 Journal of Traditional Medicines 17, 221－228, 2000

Recovering effect of Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan) on erythrocyte membrane sialidase abnormality in glucocorticoid-induced "oketsu" model mouse

Takayuki NAGAI

Oriental Medicine Research Center of the Kitasato Institute

(*Accepted October 19, 2000.*)

Abstract

When betamethasone sodium phosphate was administered intramuscularly to C3H/HeN mice at 1.6 mg/kg/day for 7 days, thrombin time was reduced and fibrinogen content was increased in the plasma. The sialidase activity against mixed gangliosides and *N*-acetylneuraminic acid (Neu5Ac) content in the erythrocyte membrane were significantly increased by the administration of the glucocorticoid. Oral administration of a Kampo medicine, Keishi-bukuryo-gan (桂枝茯苓丸 ; Gui-Zhi-Fu-Ling-Wan), used clinically for the treatment of "oketsu" (blood stagnant) state, at 2 g/kg/day to the betamethasone-treated mice for 7 days reduced the Neu5Ac content and sialidase activity in the erythrocyte membrane and fibrinogen content in the plasma to the control level. Sealed inside-out vesicle prepared from the erythrocyte of mouse showed potent sialidase activity against mixed gangliosides, but resealed ghost did not show any sialidase activity, indicating that the sialidase activity is oriented mainly in the inside of the mouse erythrocyte membrane. When betamethasone was administered intramuscularly to the mice, the sialidase activity of unsealed white ghost was increased and resealed ghost became to show the potent sialidase activity, but the sialidase activity of inside-out vesicle was decreased. Oral administration of Keishi-bukuryo-gan to the betamethasone-treated mice reduced the increased sialidase activities of unsealed white ghost and resealed ghost, but recovered the enzyme activity of inside-out vesicle. These results suggest that blood stagnation caused by intramuscular administration of betamethasone may somewhat be caused by the alteration of sialylation and asialylation of erythrocyte membrane and that oral administration of Keishi-bukuryo-gan can modulate these effects. These results also suggest that the administration of betamethasone induces the sialidase activity in the outside of erythrocyte membrane but reduces it in the inside, and the administration of Keishi-bukuryo-gan recovers localization of the enzyme activity in erythrocyte membrane of mouse.

Key words blood coagulation, erythrocyte, glucocorticoid, Gui-Zhi-Fu-Ling-Wan, Keishi-bukuryo-gan, oketsu, sialic acid, sialidase [E.C. 3.2.1.18], Yu-Xue.

Abbreviations Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan), 桂枝茯苓丸; Neu5Ac, *N*-acetylneuraminic acid; oketsu (Yu-Xue), 瘀血.

1. Introduction

"Oketsu (瘀血 ; Yu-Xue)", blood stasis or stagnant state, which is one of the pathological concepts in oriental medicine, has been defined to cause by change of blood flow and blood conditions.[1,2] This syndrome is related to several serial diseases, such as SLE, rheumatoid arthritis, cerebrovascular disease and many inflammations. Therefore biochemical elucidation of "oketsu" state expects to elucidate the action of anti-"oketsu" Kampo medicines for the treatment

〒108-8642 東京都港区白金 5-9-1
北里研究所東洋医学総合研究所 永井 隆之
5-9-1, Shirokane, Minato-ku, Tokyo 108-8642, Japan

of these diseases. Up to now, several studies were performed in order to elucidate the mechanism of "oketsu" state[3] and effects of anti- "oketsu" Kampo medicines.[4] Kohta *et al.* suggested that the erythrocyte aggregation increased in "oketsu" patients with multiple lacunar infarction[5] and a kind of anti-"oketsu" Kampo prescriptions, Keishi-bukuryo-gan (桂枝茯苓丸; Gui-Zhi-Fu-Ling-Wan), had a pharmacologic action to reverse the acceleration of erythrocyte aggregation in the "oketsu" patients.[6] Hikiami *et al.* suggested that erythrocyte deformability was related to the "oketsu" state and that the deterioration of erythrocyte deformability in the "oketsu" state was due to the decreased erythrocyte viscoelasticity.[7]

It is known that an "oketsu" like state appears with the administration of glucocorticoid in clinical.[8] Tani *et al.* reported that intramuscular administration of betamethasone induced hyperviscosity of blood and hypercoagulability in rats,[9,10] however, Keishi-bukuryo-gan repaired the enhancement of blood coagulation caused by injection of glucocorticoid to rats.[10] It has been reported that aggregation of erythrocytes was affected by the negative charge of sialic acid residues on the surface membrane,[11,12] and that the negative charge on the erythrocyte surface was increased by the injection of dexamethasone to the rat.[8] The serum sialic acid level is also known to enhance in patients with "oketsu" state.[13] These results indicate that metabolism of sialic acid residues in blood may be altered in "oketsu" state and administration of glucocorticoid. Previously, we found that predominant sialidase activity is present in the erythrocyte membrane of rabbit blood when ganglioside was used as substrate.[14]

