Inhibitory Effects of *Atractylodis Lanceae Rhizoma* and *Poria* on Collagen- or Thromboxane A₂-Induced Aggregation in Rabbit Platelets

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Kami-shoyo-san (Jia-Wei-Xiao-Yao-San), Toki-shakuyaku-san (Dang-Gui-Shao-Yao-San) and Toki-shigyaku-ka-goshuyu-shokyo-to (Dang-Gui-Si-Ni-Jia-Wu-Zhu-Yu-Sheng-Jiang-Tang) are Kampo (traditional Chinese) medicines which are traditionally and effectively used for the treatment of chilly sensation (*Hiesho*) in Japan, but the active components and their detailed mechanisms have not yet been clarified. Etiologies of *Hiesho* include poor peripheral blood circulation and platelet aggregability contributes to peripheral blood circulation; therefore, we investigated the effect of Kampo medicines on platelet aggregation using rabbit platelets *in vitro*. Collagen and U46619, a thromboxane A₂ receptor agonist, caused rabbit platelet aggregation, which was potently inhibited by pretreatment of platelets with Kami-shoyo-san and Toki-shakuyaku-san *in vitro*. Toki-shigyaku-ka-goshuyu-shokyo-to, however, did not significantly inhibit collagen- or U46619-induced platelet aggregation. Therefore, we examined the effect on platelet aggregation of two herbal medicines, *Atractylodis Lanceae Rhizoma* and *Poria*, both of which are contained in Kami-shoyo-san and Toki-shakuyaku-san but not in Toki-shigyaku-ka-goshuyu-shokyo-to. As the results indicate, *Atractylodis Lanceae Rhizoma* inhibited platelet aggregation induced by collagen but not by U46619. *Poria* effectively inhibited U46619-induced platelet aggregation and it partially inhibited collagen-induced platelet aggregation. On the other hand, *Atractylodis Lanceae Rhizoma* and *Poria* did not inhibit adrenaline/adenosine diphosphate- or adrenaline/serotonin-induced platelet aggregation. These results suggest the possibility that the inhibition of platelet aggregation by two Kampo medicines, Kami-shoyo-san and Toki-shakuyaku-san, is one of the mechanisms underlying the improvement of *Hiesho*. Furthermore, *Atractylodis Lanceae Rhizoma* and *Poria* are possible herbal medicines for the inhibition of platelet aggregation.

Key words platelet aggregation; *Atractylodis Lanceae Rhizoma*; *Poria*

Chilly sensation (*Hiesho*) is a syndrome which is defined locally in Eastern medicine. Patients with chilly sensation predominantly experience a sensation of being cold in the extremities when healthy people do not. Patients also tend to have diverse symptoms such as headache, diarrhea and back stiffness. Chilly sensation has not been dealt with in Western medicine for a long time because no diagnostic criteria exist. But recently, a focus has occurred towards the treatment of chilly sensation because the number of patients have increased due to strong air conditioning in offices and other factors.¹⁾ The factors responsible for chilly sensation are assumed to be poor peripheral blood circulation and impaired metabolism.¹⁾ Peripheral blood circulation is influenced by the activation of the sympathetic nervous system through the stimulation on α -adrenoceptors of vascular smooth muscle cells of the small artery.²⁾ Therefore, the activity of sympathetic neurons may be important for chilly sensation. Several Kampo (traditional Chinese) medicines are traditionally used for the treatment of chilly sensation in the East and are believed to be effective. They are, however, not often currently used in practice because their detailed mechanisms of action and effective components have not yet been determined. We previously showed that ethanol extract of *Piper longum* L., a plant which is used for the treatment of chilly sensation, potently inhibited platelet aggregation^{3,4)} and therefore led us to investigate if Kampo medicines used for the treatment of chilly sensation contained inhibitory components on platelet aggregation.

Platelets which play a role in thrombus formation, hemostasis and regeneration of vessels respond to a wide variety of stimuli, such as 5-hydroxytryptamine (5-HT),^{5,6)} adrenaline,^{7–9)} adenosine diphosphate (ADP),¹⁰⁾ thromboxane A₂ (TXA₂)¹¹⁾ and collagen.¹²⁾ Platelets respond to the physical or chemical stimuli by undergoing adhesion, shape change, secretion and aggregation. Such a series of responses ends in the formation of a haemostatic plug or thrombus, showing the reduction of peripheral blood circulation. Furthermore, platelets undergo cold-induced activation when they are chilled below 20 °C,¹³⁾ suggesting that platelets may be involved in chilly sensation.

