The normalizing activity of Keishi-bukuryo-gan on betamethasone-induced erythrocyte sialidase abnormality in mice

Takayuki NAGAI and Haruki YAMADA*

Oriental Medicine Research Center of the Kitasato Institute

(*Received July 15, 1996. Accepted September 3, 1996.*)

Abstract

Sealed inside-out vesicle prepared from the erythrocyte of C3H/HeN mice showed potent sialidase activity against mixed gangliosides, but resealed ghost did not show any sialidase activity, indicating that the sialidase activity is oriented mainly on the inside of the mouse erythrocyte membrane. When betamethasone sodium phosphate was administered intramuscularly to the mice at 1.6 mg/kg/day for 7 days, the sialidase activity of unsealed white ghost was increased and resealed ghost became to show the potent enzyme activity, but the sialidase activity of inside-out vesicle was decreased. When Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan), used clinically for the treatment of *'Oketsu'* (blood stagnation) state, was administered orally to the betamethasone-treated mice at 2 g/kg/day for 7 days, the increased sialidase activities of unsealed white ghost and resealed ghost were reduced, but the enzyme activity of inside-out vesicle was recovered. These results suggest that *i.m.* administration of betamethasone induces the sialidase activity on the outside of erythrocyte membrane but reduces on the inside, and oral administration of Keishi-bukuryo-gan recover localization of the enzyme activity in erythrocyte membrane of mice.

When betamethasone (1.6 mg/kg) was administered *i.m.* to the mice at a time, sialidase activity in the erythrocyte membrane (white ghost) was significantly increased at 6 h after the injection, but oral administration of Keishi-bukuryo-gan (2 g/kg) decreased the enzyme activity at 18 h. The *N*-glycolylneuraminic acid (Neu5Gc) content in the erythrocyte membrane was decreased from 6 h to 24 h after the injection of betamethasone, but the Neu5Gc content was recovered to normal level by oral administration of Keishi-bukuryo-gan.

Key words erythrocyte, glucocorticoid, Keishi-bukuryo-gan, sialidase [E.C. 3.2.1.18].

Abbreviations DMB, 1,2-diamino-4,5-methylenedioxybenzene ; Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan), 桂枝茯苓丸 ; Neu5Ac, *N*-acetyl-neuraminic acid ; Neu5Gc, *N*-glycolylneuraminic acid ; *Oketsu* (Yu-Xue), 瘀血.

Introduction

It has been reported that removal of sialic acid residues from blood cells and serum glycoproteins play important roles in several biological processes.[1,2] The content of surface-bound sialic acid may depend on the age of the erythrocyte ;[3] removal of sialic acid from erythrocyte[4] and serum glycoproteins[5] leads to a drastic decrease of their half-life in the circulation. There are certain indications that sickle cell anemia,[6] thalassemia[7] and paroxysmal nocturnal hemoglobinuria[8] are related to a reduced metabolism of sialic acid from erythrocytes. The blood sialic acid level is also known to change in *'Oketsu'*, blood stasis or stagnant syndrome,[9] which is one of the pathological concepts in oriental medicine. These observations indicate that membrane-bound sialic acids in eryth-

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

rocyte play an important role in the adhesion, aggregation and clotting of blood.

Glucocorticoid is known to cause several adverse reactions in the blood-vascular system, digestive system, central nervous system and so on when it was administered to patients for a long time. Reduction of these adverse reactions of glucocorticoid is a very important subject in modern drug therapy. Previously, we found that predominant sialidase activity is present in the erythrocyte membrane of the rabbit blood when ganglioside was used as substrate.[10] Recently we also reported that the sialidase activity against gangliosides and *N*-acetylneuraminic acid content in the erythrocyte membrane were significantly increased by the administration of betamethasone, and that oral administration of a kind of anti-*'Oketsu'* Kampo prescriptions, "Keishi-bukuryo-gan (Gui-Zhi-Fu-Ling-Wan)", to the betamethasone treated mice, reduced the sialidase activity to the control level.[11]

The present paper 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 mice as a kind of *'Oketsu'* animal model.