Therefore we studied sialidase activity and sialic acid content of erythrocyte membrane in betamethasone-administered mouse as "oketsu" like state model. We also studied the effect of an anti-"oketsu" Kampo prescription, Keishi-bukuryo-gan, on the metabolism of sialic acid residues in erythrocyte membrane of the betamethasone-administered mouse. The present paper also describes the effects of betamethasone and Keishi-bukuryo-gan on orientation of the sialidase activity in mouse erythrocyte membrane in order to elucidate the mechanism of the recovering effect of Keishi-bukuryo-gan on the glucocorticoid-treated mouse.

2. Effects of glucocorticoid on blood coagulation, and sialidase activity and sialic acid content of erythrocyte membrane in mouse

Male C3H/HeN mice (7 weeks old) were injected with betamethasone sodium phosphate solution at 0.8 or 1.6 mg/kg/day intramuscularly for 7 days. After fasting overnight, the blood was taken from the retro-orbital plexus of the mouse, and plasma was prepared. The thrombin time and fibrinogen content of plasma were determined according to the methods described by Tani *et al.*[9,10] As a result, the plasma from betamethasone-administered mice showed shorter thrombin time than that of saline-administered control mice.[15] The plasma from betamethasone-treated mice (1.6 mg/kg/day) showed also higher fibrinogen content than that of saline-administered control mice (Fig. 1).[15] These results indicate that administration of betamethasone to mice enhances the blood coagulation system.

A membrane fraction was prepared from mouse

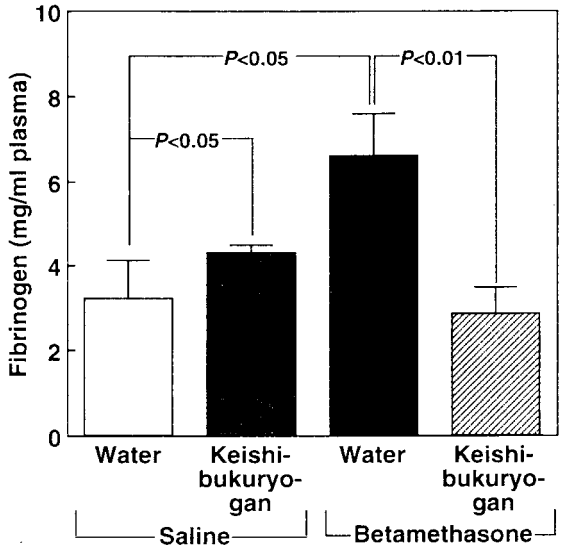

Fig. 1 Effects of betamethasone and Keishi-bukuryo-gan on fibrinogen contents of mouse plasma. C3H/HeN mice were treated *i.m.* with betamethasone (1.6 mg/kg/day) or saline and *p.o.* with Keishi-bukuryo-gan (2 g/kg/day) or water for 7 days. Values are expressed as the mean±S.E. (n=5-8).

erythrocytes and its sialidase activity was measured. After incubation of the erythrocyte membrane with substrate at 37°C for 4 h at pH 4.5, the sialic acid released from substrate was determined by fluorometric HPLC method.[16] When mice were treated with betamethasone (1.6 mg/kg/day) intramuscularly for 7 days, sialidase activity of erythrocyte membrane for bovine brain mixed gangliosides increased significantly in comparison with that of saline-treated control mice (Fig. 2).[15] The sialidase activity against mixed gangliosides was increased from one day after the administration of glucocorticoid.[15] Whereas sialidase activities against fetal bovine serum fetuin and bovine colostrum sialyllactose were also increased significantly at 5 and 7 days after the administration of betamethasone.[15] Sialic acid content of erythrocyte membrane was also determined by fluorometric HPLC method. The *N*-acetylneuraminic acid (Neu5Ac) content of erythrocyte membrane was significantly increased by the administration of betamethasone.[15]

It is known that the administration of glucocorticoid for a long period causes several adverse reactions clinically.[7] Tani *et al.* reported the injection of betamethasone induced "oketsu"-like state such as hyperviscosity of blood, hyperlipemia and hypercoagulability in rats.[9,10] Several studies have reported that negative charge, which may be due to sialic acid residues, on erythrocyte surface increased in the dexamethasone injected rats,[8] and that the sialic acid residues on the human erythrocytes affected the aggregation of the cells.[11,12] These results suggest that blood stagnation caused by glucocorticoid administration may be somewhat caused by the alteration of sialylation and asialylation of erythrocyte membrane.

