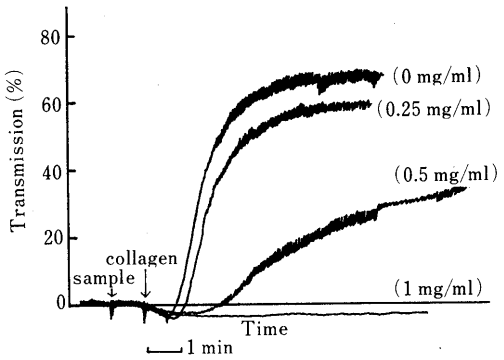

Fig. 1. Inhibition by Green Tea Extract of Platelet Aggregation Induced by Collagen

Washed rabbit platelets were preincubated with green tea extract for 1 min at 37°C and exposed to collagen (25 μg/ml). The final concentration of each sample is shown in parentheses.

Characterization of the Inhibitory Action of EGCG on Platelet Aggregation We studied the inhibitory action of EGCG, which is the major component of fr. 4 (EGCG: 56.7%) and fr. 5 (EGCG: 75.8%). First the effects of stimulants on the inhibitory action of EGCG were examined (Fig. 4). In collagen-induced platelet aggregation, EGCG prolonged the lag time and reduced the submaximal aggregation in a dose dependent manner. At the concentration of 0.2 mg/ml, aggregation did not occur. EGCG also inhibited thrombin- and PAF-induced aggregation. This is different from inhibition by aspirin, an anti-platelet drug widely used for clinical purposes, which does not inhibit PAF-induced aggregation.[10] EGCG may disturb the common pathway of aggregation by these stimulants.

The inhibitory activity of EGCG on collagen-induced aggregation was compared with that of aspirin, IC_{50} values of EGCG and aspirin were 0.11 mM (0.057 mg/ml) and 0.23 mM (0.042 mg/ml), respectively. Thus, the activity of EGCG was comparable to that of aspirin (Table I).

Next the effect of incubation time was investigated. As shown in Fig. 5a, the inhibitory effect of EGCG was dependent on preincubation time with platelets, that is, the degree of inhibition increased with preincubation time. This suggested that EGCG directly interacts with platelets, but not with stimulants, to suppress platelet aggregation.

Next EGCG was added to activated platelets. When EGCG was added 15, 30 and 45 s after addition of collagen, the aggregation was suppressed immediately (Fig. 5b).

Figure 6 shows the effect of EGCG on the cAMP levels in the platelets. Cellular cAMP level was not elevated by

Table I. Inhibitory Effects of EGCG and Aspirin on Platelet Aggregation

Aggregating agent	IC_{50}[a] mM (mg/ml) EGCG	Aspirin
Collagen	0.11 (0.057)	0.23 (0.042)
Thrombin	0.48 (0.21)	n.d.[b]
PAF	0.056 (0.025)	n.d.[b]

a) Means of 2—5 different determinations. b) Not determined.

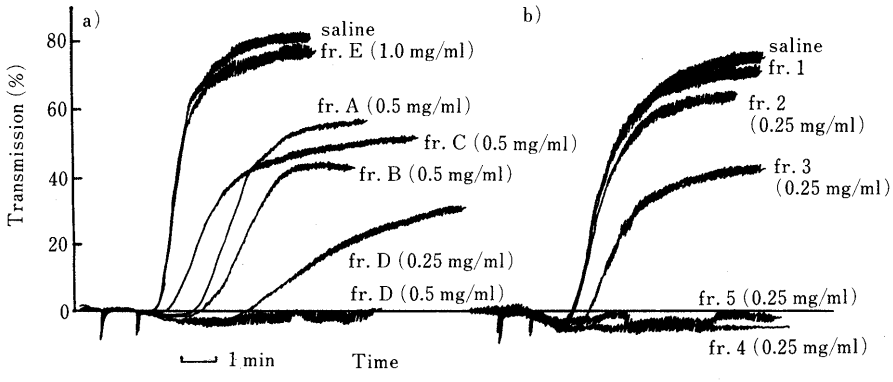

Fig. 2. Influence of Fractions of Green Tea Extract on Platelet Aggregation by Collagen

Washed rabbit platelets were preincubated with fractions of green tea extract (see Chart 1) for 1 min at 37°C and exposed to collagen (25 μg/ml). The final concentration of each sample is shown in parentheses.

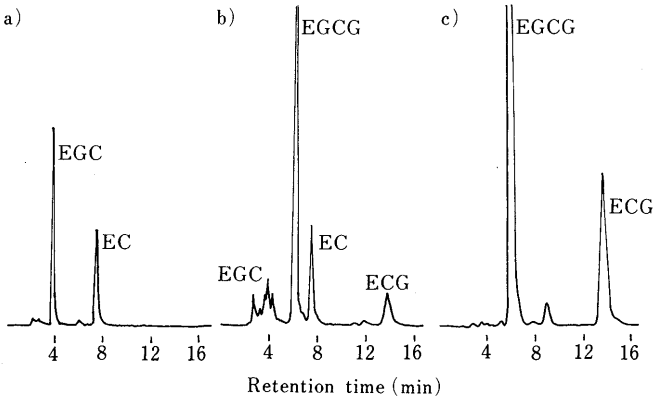

Fig. 3. High Performance Liquid Chromatogram of Frs. 3, 4, and 5

Frs. 3(a), 4(b), and 5(c), separated by Sephadex LH-20 chromatography, were analyzed by HPLC under the following conditions: column, CAPCEL PACK C_{18} (Shiseido); mobile phase, methanol/water (22 : 78) containing 0.05% H_3PO_4; detection, UV 280 nm. (−)-Epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG) were identified with reference to the literature (ref. 7).

