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-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 weak 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.

Kampo (traditional Chinese) medicines are traditionally used in Eastern medicine. Patients also tend to 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 weak stiffness. Patients with chilly sensation have in many cases been considered to have poor peripheral blood circulation. 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.

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), adrenalin, adenosine diphosphate (ADP), thromboxane A2 (TXA2) and collagen. 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, 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. TXA2 is a metabolite of arachidonic acid with a chemical half-life of about 30s. When TXA2 is released from activated platelets, it binds to TXA2 receptors to cause platelet shape change and aggregation as a positive feedback mediator.

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. 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.
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, Atractylodis 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-9α,11α-epoxymethano-prostaglandin F₂α (U46619) was obtained from Cayman Chemical (Ann Arbor, U.S.A.). Collagen (Collagenreagent Horn) 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 (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×g for 12 min to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 90×g to remove contaminated erythrocytes and leukocytes then centrifuged at 450×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 3×10⁸ 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 (3×10⁸ 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±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 µg/ml) or U46619 (3 µM) caused rabbit platelet aggregation, a preincubation with extract from Kami-shoyo-san, at a concentration of 500 µg/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 µg/ml and 300 µg/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 µg/ml and 87 µg/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 µg/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 Tokishakuyaku-san, but not in Toki-shigyaku-ka-goshuyu-shokyo-to, were candidates of herbal medicines that sup-
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**

To identify effective herbal medicines, we examined the effects of *Atractylodis Lanceae Rhizoma* or *Poria* extracts on collagen- or U46619-induced platelet aggregation (Fig. 3). *Atractylodis Lanceae Rhizoma* inhibited collagen-induced platelet aggregation in a concentration-dependent manner with an IC50 value of around 320 μg/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 IC50 value of approximately 26 μg/ml and showed only a partial effect on collagen-induced platelet aggregation with an IC50 value of about 56 μg/ml.

**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 μg/ml), *Poria* extract (■, 10—1000 μg/ml) or DMSO (control) was preincubated for 5 min before the addition of (A) 3 μg/ml collagen or (B) 3 μM U46619 in the presence of 1 mM CaCl2. Results are shown as mean±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±S.E.M. of four individual experiments (∗p<0.01 compared with control).

**Table 1. Contents of Herbal Components in Each Kampo Medicine**

<table>
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<tr>
<th>Ingredient</th>
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<th>B</th>
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<td><em>Angelicae Radix</em></td>
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<tr>
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<tr>
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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/ADP18) or adrenaline/5-HT19) (Fig. 4). As the results, *Atractylodis Lanceae Rhizoma* and *Poria* at a concentration of 300 μg/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
Kampo medicines, Kami-shoyo-san, Toki-shakuyaku-san and Toki-shigayaku-ka-goshuyu-shokyo-to, inhibit 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 Portia, the components of Kami-shoyo-san and Toki-shakuyaku-san, inhibit platelet aggregation. Previously, Iwashita et al. found that ethanol extract of Piper longum L., a plant which has been used for the treatment of chilly sensation, potently inhibited U46619-induced platelet aggregation. From the reports and present study, it is 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-shigayaku-ka-goshuyu-shokyo-to, which is also an effective Kampo medicine used for chilly sensation, did not sufficiently inhibit platelet aggregation. Thus Toki-shigayaku-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-shigayaku-ka-goshuyu-shokyo-to, relaxes vascular smooth muscle. 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 Portia, in common. They are known to have various effects such as an anti-inflammatory action and an anti-tumor effect. Furthermore, they may be candidate components having an inhibitory effect on platelet aggregation, because they are not present in Toki-shigayaku-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 Portia 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 TXA2 from platelets. The signaling pathway that might be the target of Atractylodis Lanceae Rhizoma is the activation of PLA2, and generation of TXA2. On the other hand, Portia completely inhibited U46619-induced platelet aggregation, but only partially inhibited collagen-induced aggregation. This result suggests that Portia primarily inhibits downstream of the activation of TXA2 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 TXA2, as collagen induced platelet aggregation to a small extent in the presence of SQ29548, an antagonist for the TXA2 receptor (Iwashita et al., unpublished observation). Therefore, we investigated the effect of Portia on platelet aggregation induced by stimulants other than TXA2. However, Portia did not inhibit either adrenaline/ADP- or adrenaline/5-HT-induced platelet aggregation, suggesting that Portia specifically suppressed TXA2 receptor-mediated platelet aggregation.

Furthermore, Portia did not suppress the platelet shape-change when platelets were stimulated with U46619 (Nasu et al., unpublished observation). This suggests that Portia suppresses the G4-mediated signaling pathway which covers the majority of platelet aggregation, but does not influence the G12/13-mediated signaling pathway which mainly mediates the change in platelet shape.

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 Portia are the herbal medicines that may possibly be used for the inhibition of platelet aggregation.

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