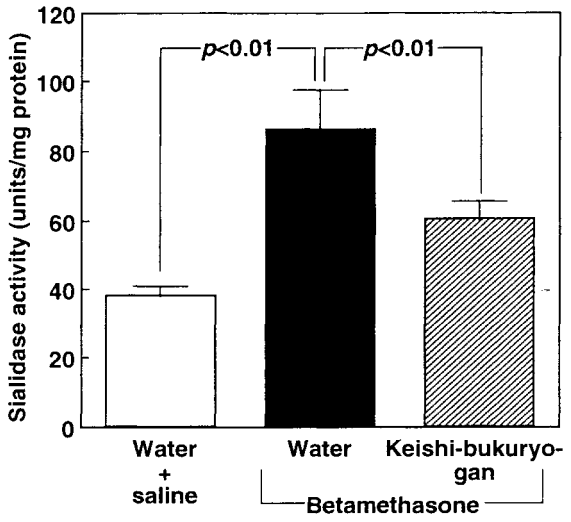

Fig. 2 Effects of betamethasone and Keishi-bukuryo-gan on sialidase activity of mouse erythrocyte membrane. C3H/HeN mice were treated with betamethasone or saline and Keishi-bukuryo-gan or water as described in the legend of Fig. 1. Sialidase activity of erythrocyte membrane against bovine brain mixed gangliosides was measured with fluorometric HPLC method at 7 days after the first administration. One unit of sialidase activity was defined as the amount of enzyme which catalyzed the release of 1 nmol sialic acid/h. Values represent mean± S.E. (n=8).

Fibrinogen is one of the plasma sialoglycoproteins, and acts as a blood coagulation factor. Fibrinogen is hydrolyzed by thrombin to form a fibrin network, and functions of the protein related to this process are markedly decreased by asialylation; the thrombin time is shortened, and tendency for the aggregation of fibrin increased.[17] Fibrin polymerization is disturbed by increased sialylation of the fibrin molecule.[17] These results suggest that the hypercoagulability of plasma such as shortened thrombin time and increased fibrinogen content by betamethasone may be caused by altered sialylation of fibrinogen. Effect of glucocorticoid on the sialylation and asialylation of plasma proteins related to blood coagulation will be investigated in our further study.

The increases in sialidase activity and sialic acid content of erythrocyte membrane in the present study seem in conflict with each other. Sialyltransferase [E. C. 2.4.99.1], which catalyzes the transfer of sialic acid into the asialoglycosidic chains of glycoproteins and glycolipids, exists predominantly as a membrane-bound form within the Golgi apparatus of mammalian cells.[18] But a soluble form of the enzyme also exists and has been detected in the serum.[19,20] Wang *et al.*[21] and Harder *et al.*[22] reported that the sialyltransferase was induced and accelerated the release from the cells by the treatment of dexamethasone in the cultured hepatocytes. These results suggest the possibility that sialyltransferase is released in serum from hepatocytes of mice by the betamethasone treatment. The role of sialyltransferase in the serum is unclear, but the enzyme in the serum may be opposed to the sialidase activity of erythrocyte membrane when the glucocorticoid was administered.

The present paper showed that hypercoagulability of plasma was induced and the sialic acid (Neu5Ac) content of erythrocyte membrane was increased in the betamethasone administered mice, simultaneously. Qin *et al.* reported that the blood sialic acid level increased in "oketsu" patients.[13] These results suggest the possibility that sialic acid content increases in the erythrocyte membrane of "oketsu" patients. The measurement of sialic acid content and sialidase activity in erythrocyte membrane of "oketsu" patients are now in progress.

3. Effects of Keishi-bukuryo-gan on fibrinogen contents of plasma, and sialidase activity and sialic acid content of erythrocyte membrane in glucocorticoid administered mouse

Keishi-bukuryo-gan was prepared according to the prescription book of Oriental Medicine Research Center of the Kitasato Institute as follows : mixture of crude drugs for one day dosage consisting of Cinnamomi Cortex (4.0 g, bark of *Cinnamomum cassia* BLUME), Poria (4.0 g, sclerotium of *Poria cocos* (FR.) WOLF), Moutan Radicis Cortex (4.0 g, root bark of *Paeonia suffruticosa* ANDREWS), Persicae Semen (4.0 g, kernel of *Prunus persica* (L.) BATSCH) and Paeoniae Radix (4.0 g, root of *Paeonia lactiflora* PALLAS) was decocted with 600 ml of water to half volume. The supernatant of the extract was lyophilized and suspended in water. C3H/HeN mice were administered betamethasone at 1.6 mg/kg/day intramuscularly and hot water extract of Keishi-bukuryo-gan (2 g/kg/day) orally for 7 days, and fibrinogen content of the plasma was measured. The increased plasma fibrinogen content by glucocorticoid was reduced to the control level by oral administration of Keishi-bukuryo-gan (Fig. 1).[15] In contrast, when Keishi-bukuryo-gan was administered to control mice, the plasma fibrinogen content was increased slightly (Fig. 1).[15] The increased sialidase activity of erythrocyte membrane by the administration of betamethasone was reduced to control level by oral administration of Keishi-bukuryo-gan (Fig. 2).[15] The increased sialic acid (Neu5Ac) contents of erythrocyte membrane by the administration of betamethasone was also reduced to control level by oral administration of Keishi-bukuryo-gan, significantly.[15]

