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Pharmacological Study on *Panax ginseng* C. A. MEYER. III.[1] Effects of Red Ginseng on Experimental Disseminated Intravascular Coagulation. (2). Effects of Ginsenosides on Blood Coagulative and Fibrinolytic Systems

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The activities of seven ginsenosides isolated from Red Ginseng on blood coagulative and fibrinolytic systems closely related to disseminated intravascular coagulation were investigated in *in vitro* models and compared with those of standard agents (aspirin, heparin and dextran sulphate).

Ginsenoside-Rg_2 showed strong inhibitory activity on platelet aggregation induced by three aggregating agents (endotoxin, collagen and arachidonic acid) as compared with aspirin at a concentration of 1.0 mM. Ginsenoside-Ro inhibited the conversion of fibrinogen to fibrin induced by thrombin at concentrations of 0.1 to 1.0 mM. Ginsenoside-Ro, -Rb_1, -Rb_2, -Rc, -Re, -Rg_1 and -Rg_2 may promote the action of urokinase in the fibrinolytic system on the basis of its action on plasminogen-containing fibrin plates.

Keywords——*Panax ginseng*; Red Ginseng; ginsenoside; blood coagulation; fibrinolysis

Red Ginseng (the steamed and dried root of *Panax ginseng* C. A. MEYER) has been used for treatment of weak constitution, ulcer, cold symptoms or anemia as an analeptic, a stomachic and an erythropoietic in the traditional Chinese system of medicine.

In the previous paper,[2] we reported that a 70% methanolic extract of Red Ginseng showed an inhibitory effect against experimental disseminated intravascular coagulation (DIC) in rats; it also inhibited platelet aggregation induced by various aggregating agents, and the conversion of fibrinogen to fibrin induced by thrombin. In addition, a 70% methanolic extract promoted the fibrinolytic system in fibrin plates. These inhibitory and promoting effects were found in the *n*-BuOH-soluble portion containing saponins fractionated from the 70% methanolic extract.

The purpose of the present investigation was to clarify the active principles of the *n*-BuOH-soluble portion on the blood coagulative and fibrinolytic systems using *in vitro* models.

Materials and Methods

Materials——Ginsenoside-Ro, -Rb_1, -Rb_2, -Rc, -Re, -Rg_1 and -Rg_2 were obtained from Red Ginseng as reported previously.[3] Thrombin was from Mochida Ltd., Japan, urokinase from Midorijuji Ltd., Japan, collagen, arachidonic acid and plasminogen from Sigma Chemical Co., U.S.A., and plasminogen-containing and free fibrinogen from Nakarai Chemical Ltd., Japan.

Animals——Male Wistar–King strain rats weighing 150—200 g were used for the experiments involving blood platelet aggregation. They were fed a standard diet (Nihon Clea, Japan) for at least 7 d. They were fasted for 24 h before the start of the experiments.

Blood Platelet Aggregation Test——Whole blood samples were collected from the heart of pentobarbital-anesthetized rats. Nine ml of the blood and 1 ml of heparin solution (10 U/ml) were transferred into a plastic tube, and centrifuged at 1000 rpm for 10 min to obtain platelet-rich plasma (PRP). PRP was removed with a siliconized pipet, and stored in a plastic test tube with a screw cap. The remaining red cell precipitate of the blood samples was further centrifuged at 3000 rpm for 30 min to give platelet-poor plasma (PPP), which was used as a maximal transmittance standard.

The platelet aggregation test described by Born and Gross[4] was performed with endotoxin (500 μg/ml), collagen (500 μg/ml) and arachidonic acid (50 mM) as aggregating agents. A 0.2 ml aliquot of PRP was placed in a test tube and the content was stirred at 1200 rpm for 1 min at 37 °C, then a 10 μl aliquot of a test solution was added. After 1 min, an aggregating agent was added to the reaction mixture. Changes in the light transmittance of the reaction mixture were continuously recorded with a Husm system platelet aggregometer (Rika Electric Co., Japan) and the transmission at the maximal aggregation after the addition of an aggregating agent was recorded. Platelet aggregation was expressed as the percent increase in the transmittance taking the transmittance of a control mixture containing no test solution as zero. An anti-platelet aggregating agent, aspirin, was used as a standard drug.