Collagen mainly binds to glycoprotein VI (GPVI) and leads to activation of phospholipase A₂ (PLA₂), followed by generation and release of TXA₂ from platelets.¹⁴⁾ TXA₂ is a metabolite of arachidonic acid with a chemical half-life of about 30 s. When TXA₂ is released from activated platelets, it binds to TXA₂ receptors to cause platelet shape change and aggregation¹⁵⁾ as a positive feedback mediator.¹⁶⁾

Kampo medicines, which are used in the East, are composed of several different herbal medicines and each herbal medicine contains a number of chemical substances, with the sum total effects of these substances producing the effect when these medicines are used.¹⁷⁾ Kampo medicines, therefore, have unique effects that improve ailments, although these effects are not immediate. Since Kampo medicines have been used for a long period, it is believed that they are safe. Even so, Kampo medicines are not generally used now

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because of lack of scientific evidence demonstrating their effectiveness.

In the present study, we examined the effects on rabbit platelet aggregation of three Kampo medicines, Kami-shoyo-san (Jia-Wei-Xiao-Yao-San), Toki-shakuyaku-san (Dang-Gui-Shao-Yao-San) and Toki-shigyaku-ka-goshuyu-shokyo-to (Dang-Gui-Si-Ni-Jia-Wu-Zhu-Yu-Sheng-Jiang-Tang), which are frequently used for the treatment of chilly sensation (*Hiesho*). Moreover, we examined the effects on platelet aggregation of two herbal medicines, *Atractylodes Lanceae Rhizoma* and *Poria*, which are found in Kami-shoyo-san and Toki-shakuyaku-san, but not in Toki-shigyaku-ka-goshuyu-shokyo-to.

MATERIALS AND METHODS

Materials 9,11-Dideoxy-9 α ,11 α -epoxymethano-prostaglandin F₂ α (U46619) was obtained from Cayman Chemical (Ann Arbor, U.S.A.). Collagen (Collagenreagent Horm) was from Nycomed Pharma GMBH (Marburg, Germany). Thrombin was from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were of reagent grade or the highest quality available.

Kampo Medicines and Herbal Medicines Kampo medicines and herbal medicines were obtained from Tsumura & Co. (Tokyo, Japan). Kampo medicines and herbal medicines were dissolved in dimethylsulfoxide (DMSO; at a concentration of less than 1%).

Preparation of Washed Platelets Washed platelets were prepared from male rabbits (Japanese white rabbits weighing about 3.0–4.0 kg), as described previously.^{3,4} Fresh blood was collected into plastic tubes containing acid citrate dextrose solution (1/6 volume of blood), composed of citric acid (65 mM), trisodium citrate (85 mM) and dextrose (2%). The blood was then centrifuged at 250 $\times g$ for 12 min to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 90 $\times g$ to remove contaminated erythrocytes and leukocytes then centrifuged at 450 $\times g$ for 15 min at room temperature (20–25 °C). The pellet was washed twice with Tyrode/N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) solution (NaCl 138.3 mM, KCl 2.68 mM, MgCl₂ 1.0 mM, NaHCO₃ 4.0 mM, HEPES 10 mM, glucose 0.1% and bovine serum albumin 0.35% at pH 6.35). The resultant pellet was suspended in the second Tyrode/HEPES solution (pH 7.35) with a final density of 3 $\times 10^8$ platelets/ml. All experimental procedures were performed in accordance with the guidelines of the Animal Experimentation Committee of Tohoku University.

Determination of Platelet Aggregation Platelet aggregation was determined by a standard turbidimetric method using an aggregometer (PAM-6C, Merbanix, Tokyo, Japan), as previously described.^{3,4} Platelet aggregation was expressed as an increase in light transmission. The levels of light transmission were calibrated as 0% for a platelet suspension and 100% for the Tyrode/HEPES solution (pH 7.35). Platelet suspension (3 $\times 10^8$ platelets/ml, 0.3 ml) in a cuvette was preincubated at 37 °C for 3 min under continuous stirring at 1000 rpm. CaCl₂ was then added at a final concentration of 1 mM for 3 min. After the preincubation of Kampo medicine extracts or Herbal medicine extracts for 5 min, platelet aggregation was initiated by the addition of U46619,

collagen or thrombin and monitored for 10 min.