Keishi-bukuryo-gan is thought to be one of the most important prescriptions for improving the "oketsu" state, and its effects have been studied in "oketsu" patients.[23] The present results show that Keishi-bukuryo-gan suppressed the increases of plasma fibrinogen content and erythrocyte membrane sialidase activity caused by betamethasone in mice. These results suggest the possibility that the anti-"oketsu" effect of Keishi-bukuryo-gan may recover the "oketsu" state partly by the reduction of erythrocyte membrane sialidase activity. But this hypothesis must be estimated by measuring sialidase activity of erythrocyte membrane obtained from "oketsu" state patients before and after administration of Keishi-bukuryo-gan.

4. Effects of glucocorticoid and Keishi-bukuryo-gan on the orientation of sialidase activity in erythrocyte membrane of mouse

The orientation of sialidase activity in erythrocyte membrane of C3H/HeN mouse was determined by measuring the sialidase activities of intact erythrocyte, unsealed white ghost, resealed ghost and sealed inside-out vesicle against mixed gangliosides in the absence of detergent. Resealed ghosts were prepared according to the method of Funder and Wieth.[24] Inside-out vesicles were prepared by the procedure of Steck and Kant.[25] As shown in Fig. 3, inside-out vesicle showed potent sialidase activity and unsealed white ghost showed significant activity, but resealed ghost showed no activity.[26] Intact erythrocyte also had no activity (data not shown). These results indicate that the sialidase activity against mixed gangliosides is present in the inside of mouse erythrocyte membrane.

Previously, we have reported that rabbit erythrocyte unsealed ghost and sealed inside-out vesicle showed sialidase activity against gangliosides, while intact erythrocyte and resealed ghost had no activity, indicating that the sialidase activity for gangliosides is located in the inside of the erythrocyte membrane.[14] These results indicate that the sialidase activity for gangliosides is mainly located in the inside of mouse erythrocyte membrane. Chiarini *et al.* repor-