Materials and Methods

Materials : Bovine brain mixed gangliosides (type III) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) as standard were obtained from Nacalai Tesque Inc. (Kyoto, Japan) and Sigma, respectively. 1,2-Diamino-4,5-methylenedioxybenzene (DMB) dihydrochloride was obtained from Dojindo Labs (Kumamoto, Japan). Medicinal plants used for preparation of Keishi-bukuryo-gan was purchased from Uchida Wakan-yaku Co. Ltd. (Tokyo, Japan). 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. After the extract was centrifuged at 7500 rpm for 30 min, the supernatant was lyophilized.

Administrations of betamethasone and Keishi-bukuryo-gan to mice : Male C3H/HeN mice, 7 weeks old (Japan SLC Co., Ltd., Hamamatsu, Japan) were used in all experiments, and were allowed food and water *ad libitum*. Betamethasone sodium phosphate solution (Rinderon® ; Shionogi & Co., Ltd., Osaka, Japan) was injected intramuscularly to the mice at 1.6 mg/kg. Blood was taken from the mouse. Keishi-bukuryo-gan lyophilizate was suspended in water (80 mg/ml), and then the suspension was orally administered to the mice at 2 g/kg using sonde.

Preparation of erythrocyte membrane (white ghost) : Blood was drawn from the retro-orbital plexus of mice, and collected into tubes containing 0.1 vol. of 3.8 % sodium citrate. Erythrocytes were collected by centrifugation at 400×g for 20 min and washed three times with saline. Each time the buffy coat was carefully aspirated from the surface of the pellet. The fresh erythrocyte was hemolyzed by repeated washing with 20 mM Tris-HCl, pH 7.4. The erythrocyte ghost was then pelleted by centrifugation at 20000×g for 40 min.

Preparation of mouse resealed ghost and inside-out vesicle : Resealed ghosts were prepared according to the method of Funder and Wieth.[12] Inside-out vesicles were prepared by the following procedure of Steck and Kant.[13] Resealed and inside-out conditions of these preparations were confirmed by determining activities of acetylcholinesterase (maker of the exterior of erythrocyte) and NADH-cytochrome *c* oxidoreductase (maker of the inner face of the membrane).

Assay of sialic acid by fluorometric HPLC method :[14] DMB (7.0 mM) was dissolved in water containing 18 mM sodium hydrosulfite and 1.0 M β-mercaptoethanol. An aliquot (5 μl) of a sample containing sialic acid was mixed with 0.025 N HCl (200 μl) in a screw-capped 1.5 ml vial. The vial was closed and heated at 80°C for 1 h to hydrolyze the

sample. After cooling, the DMB solution (200 μl) was added, and the mixture was incubated at 60°C for 2.5 h in the dark and then cooled in ice water to stop the reaction. An aliquot (20 μl) of the resulting solution was injected into the HPLC column. HPLC was performed in the isocractic mode on a Tomsorb C18 column (150×4.6 mm *i.d.* ; particle size 5 μm) with methanol-acetonitrile-water (3:1:10, v/v/v) as mobile phase at a flow rate of 1 ml/min and fluorescence was detected at excitation wavelength of 373 nm and an emission wavelength of 448 nm.

Assay of sialidase activity : The standard assay mixture contained 100 nmol substrate as bound sialic acid and the enzyme in 0.2 ml of 0.1 M potassium acetate buffer, pH 4.5. After incubation at 37°C for 4 h, the reaction was stopped by immediate cooling, and the reaction mixture was added with 50 μl of 10 % (w/v) human serum albumin, and then 0.85 ml of water. High molecular weight substances in the reaction mixture were precipitated by addition of 100 μl of 100 % trichloroacetic acid, and then centrifuged at 15000×g for 3 min at 4°C. The sialic acid in the supernatant was determined by fluorometric HPLC method as described above. One unit of sialidase was defined as the amount of enzyme which catalyzed the release of 1 nmol sialic acid/h.

Statistics : Comparisons between experimental groups were evaluated by ANOVA followed Fisher's PLSD procedure. Probability (p) values <0.05 were considered significant.

