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In vitro and *in vivo* effect of ginseng saponis, major components of Korean red ginseng on human platelet aggregation and arachidonic acid metabolism

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Abstract

Effect of ginseng saponins, major components of Korean red ginseng on human platelet aggregation was studied. Among seven ginseng saponins, only ginsenoside Rg_1 had potent anti-aggregatory action *in vitro* when platelets were stimulated with collagen and arachidonic acid (AA). The conversion of exogenously added [^{14}C]AA to [^{14}C]thromboxane B_2 (TXB_2) in washed platelets was not affected by the addition of ginsenoside Rg_1. Ginsenoside Rg_1 inhibited 9,11-epithio-11,12-methano-TXA_2 (STA_2)-induced platelet aggregation while the specific binding of [^{3}H]SQ29548 to human platelet was not affected. These results suggest that ginsenoside Rg_1 might inhibit platelet aggregation most probably by the inpairment of TXA_2-mediated pathway at postreceptor sites. Oral administration of 50 mg of ginsenoside Rg_1 to healthy subjects significantly reduced platelet aggregation induced by collagen, AA and STA_2. The present study indicates that ginsenoside Rg_1 has a potent anti-aggregatory action both *in vivo* and *in vitro*, and therefore ginsenoside Rg_1 may be beneficial for the prevention and the treatment of thrombotic cardiovascular disorders.

Key words Korean red ginseng, ginseng saponin, platelet aggregation, arachidonic acid metabolism, TXA_2 receptor.

Abbreviations AA, arachidonic acid ; cAMP, adenosine 3′, 5′-cyclic monophosphate ; cGMP, guanosine 3′,5′- cyclic monophosphate ; 12 - HETE, 12 - hydroxyeicosatetraenoic acid ; HHT, 12-hydroxyheptadecatrienoic acid ; HPLC, high performance liquid chromatography ; PGI_2-Na, prostacyclin-Na ; PPP, platelet poor plasma ; PRP, platelet rich plasma ; STA_2, 9,11-epithio-11,12-methano-TXA_2 ; TLC, thin layer chromatography ; TXA_2, thromboxane A_2 ; TXB_2, thromboxane B_2.

Introduction

Korean red ginseng (*Panax ginseng* C.A. MEYER) has been widely used in Chinese medicine for the treatment of various diseases including thrombotic cardiovascular disorders.[1,2] The structures of ginseng saponins, major components of Korean red ginseng, have been intensively investigated and determined by Shibata and his coworkers[3-5] (Fig. 1). It has been reported that ginseng saponins had an anti-platelet aggregatory effect *in vitro*.[6] However no detailed study on anti-aggregatory effects of ginseng saponins has been reported yet.

Recent studies on arachidonic acid (AA) metabolism and platelet function indicate that thromboxane A_2 (TXA_2) is the most important AA metabolite for platelet aggregation because of its potent proaggregatory effect.[7,8] TXA_2 is said to bind to its receptor on platelets and evoke postreceptor events, which lead to platelet aggregation.[9]

In order to get further insight into the anti-aggregatory effect of ginseng saponins, we investigated the *in vitro* effect of ginseng saponins on

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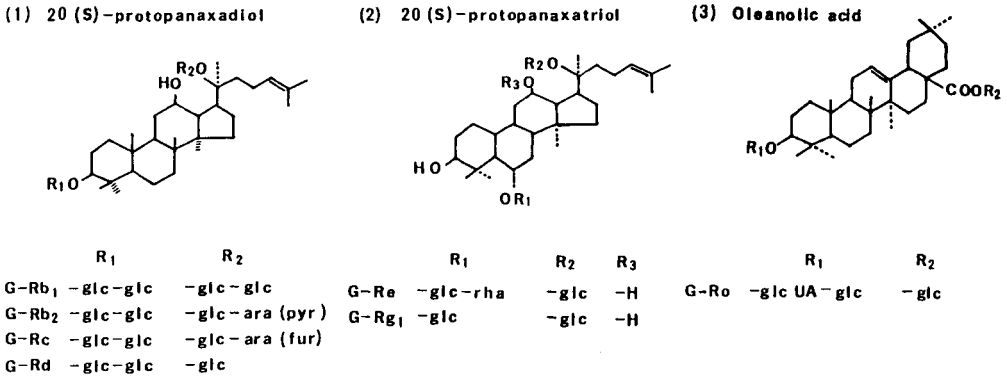

Fig. 1 Structure of ginseng saponins.

the metabolism of AA to TXA_2 in platelets and TXA_2-mediated pathway which led to platelet aggregation. Furthermore, a healthy volunteer study was performed in order to determine the *in vivo* effect of ginseng saponins on human platelet aggregation.