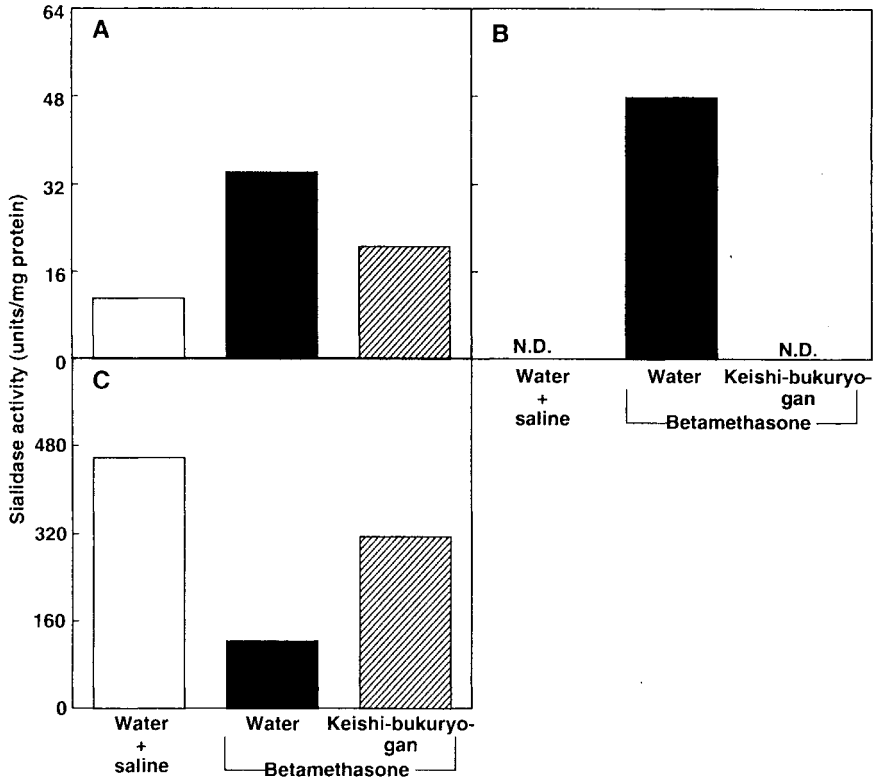

Fig. 3 Effects of betamethasone and Keishi-bukuryo-gan on orientation of sialidase activity in mouse erythrocyte membrane. C3H/HeN mice were treated with betamethasone or saline and Keishi-bukuryo-gan or water as described in the legend of Fig. 1. At the next day of final treatment, sialidase activities of the unsealed white ghost (A), resealed ghosts (B) and sealed inside-out vesicles (C) prepared from pooled erythrocytes ($n=8$) were assayed with bovine brain mixed gangliosides as substrate in the absence of detergents. One unit of sialidase activity was defined as the amount of enzyme which catalyzed the release of 1 nmol sialic acid/h; N.D.=not detectable.

ted that 10-12 % of 4-methylumbelliferyl-Neu5Ac-hydrolyzing sialidase was released from intact human erythrocyte by treatment with phosphatidylinositol-specific phospholipase C from *Bacillus cereus*, indicating that human erythrocyte membrane sialidase was partly located in the outer surface.[27] These observations also suggest the possibility that the remaining human erythrocyte membrane sialidase is present in the inside of the erythrocyte membrane. Orientation of sialidase in cell membrane may be related to the regulation of sialidase activity in erythrocyte cells. If the enzyme activity is present on the external surface of membrane, sialoglycoconjugates in erythrocyte plasma membrane may be hydrolyzed by the sialidase, because sialosugar chains of glycoconjugates are present on the external surface of membrane.

When mice were administered with betamethasone (1.6 mg/kg/day) intramuscularly for 7 days, sialidase activity of resealed ghost became detectable, and unsealed white ghost increased the enzyme activity (Fig. 3A and B).[26] But sialidase activity of sealed inside-out vesicle from betamethasone-administered mice decreased in comparison with that of control mice (Fig. 3C). These results suggest the possibility that the sialidase activity against gangliosides is induced in outside but reduced in inside of mouse erythrocyte membrane when betamethasone was administered. The sialidase activity which appeared in the outside of erythrocyte membrane may modulate the sialoglycoconjugates which is present on the outer surface of the cells. Several studies have reported that the sialic acid residues on the human erythrocyte affect microcirculatory disturbance through the aggregation of the erythrocyte cells.[11,12] These results suggest that blood stagnation caused by glucocorticoid administration may be somewhat

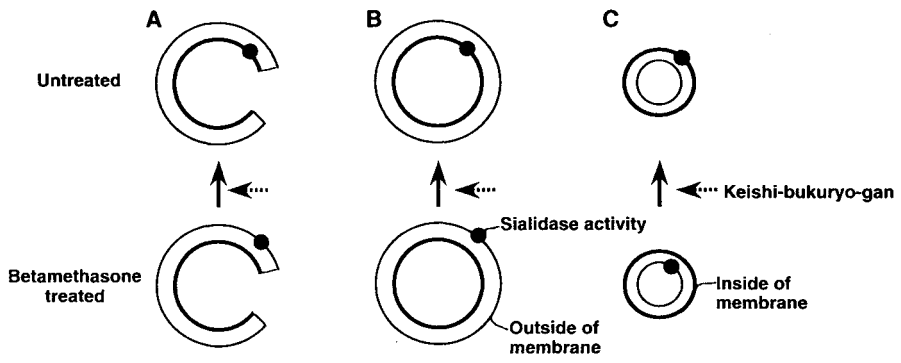

Fig. 4 Hypothetic structure models of erythrocyte membrane preparations, unsealed white ghost (A), resealed ghost (B) and sealed inside-out vesicle (C), and localization of sialidase activity in the membrane with or without betamethasone and Keishi-bukuryo-gan treatments.
●, sialidase activity; ——, inside of erythrocyte membrane; ——, outside of erythrocyte membrane.

caused by the altered localization of sialidase activity in erythrocyte membrane. While the increased sialidase activity of resealed ghost and unsealed white ghost by the administration of betamethasone were reduced to control level by oral administration of Keishi-bukuryo-gan (2 g/kg/day, 7 days), and the decreased enzyme activity of inside-out vesicles by the administration of steroid was also recovered (Fig. 3).[26] These results suggest that oral administration of Keishi-bukuryo-gan repairs abnormal localization of the sialidase activity in erythrocyte membrane of mouse caused by glucocorticoid (Fig. 4).