Results

Orientation of sialidase activity in erythrocyte membrane

The orientation of sialidase activity in erythrocyte membrane of C3H/HeN mice 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. As shown in Fig. 1. inside-out vesicle showed strong sialidase activity and unsealed white ghost showed significant activity, but resealed ghost showed no activity. Intact erythrocyte also had no activity (data not shown). These results indicate that the sialidase activity against mixed gangliosides is present on the inside of mouse erythrocyte membrane.

Effects of betamethasone and Keishi-bukuryo-gan on orientation of sialidase activity in erythrocyte membrane

When the 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. 1A and B). But sialidase activity of sealed inside-out vesicle from betamethasone-administered mice decreased in comparison with that of control mice (Fig. 1C). These results suggest the possibility that the sialidase activity against gangliosides is induced outside but reduced inside of mouse erythrocyte membrane by the administration of betamethasone. 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. 1). These results suggest that oral administration of Keishi-bukuryo-gan repaired localization of the sialidase activity on erythrocyte membrane of mice.

Time dependent effects of betamethasone and Keishi-bukuryo-gan on sialidase activity of erythrocyte membrane

The mice were injected with betamethasone (1.6 mg/kg) intramuscularly, and the blood was taken from the retro-orbital plexus of the mice from 0 h to 24 h after the administration of glucocorticoid. Then, the sialidase activity of erythrocyte membrane fraction was measured. At 6 h after the administration of betamethasone to the mice, the sialidase activity in erythrocyte membrane for ganglioside increased significantly in comparison with before administration (0 h) of the glucocorticoid (Fig. 2). After 6 h, the sialidase activity was decreased gradually but not until control level. Daily variation of sialidase activity in erythrocyte membrane was not observed in saline-treated control mice (data not shown). When Keishi-bukuryo-gan (2 g/kg) was administered orally to betamethasone-treated mice at the same time, sialidase activity in erythrocyte membrane increased

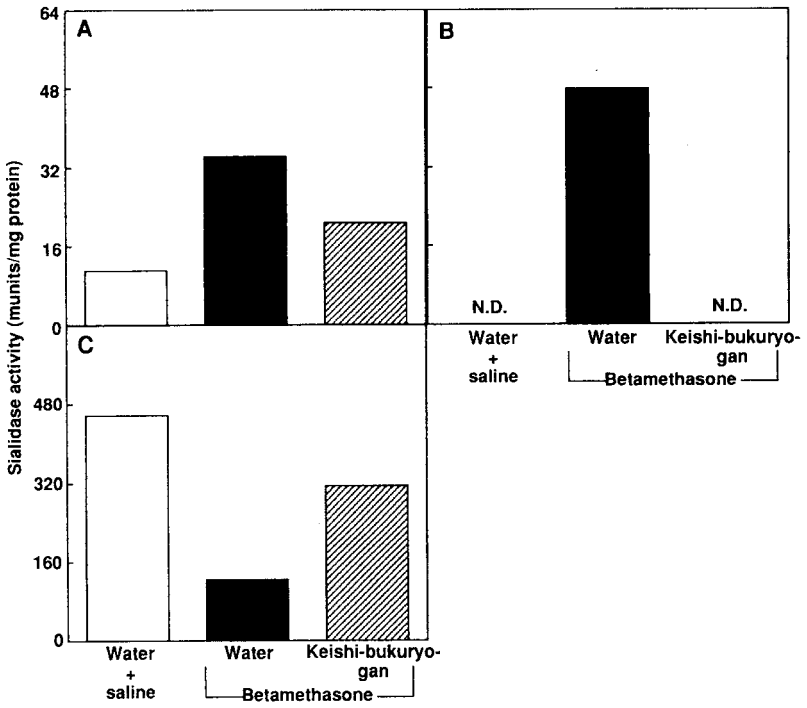

Fig. 1 Effects of betamethasone and Keishi-bukuryo-gan on orientation of sialidase activity in mouse erythrocyte membrane. C3H/HeN mice were treated *i.m.* with betamethasone (1.6 mg/kg/day) or saline for 7 days. Mice were also treated *p.o.* with Keishi-bukuryo-gan (2 g/kg/day) or water at the same time of betamethasone administration. At the next day of final treatment, sialidase activities of the unsealed white ghosts (A), resealed ghosts (B) and sealed inside-out vesicles (C) prepared from pooled erythrocytes (n=8) as described in Materials and Methods 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.