Thrombin-Induced Conversion of Fibrinogen to Fibrin——Fibrinogen (500 mg) was dissolved in 100 ml of 0.05 M NaCl containing 0.05 M Tris acetate buffer (pH 7.4). A test solution (0.1 ml) was added to 1.8 ml of the fibrinogen solution with stirring. After 1 min, 0.1 ml of thrombin solution (0.2 U/ml) was added to the mixture and the whole was gently stirred until a fibrin clot appeared. The time required for clotting was recorded. An anti-thrombin agent, heparin, was used as a standard drug.

Fibrin Plates——Fibrin plates were prepared by the method of Noren *et al.*[5] One % agarose solution in phosphate-buffered saline (0.01 M phosphate buffer (pH 7.8) in 0.15 M NaCl) was kept at 45—50 °C in a water bath. Agarose solutions of plasminogen-containing fibrinogen and of plasminogen-free fibrinogen were prepared by dissolving 166 mg of plasminogen-containing fibrinogen and 200 mg of plasminogen-free fibrinogen, respectively, in 100 ml aliquots of agarose solution at 31 °C. A 10 ml aliquot of one of these solutions and 0.1 ml of thrombin (10 U/ml) solution were quickly mixed in a test tube, and the contents were immediately poured into a Petri dish. Then, five wells of 5 mm diameter were made in each fibrin-agar plate.

Activation of Plasminogen——The experiments on activation of plasminogen, promotion of urokinase action and activation of plasmin were performed by the method of Astrup and Mullertz.[6] The test solution (20 μl) prepared at the required concentration was placed in each hole in the plasminogen-containing fibrin plate. The plates were incubated at 31 °C for 20 h, and the lysed area was measured. The effect of samples on the activation of plasminogen was assessed by comparing the lysed area with that of the control. The control was 20 μl of phosphate buffer.

Promotion of Urokinase Action——A test solution (0.1 ml) and urokinase solution (0.1 ml, 100 U/ml) were mixed, and 20 μl of the mixture was added to each of the wells in a plasminogen-containing fibrin plate. Twenty μl of a mixture of phosphate buffer (0.1 ml) and urokinase solution (0.1 ml, 100 U/ml) was used as a control mixture. The plates were incubated at 31 °C for 20 h. Transparent rings appeared where the fibrin lysis had occurred. The lysed area was measured. The activating effect of test samples on this fibrinolytic system was assessed by comparing the lysed area with that of the control. The activity was expressed as the concentration which activated the lysis by 50% (AC_{50}: mM). Dextran sulphate was used as a standard drug.

Activation of Plasmin——Urokinase solution (0.5 ml, 100 U/ml) and plasminogen solution (0.5 ml, 0.5 mg/ml) were mixed and incubated at 28 °C for 30 min. To the incubated solution (0.1 ml) was added a test solution (0.1 ml) of an appropriate concentration. Then, 20 μl of the mixture was put into each well in a plasminogen-free fibrin plate. Twenty μl of phosphate buffer was used as a control. The plates were incubated at 37 °C for 18 h, and the lysed area was measured. The activating effect of samples on this fibrinolytic system was assessed by comparison of the lysed area with that of the control.

Results

Endotoxin-Induced Blood Platelet Aggregation

As shown in Table I, aspirin at a concentration of 1.0 mM had no effect on the endotoxin-induced blood platelet aggregation. Ginsenoside-Rb_1, and -Rg_2 inhibited the endotoxin-induced blood platelet aggregation at a concentration of 1.0 mM.

Collagen-Induced Blood Platelet Aggregation

As shown in Table II, ginsenoside-Rb_1, -Rg_1 and -Rg_2, as well as the active control agent, aspirin, inhibited the collagen-induced blood platelet aggregation at a concentration of 1.0 mM.