Materials and Methods

Chemicals : Arachidonic acid (99% pure, AA) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Collagen was purchased from Hormonchemie (Munich, West Germany). Prostacyclin-Na (PGI_2-Na) and 9,11-epithio-11,12-methano-TXA_2 (STA_2) was a generous gift from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). [5,6-^{3}H(N)]SQ29548 (40 Ci/mmol) and [1-^{14}C]arachidonic acid ([^{14}C-AA]) (10 μCi/ml EtOH) was purchased from New England Nuclear (Boston, MA, U.S.A.). SQ29548 was a gift from Squibb Institute (Princeton, NJ, U.S.A.). Silica gel 60A LK6D plates were obtained from Whatman Manufacturing Inc. (Clifton, NJ, U.S.A.). Other chemicals were of special grade and purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan).

Ginseng saponins (Rb_1, Rb_2, Rc, Rd, Re, Rg_1 and Ro) used in this experiment were isolated from the Red Ginseng Root by the procedure of Shibata and his co-workers.[3-5] These ginseng saponins were kindly supplied from Japan Korea Red Ginseng Co., Ltd. (Kobe, Japan) and Korea Ginseng and Tobacco Research Institute (Daejeon, Korea). The purities of these ginseng saponins were greater than 98% based on the analysis by high performance liquid chromatography (HPLC).

Platelet aggregation : Citrated venous blood was obtained from healthy volunteers who had not taken any drugs for at least 2 weeks. Platelet aggregation study was carried out according to the method as previously reported.[10] When the *in vitro* effect of ginseng saponins was investigated, platelet rich plasma (PRP) (3×10^8/ml) was incubated with various concentrations of ginseng saponins in ethanol (final concentration 0.4%) for 3 min at 37℃ and then stimulated by an aggregant for 5 min using a 4 channel aggregometer HEMA TRACER 1 (Nico Bioscience, Tokyo, Japan). The change in light transmission after the addition of aggregants was recorded as a percent value to determine the maximal aggregation.

Metabolism of exogenous [^{14}C] arachidonic acid by washed human platelets : Washed human platelets (10^9/ml) were prepared according to the method as previously reported.[10] Washed platelet suspensions were preincubated with various concentrations of ginsenoside Rg_1 dissolved in ethanol (final concentration 0.4%) for 3 min at 37℃ and then further incubated with [^{14}C]AA (0.1 μCi). The reaction was carried out for 5 min at 37℃ and terminated by the addition of 2 M citrate. The metabolites of [^{14}C]AA were extracted by ice-cold ethylacetate and separated by thin layer chromatography (TLC) as previously

repored.[10] The areas corresponding to AA, TXB_2, 12-hydroxyheptadecatrienoic acid (HHT) and 12-hydroxyeicosatetraenoic acid (12-HETE) were detected by autoradiography, scraped off and counted in a liquid scintillation counter (LKB, Rack β, Wallac, Turku, Finland).

Displacement of the specific [^3H]SQ29548 binding to human platelets by ginsenoside Rg_1 and STA_2 : Binding studies were performed as described by Narumiya *et al.*[11] Washed human platelets (10^9/ml) were suspended in the suspension buffer (138 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 25 mM Tris-HCl, pH 7.5) and incubated with 0.5 nM [^{3}H]SQ29548 (0.004 μCi) at 37℃ for 15 min in the presence of various concentrations of ginsenoside Rg_1 and STA_2. Non-specific binding was obtained by displacing bound radioactivity with 5 μM SQ29548. The reaction was terminated by centrifugation and the supernatant was rapidly removed, and the pellet was washed 1 ml of the suspension buffer. The radioactivity in the pellet was measured in a liquid scintillation counter.

Measurement of basal levels of adenosine 3', 5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) in washed human platelets : A washed platelet suspension prepared as mentioned above was also used for the determination of cAMP and cGMP contents in washed platelets as previously reported.[10] A washed platelet suspension (10^9/ml) was incubated with various concentrations of ginsenoside Rg_1 in ethanol (final concentration 0.4%) and incubated at 30℃ for 5 min. Then, precooled 0.1 N HCl was added. After freezing and thawing 3 times, the reaction mixture was centrifuged at $1800 \times g$ for 30 min at 4℃. Cyclic AMP and cGMP in the supernatant fluid were succinylated quantitatively and were then measured by use of commercially available kits (Yamasa Shoyu Co., Ltd., Chiba, Japan) according to the method of Kunitada *et al.*[12]