Data Analysis Data are expressed as mean $\pm$ S.E.M. Significant differences ($p < 0.05$) were determined by Tukey–Kramer test or Scheffe test.

RESULTS

Effects of Kampo Medicine Extracts on Collagen- or U46619-Induced Platelet Aggregation First, we examined the effects of Kampo medicine extracts on collagen- or U46619-induced platelet aggregation. While collagen (3 μ g/ml) or U46619 (3 μ M) caused rabbit platelet aggregation, a preincubation with extract from Kami-shoyo-san, at a concentration of 500 μ g/ml, significantly prevented collagen- or U46619-induced aggregation (Figs. 1A, B). The extracts of Kami-shoyo-san and Toki-shakuyaku-san inhibited collagen-induced platelet aggregation in a concentration-dependent manner with IC₅₀ values of approximately 130 μ g/ml and 300 μ g/ml, respectively (Fig. 2A). Kami-shoyo-san and Toki-shakuyaku-san also inhibited U46619-induced platelet aggregation in a concentration-dependent manner with IC₅₀ values of approximately 79 μ g/ml and 87 μ g/ml, respectively (Fig. 2B). In contrast, Toki-shigyaku-ka-goshuyu-shokyo-to did not show potent inhibition of U46619- or collagen-induced platelet aggregation (Figs. 2A, B). We also examined the effects of extracts from three Kampo medicines (500 μ g/ml) on thrombin (0.03 U/ml)-induced platelet aggregation, but sufficient inhibitory effects were not observed (data not shown).

Contents of Herbal Components in Each Kampo Medicine Each Kampo medicine is composed of several different herbal medicines (Table 1). We thought that the components contained in both Kami-shoyo-san and Toki-shakuyaku-san, but not in Toki-shigyaku-ka-goshuyu-shokyo-to, were candidates of herbal medicines that sup-

Fig. 1. Effect of Kami-shoyo-san Extract on Collagen- or U46619-Induced Platelet Aggregation

Representative traces of (A) collagen- or (B) U46619-induced platelet aggregation with or without Kami-shoyo-san extract. Kami-shoyo-san extract (500 μ g/ml) or DMSO (final concentration of 1%) was preincubated for 5 min before the addition of U46619 (3 μ M) or collagen (3 μ g/ml) in the presence of 1 mM CaCl₂, as shown with an arrow.

Fig. 2. Concentration-Dependency of Kampo Medicine Extracts in Inhibition of Collagen- or U46619-Induced Platelet Aggregation

Kami-shoyo-san extract (◆; 20–1000 $\mu\text{g/ml}$), Toki-shakuyaku-san extract (■; 50–1000 $\mu\text{g/ml}$), Toki-shigyaku-ka-goshuyu-shokyo-to extract (▲; 100–1000 $\mu\text{g/ml}$) or DMSO (control) was preincubated for 5 min before the addition of (A) 3 $\mu\text{g/ml}$ collagen or (B) 3 μM U46619 in the presence of 1 mM CaCl_2 . The aggregation in the presence of Kampo medicine extracts was expressed as % of that in the absence of the extract. Results are shown as mean $\pm$ S.E.M. of four individual experiments (* $p < 0.05$ compared with control, # $p < 0.01$ compared with control).

Table 1. Contents of Herbal Components in Each Kampo Medicine

	Content (% of total)		
	A	B	C
<i>Angelicae Radix</i>	12.0	12.0	13.0
<i>Paoniae Radix</i>	12.0	24.0	13.0
<i>Atractylodis Lanceae Rhizoma</i>	12.0	18.0	0
<i>Poria</i>	12.0	18.0	0
<i>Bupleuri Radix</i>	16.0	0	0
<i>Moutan Radicis Cortex</i>	12.0	0	0
<i>Gardeniae Fructus</i>	8.0	0	0
<i>Glycyrrhizae Radix</i>	8.0	0	8.7
<i>Zingiberis Rhizoma</i>	4.0	0	4.3
<i>Menthae Herba</i>	4.0	0	0
<i>Alismatis Rhizoma</i>	0	16.0	0
<i>Cnidii Rhizoma</i>	0	12.0	0
<i>Zizyphi Fructus</i>	0	0	21.7
<i>Cinnamomi Cortex</i>	0	0	13.0
<i>Asiasari Radix</i>	0	0	13.0
<i>Evodiae Fructus</i>	0	0	4.3
<i>Akebia Stem</i>	0	0	8.7

A, Kami-shoyo-san; B, Toki-shakuyaku-san; C, Toki-shigyaku-ka-goshuyu-shokyo-to.

pressed collagen- or U46619-induced platelet aggregation. *Atractylodis Lanceae Rhizoma* and *Poria* were selected as the candidates that may inhibit platelet aggregation (Table 1).