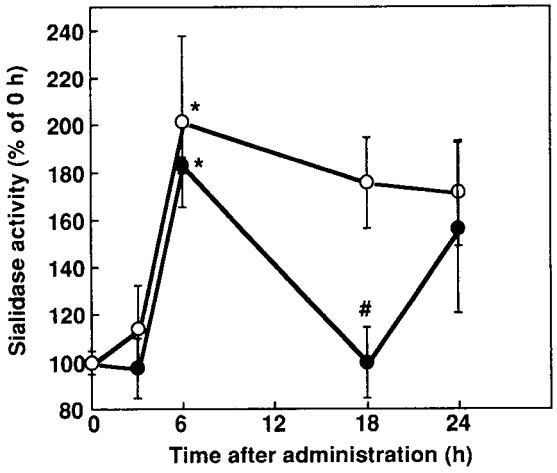

Fig. 2 Time-dependent effects of betamethasone and Keishi-bukuryo-gan on sialidase activity of mouse erythrocyte membrane. C3H / HeN mice were treated with betamethasone (1.6 mg/kg, *i.m.*) (○), or betamethasone and Keishi-bukuryo-gan (2 g/kg, *p.o.*) (●). Sialidase activity of erythrocyte membrane against ganglioside was determined at 0,3,6,18 and 24 h after the administrations. Values represent mean±S.E. (n=3). *, # ; Significant differences at $p<0.05$ from 0 h and betamethasone group, respectively.

significantly like as betamethasone-treated mice at 6 h after administration. Whereas, sialidase activity of erythrocyte membrane from betamethasone and Keishi-bukuryo-gan treated mice decreased to control level at 18 h after administration (Fig. 2). However, the sialidase activity was increased at 24 h after administration of Keishi-bukuryo-gan. It may be caused by excretion and degradation of the active ingredients in Keishi-bukuryo-gan at 24 h.

Effects of betamethasone and Keishi-bukuryo-gan on sialic acid content of erythrocyte membrane

Sialic acid content of erythrocyte membrane was determined by fluorometric HPLC method. When the mice were injected with betamethasone (1.6 mg/kg) intramuscularly, Neu5Gc content in erythrocyte membrane was decreased from 6 h to 24 h after administration of glucocorticoid (Fig. 3), but Neu5Ac content was not changed until 24 h (data not shown). However, when Keishi-bukuryo-gan (2 g/kg) was ad-

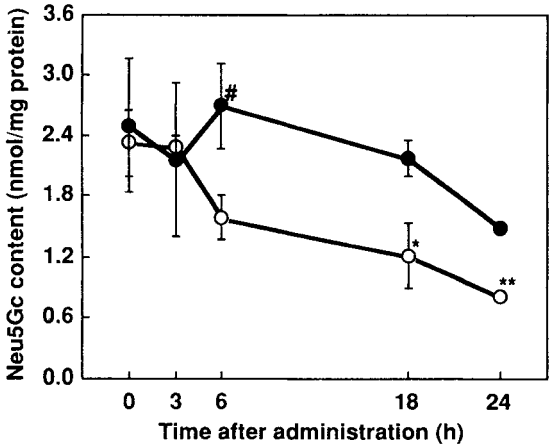

Fig. 3 Time-dependent effects of betamethasone and Keishi-bukuryo-gan on sialic acid content of mouse erythrocyte membrane. C3H / HeN mice were treated with betamethasone (○), or betamethasone and Keishi-bukuryo-gan (●) as described in legend of Fig. 2. *N*-glycolylneuraminic acid (Neu5Gc) content of erythrocyte membrane was measured with fluorometric HPLC method at 0,3,6,18 and 24 h after the administrations. Values represent mean±S.E. (n=3).
*,** ; Significant differences at $p<0.05$ and $p<0.01$ from 0 h, respectively.
; Significant difference at $p<0.05$ from betamethasone group.

ministered orally to the betamethasone treated mice at the same time, Neu5Gc content in erythrocyte membrane did not decrease significantly until 24 h after the administration.