Experimental design of healthy volunteer study : Six healthy male subjects who had not taken any drugs for at least 2 weeks were used in the present experiment. Their mean age was 30 years (range 24−38). They had a mean body weight of 61 kg. Consent was obtained from individuals. The subjects ingested 50 mg of ginsenoside Rg_1 after 12 hours fasting. Citrated venous blood was obtained at the beginning of the experiment and 3 hours after the administration. PRP and plateler poor plasma (PPP) were prepared and platelet aggregation study was carried out as described above. The following aggregants were used ; 0.25, 0.5, 0.75 and 1.0 μg/ml of collagen and 200, 300, 400, 500 and 600 μM of AA and 0.25, 0.5, 0.75 and 1.0 μM of STA_2. Threshold dose of an aggregant for platelet aggregation was expressed by an observed value of the minimum concentration of the aggregant that would induce more than 50% aggregation. Threshold dose of each aggregant was determined at the beginning of the experiment and a percent value of platelet aggregation stimulated by the threshold dose of an aggregant was recorded before and after administration of ginsenoside Rg_1.

Statistical analysis : Comparisons of the results obtained before and after the ingestion of ginsenoside Rg_1 were made using the paired Student's t-test. Other comparisons were made using the non-paired Student's t-test.

Results

In vitro effect of ginseng saponins on human platelet aggregation

As shown in Fig. 2(a), ginsenoside Rg_1 dose-dependently inhibited collagen-induced platelet aggregation, while other ginseng saponins up to 500 μM had no effect on collagen-induced platelet aggregation (data not shown). As shown in Fig. 2(b), ginsenoside Rg_1 dose-dependently inhibited AA-induced platelet aggregation and aggregation was completely abolished at concentration about 200 μM. On the other hand, other ginseng saponins up to 500 μM had no effect on AA-induced platelet aggregation (data not shown). As shown in Fig. 2(c), ginsenoside Rg_1 dose-dependently inhibited STA_2-induced platelet aggregation, while other ginseng saponins up to 500 μM had no effect on STA_2-induced platelet aggregation (data not shown).

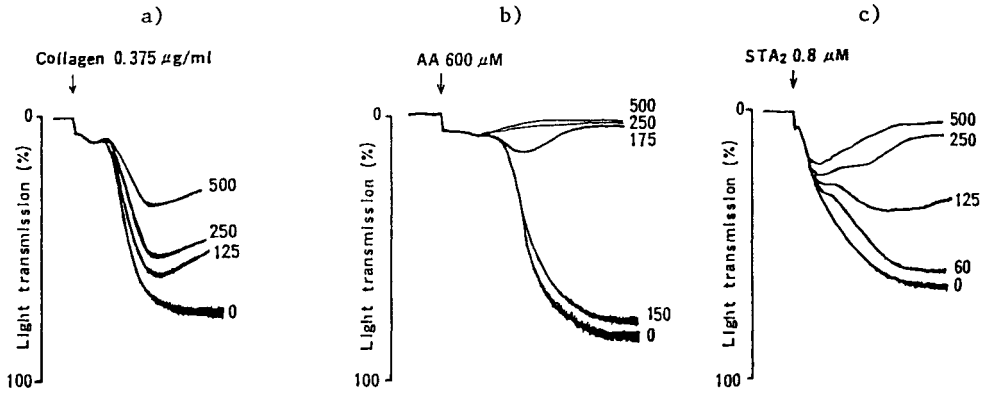

Fig. 2 Effect of ginsenoside Rg_1 on platelet aggregation of PRP.
The platelets were preincubated with ginsenoside Rg_1 (the final concentration in μM for each curve) for 3 min before the stimulation.

Table I Effect of ginsenoside Rg_1 on the metabolism of exogenously added [^{14}C]AA by washed human platelets.

Concentration of ginsenoside Rg_1 (μM)	[^{14}C]TXB_2 (% of control)	[^{14}C]12-HETE (% of control)
0	100.0±2.0	100.0± 4.2
50	102.0±3.7	95.0± 9.4
100	99.2±0.5	100.0± 8.5
250	100.8±5.6	101.0± 1.7
500	97.3±3.7	101.0±16.3

Washed platelets (10^9/ml) were incubated with [^{14}C]AA for 5 min in the presence or absence of ginsenoside Rg_1. After incubation, AA metabolites were extracted and analyzed as described in Materials and Methods. Values are means±S.D. obtained from three experiments.