It is known that "oketsu"-like state appears with the administration of glucocorticoid in clinical[22] and animal experiments with rat.[9,10] Kohta *et al.* reported that erythrocyte aggregability increased significantly in severely affected "oketsu" group of multiple lacunar infarction patients divided according to the diagnostic criteria.[5] They also discussed the possibility of the relationship between a pathophysiological disturbance on the erythrocyte membrane surface, such as reduced negative charge, and erythrocyte aggregability in the severe "oketsu" state. It is known that negative charge on the erythrocyte membrane surface is due to carboxyl groups of sialic acid residue. These results suggest that the sialidase activity in the outside of erythrocyte membrane induced by the betamethasone may be related to the erythrocyte aggregability in "oketsu" state. Present results showed that the oral administration of Keishi-bukuryo-gan recovered the altered localization of the sialidase activity in erythrocyte membrane of mouse caused by intramuscular administration of betamethasone. These results suggest the possibility that anti-"oketsu" effect of Keishi-bukuryo-gan may recover the "oketsu" state partly by normalizing the localization of sialidase activity in erythrocyte membrane. Purification of sialidase from erythrocyte membrane has made it possible to prepare an anti-sialidase antibody. This may be useful to clarify the localization of sialidase in erythrocyte membrane. Localization of sialidase in erythrocyte membrane using anti-sialidase antibody will be investigated in our further study.

5. Conclusion

The present paper showed that hypercoagulability (shortened thrombin time and increased fibrinogen content) of plasma was induced by the intramuscular administration of betamethasone to the mouse, and that oral administration of Keishi-bukuryo-gan suppressed the increase of plasma fibrinogen content (Fig. 5).[15] The present paper also indicated that the sialidase activity of erythrocyte membrane was increased in the betamethasone-administered mice and Keishi-bukuryo-gan suppressed the increase of sialidase activity.[15] These observations suggest that some parts of the sialidase activities against gangliosides, which is present in the inside of mouse

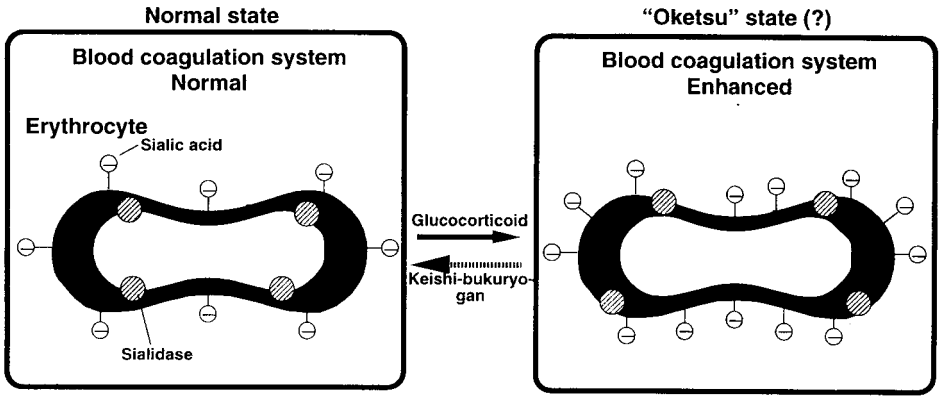

Fig. 5 Hypothetic effects of glucocorticoid and anti-"oketsu" Kampo medicine, Keishi-bukuryo-gan, on the blood.

erythrocyte membrane in normal condition, appear in the outside of the membrane by the administration of betamethasone, and that the administration of Keishi-bukuryo-gan suppresses the glucocorticoid-induced sialidase activity in the outside of membrane by recovering the localization of the enzyme activity in erythrocyte membrane of the mouse (Fig. 5).[26] These results suggest the possibility that the anti-"oketsu" effect of Keishi-bukuryo-gan may recover the "oketsu" state partly by the reduction of erythrocyte membrane sialidase activity.

In a preliminary study in collaboration with Terasawa group in Toyama Medical and Pharmaceutical University, the sialidase activity of erythrocyte membrane showed a tendency to increase in a mildly affected "oketsu" group of patients, based on the evaluation criteria ("oketsu" score),[28] with multiple lacunar infarction who visited their hospital (unpublished data). This increased sialidase activity of erythrocyte membrane was reduced to the level of a non-"oketsu" group by oral administration of Keishi-bukuryo-gan (unpublished data). These observations agreed well with the present study. Further clinical studies are required in order to estimate the relation between the "oketsu" (blood stagnant) state and sialidase in the erythrocyte membrane.

Acknowledgments

The author is grateful to Prof. K. Terasawa and his coworkers, Department of Japanese Oriental (Kampo) Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, for cooperation to provide the erythrocyte membranes of patients with multiple lacunar infarction. The author is deeply grateful to Prof. H. Yamada, Oriental Medicine Research Center, the Kitasato Institute, for his guidance in this study.