Arachidonic Acid-Induced Blood Platelet Aggregation

As shown in Table III, ginsenoside-Rg_2 and aspirin inhibited the arachidonic acid-

Table I. Effects of Ginsenosides and Aspirin on Endotoxin-Induced Blood Platelet Aggregation

Treatment	% of inhibition	
	0.5	1.0 (mM)
Ginsenoside-Ro		0
Ginsenoside-Rb_1		12.3±2.5
Ginsenoside-Rb_2		0
Ginsenoside-Rc		0
Ginsenoside-Re		5.3±2.4
Ginsenoside-Rg_1		10.2±4.3
Ginsenoside-Rg_2	15.3±3.0	37.7±3.8
Aspirin		0

Each value represents the mean±S.E. of 5 experiments.

Table II. Effects of Ginsenosides and Aspirin on Collagen-Induced Blood Platelet Aggregation

Treatment	% of inhibition	
	0.5	1.0 (mM)
Ginsenoside-Ro		0
Ginsenoside-Rb_1	7.8±3.0	22.2±3.2
Ginsenoside-Rb_2		0
Ginsenoside-Rc		0
Ginsenoside-Re		0
Ginsenoside-Rg_1		10.3±3.1
Ginsenoside-Rg_2	12.5±0.9	25.0±1.3
Aspirin	8.0±2.7	21.6±3.2

Each value represents the mean±S.E. of 5 experiments.

Table III. Effects of Ginsenosides and Aspirin on Arachidonic Acid-Induced Blood Platelet Aggregation

Treatment	% of inhibition	
	0.5	1.0 (mM)
Ginsenoside-Ro		10.4±3.5
Ginsenoside-Rb_1		0
Ginsenoside-Rb_2		14.3±2.3
Ginsenoside-Rc		0
Ginsenoside-Re		0
Ginsenoside-Rg_1		12.3±4.3
Ginsenoside-Rg_2	14.8±1.3	31.3±1.3
Aspirin	16.7±3.5	30.4±2.5

Each value represents the mean±S.E. of 5 experiments.

Table IV. Effects of Ginsenosides and Heparin on Conversion of Fibrinogen to Fibrin Induced by Thrombin

Treatment	Clotting time of fibrinogen (s)				
	0	0.05	0.1	0.5	1.0 (mM)[a]
Control	163±5				
Ginsenoside-Ro		185±17	208±15[c]	282±14[c]	375±12[c]
Ginsenoside-Rb_1				181±3	185±5[c]
Ginsenoside-Rb_2				170±3	180±6
Ginsenoside-Rc				176±5	162±3
Ginsenoside-Re				178±5	167±8
Ginsenoside-Rg_1				170±3	187±9[b]
Ginsenoside-Rg_2				173±3	188±3[b]
Heparin (10 U/ml)	250±3[c]				

Each value represents the mean±S.E. of 5 experiments. *a*) Concentrations of ginsenosides. Significantly different from the control, *b*) $p<0.05$, *c*) $p<0.01$.

TABLE V. Promoting Effects of Ginsenosides and Dextran Sulphate on Urokinase Action in a Plasminogen-Containing Fibrin Plate

Treatment	% of promotion					AC_{50} (mM, 95% C.L.)
	0.01	0.05	0.1	0.5	1.0 (mM)	
Ginsenoside-Ro	14.8±2.1	42.4±1.2	63.8±3.4	73.0±2.2	81.3±5.8	0.102 (0.028—0.371)
Ginsenoside-Rb_1		24.0±2.4	40.3±4.5	75.7±2.4	98.5±5.4	0.155 (0.054—0.448)
Ginsenoside-Rb_2		23.2±1.5	40.0±3.7	57.6±4.4	97.4±4.0	0.215 (0.082—0.562)
Ginsenoside-Rc		20.7±1.5	39.8±4.5	49.7±4.5	63.4±2.5	0.223 (0.063—0.785)
Ginsenoside-Re		35.7±3.8	51.4±2.7	58.7±2.1	62.4±4.9	0.174 (0.056—0.544)
Ginsenoside-Rg_1		18.4±2.0	39.4±1.7	61.0±3.6	62.7±1.0	0.240 (0.072—0.795)
Ginsenoside-Rg_2		15.2±1.4	30.7±4.0	43.0±3.4	55.0±4.2	0.255 (0.096—0.674)
Dextran sulphate	8.7±1.3	48.4±3.2	62.4±5.4	65.8±7.2	75.4±4.1	0.101 (0.022—0.482)