Effects of *Atractylodis Lanceae Rhizoma* and *Poria* Extracts on Collagen- or U46619-Induced Platelet Aggregation

Fig. 3. Concentration-Dependency of *Atractylodis Lanceae Rhizoma* or *Poria* Extracts in Inhibition of Collagen- or U46619-Induced Platelet Aggregation

Atractylodis Lanceae Rhizoma extract (◆; 30–1000 $\mu\text{g/ml}$), *Poria* extract (■; 10–1000 $\mu\text{g/ml}$) or DMSO (control) was preincubated for 5 min before the addition of (A) 3 $\mu\text{g/ml}$ collagen or (B) 3 μM U46619 in the presence of 1 mM CaCl_2 . Results are shown as mean $\pm$ S.E.M. of three (*Atractylodis Lanceae Rhizoma* extract) or six (*Poria* extract) individual experiments (* $p < 0.05$ compared with control, # $p < 0.01$ compared with control). The aggregation in the presence of herbal medicine extracts was expressed as % of that in the absence of the extract. Results are mean $\pm$ S.E.M. of four individual experiments (* $p < 0.01$ compared with control).

tion To identify effective herbal medicines, we examined the effects of *Atractylodis Lanceae Rhizoma* or *Poria* extract on collagen- or U46619-induced platelet aggregation (Fig. 3). *Atractylodis Lanceae Rhizoma* inhibited collagen-induced platelet aggregation in a concentration-dependent manner with an IC_{50} value of around 320 $\mu\text{g/ml}$, although no inhibitory effect on U46619-induced platelet aggregation was seen. *Poria* inhibited U46619-induced platelet aggregation in a concentration-dependent manner with an IC_{50} value of approximately 26 $\mu\text{g/ml}$ and showed only a partial effect on collagen-induced platelet aggregation with an IC_{50} value of about 56 $\mu\text{g/ml}$.

Effects of *Atractylodis Lanceae Rhizoma* and *Poria* Extracts on Adrenaline/Adenosine Diphosphate- or Adrenaline/Serotonin-Induced Platelet Aggregation To determine the mechanism of action of *Atractylodis Lanceae Rhizoma* or *Poria*, we examined the effects of these herbal medicines on other agonists for platelet aggregation, such as adrenaline/ADP⁽¹⁸⁾ or adrenaline/5-HT⁽¹⁹⁾ (Fig. 4). As the results, *Atractylodis Lanceae Rhizoma* and *Poria* at a concentration of 300 $\mu\text{g/ml}$ did not inhibit adrenaline (10 μM)/ADP (10 μM)- nor adrenaline (10 μM)/5-HT (10 μM)-induced platelet aggregation.

DISCUSSION

In the present study, we examined the effects of three

Fig. 4. Effects of *Atractylodis Lanceae Rhizoma* or *Poria* Extracts on Adrenaline/ADP- or Adrenaline/5-HT-Induced Platelet Aggregation

Atractylodis Lanceae Rhizoma extract (300 µg/ml), *Poria* extract (300 µg/ml) or DMSO (control) was preincubated for 5 min before the addition of 10 µM/10 µM adrenaline/ADP (Adr-ADP) or 10 µM/10 µM adrenaline/5-HT (Adr-5HT) in the presence of 1 mM CaCl_2 . The aggregation in the presence of herbal medicine extracts was expressed as % of that in the absence of the extract. Results are mean $\pm$ S.E.M. of four individual experiments.