和文抄録

糖質コルチコイドを投与して作製した瘀血病態モデルマウスにおける赤血球膜シアル酸代謝，及び桂枝茯苓丸の効果について検討を行なった。

C3H/HeN マウスにベタメタゾンを 1.6 mg/kg/day の用量で 7 日間，筋肉内投与すると，血漿のトロンビン時間の短縮及びフィブリノーゲン量の増加が認められた。また，ベタメタゾンの投与によってガングリオシドを基質としたときの赤血球膜シアリダーゼ活性及び赤血球膜の *N*-アセチルノイラミン酸含量が有意に増加した。これに対し，マウスにベタメタゾン投与と同時に「瘀血」病態の治療に臨床的に用いられている漢方薬である桂枝茯苓丸を 2 g/kg/day の用量で 7 日間，経口投与すると赤血球膜のシアル酸含量，赤血球膜シアリダーゼ活性及び血漿のフィブリノーゲン量はコントロールレベルまで低下した。マウスの赤血球から調製した unsealed ホワイトゴースト及び sealed inside-out vesicle にはガングリオシドを基質としたときのシアリダーゼ活性が認められたのに対し，resealed ゴーストは活性を示さなかった。マウスにベタメタゾンを 1.6 mg/kg/day の用量で 7 日間筋肉内投与すると，ホワイトゴースト及び resealed ゴーストのシアリダーゼ活性は上昇したのに

対し，inside-out vesicleの活性は低下した。ベタメタゾンと同時に桂枝茯苓丸を2g/kg/dayの用量で7日間経口投与すると，ベタメタゾンによって上昇したホワイトゴースト及びresealedゴーストのシアリダーゼ活性は低下し，低下したinside-out vesicleの活性は上昇した。

これらの結果より，糖質コルチコイドを投与することによって生じる血液異常の原因のひとつとして赤血球膜のシアル酸代謝の変化が関与している可能性が考えられた。また，桂枝茯苓丸はベタメタゾンによるこれらの作用を改善する効果を有することが示唆された。さらに，通常では赤血球膜の内側にのみ認められる主要シアリダーゼ活性は，ベタメタゾンを投与することによって外側の活性が上昇し，内側の活性が低下するのに対し，桂枝茯苓丸の投与によってベタメタゾンによるシアリダーゼ活性の赤血球膜における局在性の変化が抑制される可能性が示唆された。