Each value represents the mean±S.E. of 5 experiments. AC_{50}: Concentration required to promote urokinase action by 50%. C.L.: Confidence limit.

induced blood platelet aggregation at concentrations of 0.5—1.0 mM.

Conversion of Fibrinogen to Fibrin Induced by Thrombin

As shown in Table IV, the clotting time of the control without addition of any test solution was 163±5 s. The clotting time of the anti-thrombin drug, heparin, at a concentration of 10 U/ml was 250±3 s. Ginsenoside-Ro significantly prolonged the clotting time at concentrations of 0.1—1.0 mM in a dose-dependent manner. Ginsenoside-Rb_1, -Rg_1 and -Rg_2 also prolonged the clotting time at a concentration of 1.0 mM.

Activation of Plasminogen

Ginsenoside-Ro, -Rb_1, -Rb_2, -Rc, -Re, -Rg_1 and -Rg_2 and dextran sulphate had no effect on the activation of plasminogen.

Promotion of Urokinase Action

As shown in Table V, plasminogen-containing fibrin was extensively lysed by addition of a mixture of the test solution and urokinase solution as compared with the control. The promoting activity on urokinase action of a test compound was expressed as the AC_{50} value, the concentration required to promote urokinase action by 50%. The AC_{50} values of ginsenoside-Ro, -Rb_1, -Rb_2, -Rc, -Re, -Rg_1 and -Rg_2 and dextran sulphate on the promoting activity of urokinase action were 0.102, 0.155, 0.215, 0.223, 0.174, 0.240, 0.255 and 0.101 mM, respectively.

Activation of Plasmin

Ginsenoside-Ro, -Rb_1, -Rb_2, -Rc, -Re, -Rg_1 and -Rg_2 and dextran sulphate did not exhibit any effect on the activation of plasmin.

Discussion

In order to clarify the active principles in the action of Red Ginseng against experimental DIC, the effects of various components isolated from Red Ginseng, ginsenoside-Ro, -Rb_1, -Rb_2, -Rc, -Re, -Rg_1 and -Rg_2 on the blood coagulative and fibrinolytic systems were investigated *in vitro*.

Among seven ginsenosides tested for effect on *in vitro* platelet aggregation induced by the aggregating agents, ginsenoside-Rg_2 was strongly inhibitory compared with aspirin at a concentration of 1.0 mM against all three agents, endotoxin, collagen and arachidonic acid. Ginsenoside-Rg_1 also showed relatively strong inhibitory activity against all three agents.

Ginsenoside-Rb_1 was effective against endotoxin and collagen.

As regards the conversion of fibrinogen to fibrin induced by thrombin, ginsenoside-Ro exhibited significant inhibitory activity, while ginsenoside-Rb_1, -Rg_1 and -Rg_2 showed weak activity.

As regards activation of the fibrinolytic system, all the components tested showed a promoting effect. The mode of promoting effect of these components was similar to that of dextran sulphate, which promotes the conversion of plasminogen to plasmin by urokinase.

It is considered that the syndrome of DIC is an acquired hemorragic disorder combined with the pathologic consequence of fibrin deposition in the microcirculation. In the Chinese system of medicine, Red Ginseng has been experientially applied for treatment of peripheral circulatory disorder, so it is of interest to note that its components exhibited a preventive effect against experimental DIC. There is no evidence that Red Ginseng is clinically effective on thrombosis or arteriosclerosis. However, it might have some preventive or therapeutical effects based on the present findings.

Further study is in progress on the effects of these components on *in vivo* experimental DIC in rats.

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