Kampo medicines, Kami-shoyo-san, Toki-shakuyaku-san and Toki-shigyaku-ka-goshuyu-shokyo-to, on collagen- or U46619-induced rabbit platelet aggregation. These three Kampo medicines have been used for the treatment of chilly sensation (*Hiesho*) in Japan. We then found that Kami-shoyo-san and Toki-shakuyaku-san inhibited the platelet aggregation in a concentration-dependent manner, which is the first finding of its kind. This is also the first report that *Atractylodis Lanceae Rhizoma* and *Poria*, the components of Kami-shoyo-san and Toki-shakuyaku-san, inhibit platelet aggregation. Previously, Iwashita *et al.*^{3,4)} also found that ethanol extract of *Piper longum* L. or piperlongumine, one of the components of *Piper longum* L., a plant which has been used for the treatment of chilly sensation, potentially inhibited U46619-induced platelet aggregation. From the reports and present study, it can be thought that platelet aggregation and chilly sensation are tightly associated. In contrast to two Kampo medicines (Kami-shoyo-san and Toki-shakuyaku-san), Toki-shigyaku-ka-goshuyu-shokyo-to, which is also an effective Kampo medicine used for chilly sensation, did not sufficiently inhibit platelet aggregation. Thus Toki-shigyaku-ka-goshuyu-shokyo-to may have other mechanisms, such as dilation of peripheral blood vessels, which help to improve chilly sensation. In fact, *Paeoniae Radix*, one of the components of Toki-shigyaku-ka-goshuyu-shokyo-to, relaxes vascular smooth muscle.²⁰⁾ This herbal medicine is also included in Kami-shoyo-san and Toki-shakuyaku-san. Therefore, to investigate the mechanisms of Kami-shoyo-san and Toki-shakuyaku-san for chilly sensation, we have to consider not only platelet aggregation but also the dilation of peripheral blood vessels in the future.

Kami-shoyo-san and Toki-shakuyaku-san have two herbal medicines, *Atractylodis Lanceae Rhizoma* and *Poria*, in common. They are known to have various effects such as an antianoxic action²¹⁾ and an anti-tumor effect.^{22–24)} Furthermore, they may be candidate components having an inhibitory effect on platelet aggregation, because they are not present in Toki-shigyaku-ka-goshuyu-shokyo-to, which did not show an inhibitory effect on platelet aggregation sufficiently.

Then, we investigated the effects of *Atractylodis Lanceae Rhizoma* and *Poria* on collagen- or U46619-induced platelet aggregation. We found that *Atractylodis Lanceae Rhizoma* inhibited collagen-induced platelet aggregation, but not U46619-induced platelet aggregation. We also found that *Atractylodis Lanceae Rhizoma* did not inhibit adrenaline/ADP- or adrenaline/5-HT-induced platelet aggregation. These findings indicate that *Atractylodis Lanceae Rhizoma* specifically suppresses the collagen-induced signal pathway, which is upstream of the release of TXA_2 from platelets.¹⁴⁾ The signaling pathway that might be the target of *Atractylodis Lanceae Rhizoma* is the activation of PLA_2 and generation of TXA_2 . On the other hand, *Poria* completely inhibited U46619-induced platelet aggregation, but only partially inhibited collagen-induced aggregation. This result suggests that *Poria* primarily inhibits downstream of the activation of TXA_2 receptor, but not the collagen-specific pathway, as stated above. We have previously found that collagen may partly cause platelet activation without the production of TXA_2 , as collagen induced platelet aggregation to a small extent in the presence of SQ29548, an antagonist for the TXA_2 receptor (Iwashita *et al.*, unpublished observation). Therefore, we investigated the effect of *Poria* on platelet aggregation induced by stimulants other than TXA_2 . However, *Poria* did not inhibit either adrenaline/ADP- or adrenaline/5-HT-induced platelet aggregation, suggesting that *Poria* specifically suppressed TXA_2 receptor-mediated platelet aggregation.

Furthermore, *Poria* did not suppress the platelet shape-change when platelets were stimulated with U46619 (Nasu *et al.*, unpublished observation). This suggests that *Poria* suppresses the G_q -mediated signaling pathway which covers the majority of platelet aggregation,²⁵⁾ but does not influence the $\text{G}_{12/13}$ -mediated signaling pathway which mainly mediates the change in platelet shape.²⁶⁾

In conclusion, the present results suggest the possibility that the inhibition of platelet aggregation by two Kampo medicines, Kami-shoyo-san and Toki-shakuyaku-san, is one of the mechanisms involved in the improvement of *Hiesho*. Furthermore, *Atractylodis Lanceae Rhizoma* and *Poria* are the herbal medicines that may possibly be used for the inhibition of platelet aggregation.

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