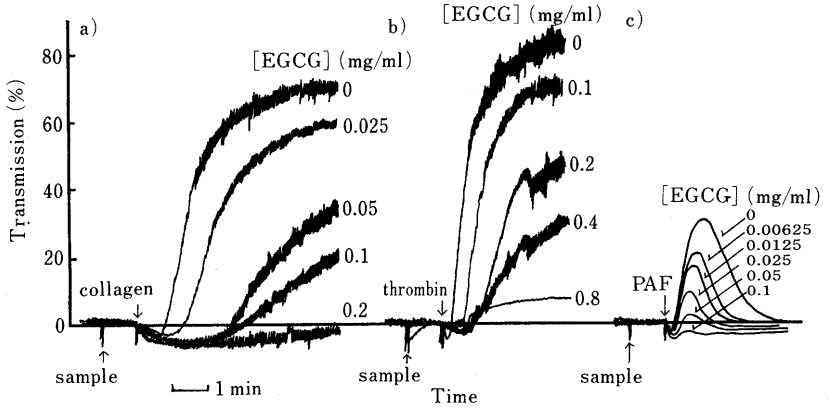

Fig. 4. Inhibition by (−)-Epigallocatechin Gallate (EGCG) of Platelet Aggregation Induced by Collagen (a), Thrombin(b) and PAF (c)

Washed rabbit platelets were preincubated with EGCG at various concentrations for 1 min at 37 °C and then exposed to collagen (25 μg/ml), thrombin (0.5 U/ml) and PAF (0.1 nM).

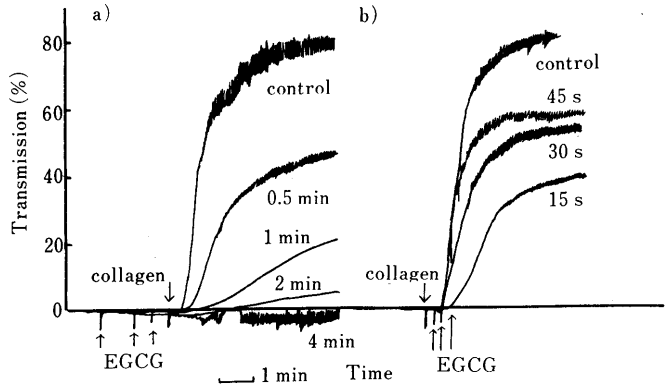

Fig. 5. Effect of Addition of (−)-Epigallocatechin Gallate (EGCG) at Various Times on Collagen-Induced Platelet Aggregation

EGCG (0.2 mg/ml) was added to platelets at the indicated times before (a) and after (b) addition of collagen (25 μg/ml).

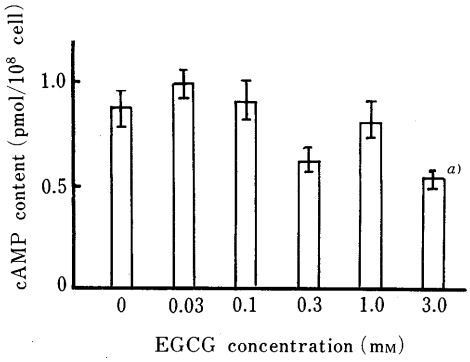

Fig. 6. Influence of (−)-Epigallocatechin Gallate (EGCG) on cAMP Level in Washed Rabbit Platelets

Platelet cAMP level was measured after incubation with EGCG or the solvent for 1 min at 37 °C. Values are expressed as mean ± S.E.M. for 5 experiments. *a)* represents significant difference ($p < 0.05$) between cAMP level in the presence of EGCG and in its absence.

incubation with EGCG, but rather tended to decrease at higher concentrations.

Many anti-aggregant drugs including several flavonoids are known to raise cAMP level in platelets.[11] Although catechin, a kind of flavanol, can be regarded as a flavonoid, its mechanism may be different from that of other flavonoids.

Various physiological properties of tea catechins have been reported, such as anti-mutagenicity,[12] anti-hypercholesterolemia[13,14] and hypotension.[15] In this study anti-aggregant activity was additionally found in tea catechins. Further studies are continuing to evaluate the *in vivo* effect of tea catechins as anti-aggregant agents.

Acknowledgement The authors wish to thank Dr. R. Goto for his helpful advice. Thanks are also due to Dr. T. Takeo for his encouragement throughout this study and to Mr. Yayabe for a gift of EGCG.

References and Notes

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