In vitro effect of ginsenoside Rg_1 on the metabolism of exogenously added [^{14}C]AA by washed human platelets

As shown in Table I, the *in vitro* addition of ginsenoside Rg_1 up to 500 μM had no significant effect on the conversion of exogenously added [^{14}C]AA to both [^{14}C]TXB_2 and [^{14}C]12-HETE. Also the conversion to [^{14}C]-HHT did not change by the addition of ginsenoside Rg_1 (data not shown).

Effect of ginsenoside Rg_1 on the specific binding of [^{3}H]SQ29548 to washed human platelets

As shown in Fig. 3, ginsenoside Rg_1 did not affect the specific binding of [^{3}H]SQ29548 to washed human platelets at concentrations between 10^{-6} M and 10^{-3} M, while STA_2 displaced [^{3}H]SQ29548 binding in a concentration-depen-

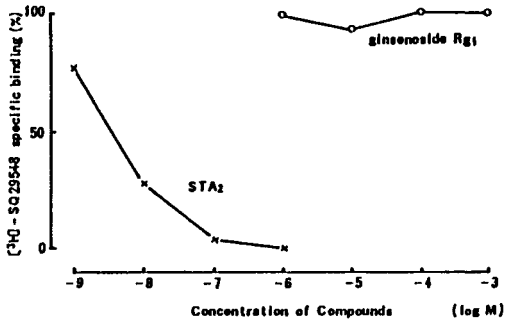

Fig. 3 Displacement of the specific [^{3}H]SQ29548 binding by ginsenoside Rg_1 and STA_2.
Each point represents the mean of three experiments.

Table II Effect of ginsenoside Rg_1 on basal levels of cAMP and cGMP in washed human platelets.

Ginsenoside Rg_1 (μM)	cAMP (pmol/10^9 platelets)	cGMP (pmol/10^9 platelets)
0	7.0±0.23	0.93±0.07
100	7.1±0.06	0.92±0.16
250	7.3±0.49	0.84±0.04
500	7.7±1.01	0.79±±0.08

Washed platelets (10^9/ml) were incubated with various concentrations of ginsenoside Rg_1 for 5 min. Assays were performed as described in Materials and Methods. Values are means±S.D. obtained from three experiments.

dent manner at concentrations between 10^{-9} M and 10^{-6} M.

In vitro effect of ginsenoside Rg_1 on basal levels of cAMP and cGMP in washed human platelets

The basal levels of cAMP and cGMP in washed human platelets was 7.0±0.23 and 0.93±0.07 pmol/10^9 platelets (mean±S.D., N=3) respectively. As shown in Table II, the *in vitro* addition of ginsenoside Rg_1 up to 500 μM did not affect basal levels of cAMP and cGMP in washed human platelets.

Effect of oral administration of ginsenoside Rg_1 on platelet aggregation in healthy subjects

Table III shows the changes in platelet aggregation before and after single oral administration of ginsenoside Rg_1. Threshold doses of collagen, AA and STA_2, which were used in the present study, were 0.6±0.2 μg/ml (mean±S.D.), 500 ± 180 μM, 0.8 ± 0.2 μM, respectively. Platelet aggregation induced by collagen, AA and STA_2 were significantly reduced by oral administration of ginsenoside Rg_1.

Table III Effect of single oral administration of ginsenoside Rg_1 on platelet aggregation.

Aggregant	Platelet aggregation (%)	
	before	3 hr
Collagen	56±15	37±20*
AA	65±11	19±30*
STA_2	70± 6	43±21*

n=6, *$p<0.05$ (mean±S.D.).

Discussion

In the present experiment, the *in vitro* addition of ginsenoside Rg_1 inhibited platelet aggregation in PRP when stimulated by collagen and AA. Similar results were obtained by Nakanishi *et al.*[13] In contrast, other ginseng saponins had no effect in inhibiting platelet aggregation. These results indicate that among ginseng saponins which were used in this experiment, only Rg_1 has anti-platelet aggregatory effect *in vitro*.