References

1) Terasawa, K. : An exploratory standard and clinical analysis of Kanpo Oketsu. *Biomed. Therapeut.* **10 supple.**, 13-19, 1983.
2) Terasawa, K. : Scientific approach to oketsu (blood stasis) syndrome. *Jap. J. Oriental Medicine* (in Japanese) **48**, 409-436, 1998.
3) Terasawa, K., Toriizuka, K., Tosa, H., Ueno, M., Hayashi, T. and Shimizu, M. : Rheological studies on "oketsu" syndrome I. The blood viscosity and diagnostic criteria. *J. Med. Pharm. Soc. WAKAN-YAKU* **3**, 98-104, 1986.
4) Tosa, H., Toriizuka, K. and Terasawa, K. : The effect of Keishi-bukuryo-gan on blood viscosity, platelet functions and blood coagulation in normal subjects. *J. Med. Pharm. Soc. WAKAN-YAKU* **4**, 172-179, 1987.
5) Kohta, K., Hiyama, Y., Terasawa, K., Hamazaki, T., Itoh, T. and Tosa, H. : Hemorheological studies of "oketsu" syndrome. -Erythrocyte aggregation in "oketsu" syndrome-. *J. Med. Pharm. Soc. WAKAN-YAKU* **9**, 221-228, 1992.
6) Kohta, K., Hikiami, H., Shimada, Y., Matsuda, H., Hamazaki, T. and Terasawa, K. : Effects of Keishi-bukuryo-gan on erythrocyte aggregability in patients with multiple old lacunar infarction. *J. Med. Pharm. Soc. WAKAN-YAKU* **10**, 251-259, 1993.
7) Hikiami, H., Kohta, K., Sekiya, N., Shimada, Y., Itoh, T. and Terasawa, K. : Erythrocyte deformability in "oketsu" syndrome and its relations to erythrocyte viscoelasticity. *J. Trad. Med.* **13**, 156-164, 1996.
8) Abe, H. : The relations between Oketsu and abnormal microcirculation. *Biomed. Therapeut.* **10 supple.**, 26-30, 1983.
9) Tani, T., Iwanaga, M., Ohno, T. and Arichi, S. : Effect of crude drugs and their prescriptions on the blood rheology affected by glucocorticoid treatment (I) : Effect of betamethasone on rat blood rheology. *Med. J. Kinki Univ.* **9**, 229-238, 1984.
10) Tani, T., Iwanaga, M., Ohno, T., Higashino, M., Kubo, M. and Arichi, S. : Effect of crude drugs and their prescriptions on the blood rheology affected by glucocorticoid treatment (II) : Effect of Keishi-bukuryo-gan on adverse reactions of betamethasone treatment. *Shoyakugaku Zasshi* **38**, 166-174, 1984.
11) Shiga, T., Maeda, N. and Kon, K. : Erythrocyte rheology. *Crit. Rev. Oncol. Hematol.* **10**, 9-48, 1990.
12) Maeda, N. : Rheology of blood. *Jap. J. Clin. Pathol.* **S91**, 82-91, 1991.
13) Qin, W.Z., Xiang, X.L., Wu, H.L., Shao, Y.D., Zhou, C.Y., Qu, B.Z. and Dan, Y.J. : *Zhonxiyijiehezhazhi* **5**, 151-154, 1985.
14) Chen, X.-G., Nagai, T. and Yamada, H. : Sialidase in rabbit blood : Characterization of sialidase purified from rabbit erythrocyte membrane. *Eur. J. Biochem.* **221**, 655-664, 1994.
15) Nagai, T., Chen, X.-G. and Yamada, H. : Enhanced sialidase activity and sialic acid content of erythrocyte membrane from betamethasone treated mice and their recovering effect of Keishi-bukuryo-gan. *J. Trad. Med.* **12**, 195-201, 1995.
16) Hara, S. Takemori, Y., Yamaguchi, M., Nakamura, M. and Ohkura, Y. : Fluorometric high-performance liquid chromatography of *N*-acetyl- and *N*-glycolylneuraminic acids and its application to their microdetermination in human and animal sera, glycoproteins, and glycolipids. *Anal. Biochem.* **164**, 138-145, 1987.
17) Reutter, W., Koettgen, E., Bauer, C. and Gerok, W. : Biological significance of sialic acids. *Cell Biol. Monogr.* **10**, 263-305, 1982.
18) Wong, S.H., Low, S.H. and Hong, W. : The 17-residue transmembrane domain of β-galactoside α-2,6-sialyltransferase is sufficient for Golgi retention. *J. Cell Biol.* **117**, 245-258, 1992.
19) Dairaku, K., Miyagi, T., Wakai, A. and Tuiki, S. : Increase in serum sialyltransferase in tumor-bearing rats: The origin and nature of the increased enzyme. *Gann* **74**, 656-662, 1983.
20) Kaplan, H.A., Woloski, B.M.R.N.J., Hellman, M. and Jamieson, J. C. : Studies on the effect of inflammation on rat liver and serum sialyltransferase. *J. Biol. Chem.* **258**, 11505-11509, 1983.
21) Wang, X.C., O'Hanlon, T.P. and Lau, J.T.Y. : Regulation of β-galactoside α2,6-sialyltransferase gene expression by dexamethasone. *J. Biol. Chem.* **264**, 1854-1859, 1989.
22) Harder, G., Jamieson, J.C. and Woloski, B.M.R.N.J. : Stimulation of release of Galβ1-4GlcNAcα2-6 sialyltransferase from the FAZA hepatoma cell line by dexamethasone and phorbol ester. *Int. J. Biochem.* **22**, 11-14, 1990.
23) Itoh, T., Terasawa, K., Kohta, K., Shibahara, N., Tosa, H. and Hiyama, Y. : Effects of Keishi-bukuryo-gan and trapidil on the microcirculation in patients with cerebro-spinal vascular disease. *J. Med. Pharm. Soc. WAKAN-YAKU* **9**, 40-46, 1992.
24) Funder, J. and Wieth, J.O. : Chloride transport in human erythrocytes and ghosts : a quantitative comparison. *J. Physiol. (London)* **262**, 679-698, 1976.
25) Steck, T.L. and Kant, J.A. : Preparation of impermeable ghosts and inside-out vesicle from human erythrocyte membranes. *Methods Enzymol.* **31**, 172-180, 1974.
26) Nagai, T. and Yamada, H. : The normalizing activity of Keishi-bukuryo-gan on betamethasone-induced erythrocyte sialidase abnormality in mice. *J. Trad. Med.* **13**, 229-236, 1996.
27) Chiarini, A., Fiorilli, A., Francesco, L.D., Venerando, B. and Tettamanti, G. : Human erythrocyte sialidase is linked to the plasma membrane by a glycosyl phosphatidylinositol anchor and partly located on the outer surface. *Glycoconjugate J.* **10**, 64-71, 1993.
28) Terasawa, K., Shinoda, H., Imadaya, A., Tosa, H., Bandoh, M. and Satoh, N. : The presentation of diagnostic criteria for "oketsu" syndrome. *J. Jap. Soc. Oriental Medicine* (in Japanese) **34**, 1-17, 1983.