It is well known that TXA_2 is a major metabolite of AA in platelets and has a potent pro-aggregatory effect. TXA_2 is proposed to play an important role in both collagen- and AA-induced platelet aggregation.[14,15] Actually, platelet aggregation induced by these aggregants is reported to be abolished by the inhibition of TXA_2 production.[14-17] Accordingly, we investigated the *in vitro* effect of ginsenoside Rg_1 on the metabolism of AA in platelets. An interesting finding was that the conversion of exogenously added [^{14}C]AA to [^{14}C]TXB_2 and [^{14}C]12-HETE was not affected by the addition of ginsenoside Rg_1. This result indicates that ginsenoside Rg_1 has virtually no effect on the metabolic pathway from AA to TXA_2. Also it raises a possibility that ginsenoside Rg_1 may inhibit platelet aggregation after TXA_2 is produced. Then the effect of ginsenoside Rg_1 on aggregatory action of TXA_2 was studied. As TXA_2 is a very unstable compound and immediately metabolized to biologically inactive TXB_2, STA_2, a stable analogue of

TXA_2, was used in the present study. STA_2 is reported to bind TXA_2 receptor on platelet membrane and activate human platelets through TXA_2 receptor-mediated pathway.[9,18] A notable finding is that the *in vitro* addition of ginsenoside Rg_1 dose-dependently inhibited platelet aggregation induced by STA_2. These results suggest that ginsenoside Rg_1 might inhibit platelet aggregation not by the inhibition of TXA_2 production, but by the inhibition of the aggregatory action of TXA_2.

TXA_2 is said to bind to its receptor on platelet membrane and thereby induce platelet aggregation. Therefore the effect of ginsenoside Rg_1 on the binding of TXA_2 to its receptor was studied. TXA_2 receptor has been investigated using various radioligands of TXA_2 agonists and antagonists.[19,20] In the present study [^{3}H]SQ29548, a specific TXA_2 receptor antagonist, was used as a ligand.[21,22] The specific binding of [^{3}H]SQ29548 to human platelets was not affected by ginsenoside Rg_1 while it was dose-dependently displaced by STA_2. These results suggest that ginsenoside Rg_1 may not impair the binding of TXA_2 to its receptor on platelet membrane. It raises a possibility that ginsenoside Rg_1 may inhibit TXA_2-induced platelet aggregation probably by impairing postreceptor events.

Agents which increase cAMP and cGMP levels in platelets have been described to inhibit platelet aggregation.[23,24] So we investigated the effect of ginsenoside Rg_1 on the basal levels of cAMP and cGMP in washed platelets. Ginsenoside Rg_1 did not affect the basal levels of both cAMP and cGMP. Therefore this possibility appears to be less likely.

These *in vitro* studies demonstrated that ginsenoside Rg_1 inhibited collagen- or AA-induced platelet aggregation and this anti-aggregatory effect of ginsenoside Rg_1 might be ascribed to the impairment of TXA_2-mediated pathway at postreceptor events, though further study is required.

Then we performed healthy volunteer study in order to clarify whether ginsenoside Rg_1 also had an anti-platelet aggregatory effect *in vivo*. Fifty mg of ginsenoside Rg_1 was orally administered to 6 healthy subjects. We previously reported that single oral administration of crude saponins of Korean red ginseng reduced platelet aggregation after 3 hours of ingestion.[25] Accordingly in the present study, platelet aggregation was determined after 3 hours of ingestion. A notable finding is that single oral administration of ginsenoside Rg_1 significantly decreased platelet aggregation induced by collagen, AA and STA_2. These results indicate that ginsenoside Rg_1 has an anti-platelet aggregatory effect not only *in vitro* but also *in vivo*.

In conclusion, the present study demonstrated that ginsenoside Rg_1 has an anti-platelet aggregatory effect both *in vitro* and *in vivo*. These results raise a possibility that ginsenoside Rg_1 may be beneficial for the prevention and the treatment of cardiovascular thrombotic disorders.

Acknowledgement

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和文抄録

高麗人参の主成分である人参サポニンの血小板凝集に対する作用を検討した。検討を加えた7つの人参サポニンの中で，ジンセノサイドRg_1だけに，アラキドン酸及びコラーゲン凝集について強力な血小板凝集抑制作用が認められた。洗浄血小板において外因性に添加された[^{14}C]アラキドン酸から[^{14}C]トロンボキサンB_2への変換は，ジンセノサイドRg_1の存在下にて影響をうけなかった。ジンセノサイドRg_1は，STA_2凝集を抑制したが[^{3}H]SQ29548のヒト血小板への特異的結合には影響をあたえなかった。これらの結果よりジンセノサイドRg_1は，TXA_2依存性の反応をおそらくレセプター以後で阻害して血小板凝集を抑制している可能性が示唆された。健常人へのジンセノサイドRg_1 50 mgの経口投与によりコラーゲン，アラキドン酸及びSTA_2による血小板凝集が有意に抑制された。今回の検討によりジンセノサイドRg_1が，*in vivo*においても*in vitro*においても血小板凝集抑制作用を持つことが明らかとなり，血栓性疾患の予防や治療に有用な薬物となる可能性が示唆された。

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