Discussion

It is known that the administration of glucocorticoid for a long period causes several adverse reactions clinically. Tani *et al.* reported that the injection of betamethasone induced hyperviscosity of blood, hyperlipemia and hypercoagulability in rats.[15,16] Previously, we reported hypercoagulability of plasma (shortened thrombin time and increased fibrinogen content) was induced in C3H/HeN mice by the administration of betamethasone.[11] Significant increases of sialidase activity and sialic acid (Neu5Ac) content on erythrocyte membrane were also observed in the betamethasone administered mice.

Previously, we reported that rabbit erythrocyte unsealed ghost and sealed inside-out vesicle showed sialidase activity against mixed gangliosides, while intact erythrocyte and resealed ghost did not, indicating that the sialidase activity for mixed gangliosides is located on the inside of the erythrocyte membrane.[10] In the present study, unsealed white ghost and sealed inside-out vesicle from C3H/HeN mouse erythrocyte showed sialidase activity against mixed gangliosides, while the resealed ghost did not show any sialidase activity. These results indicate that the sialidase activity for gangliosides is mainly located in the inside of mouse erythrocyte membrane. Chiarini *et al.* reported 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 on the outer surface.[17] These observations also suggest the possibility that the remaining human erythrocyte membrane sialidase is present on the inside of the erythrocyte membrane. Orientation of membrane 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. Consequently, deformation, hemolysis and clearance from the circulatory system of erythrocytes will occur, and *'Oketsu'*- like state also may be induced.

In the present study, unsealed white ghost and resealed ghost from the erythrocyte of betamethasone administered mice for 7 days showed higher sialidase activities against mixed gangliosides than saline injected mice, while the sialidase activity of sealed inside-out vesicle prepared from betamethasone-administered mice was decreased in comparison with control group. These results suggest that betamethasone enhances sialidase activity on the outside of the mouse erythrocyte membrane. The sialidase activity appeared on the outside of erythrocyte 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.[18,19] These results

suggest that blood stagnation caused by glucocorticoid administration may be somewhat caused by the altered localization of sialidase activity in erythrocyte membrane.

Previously, we reported that Keishi-bukuryo-gan suppressed the enhancement of sialidase activity in erythrocyte membrane caused by the betamethasone administration to mice.[11] Present results seemed that oral administration of Keishi-bukuryo-gan recovered localization of the sialidase activity on erythrocyte membrane of mice. These results suggest that Keishi-bukuryo-gan, which is often used clinically in combined therapy with glucocorticoid, partly suppresses glucocorticoid induced sialidase activity in outside of erythrocyte by recovering the localization of the enzyme activity on erythrocyte membrane (Fig. 4).

It is known that *'Oketsu'*-like state appears with the administration of glucocorticoid in clinically[20] and animal experiment with rat.[15, 16] 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.[21] They also discussed the possibility of 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 caused by sialic acid residue. These results suggest that the sialidase activity on the outside of erythrocyte membrane induced by the betamethasone may be related to the erythrocyte aggregability in *"Oketsu"* state. 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.[22] Present results showed that the oral administration of Keishi-bukuryo-gan recovered the altered localization of the sialidase activity on erythrocyte membrane of mice caused by *i.m.* administration of betamethasone. These results suggest the possibility that anti-*'Oketsu'* effect of Keishi-bukuryo-gan may recover *'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.

In the present study, sialidase activity of erythrocyte membrane appeared to increase at 6 h after *i.m.* administration of betamethasone to mice. When betamethasone was administered for 7 days to mice, the sialic acid (Neu5Ac) content of erythrocyte membrane was increased significantly.[11] In the present study, Neu5Gc content of erythrocyte membrane was decreased at 6 h after the administration of betamethasone. When the glucocorticoid was administered for 7 days, Neu5Ac content of erythrocyte

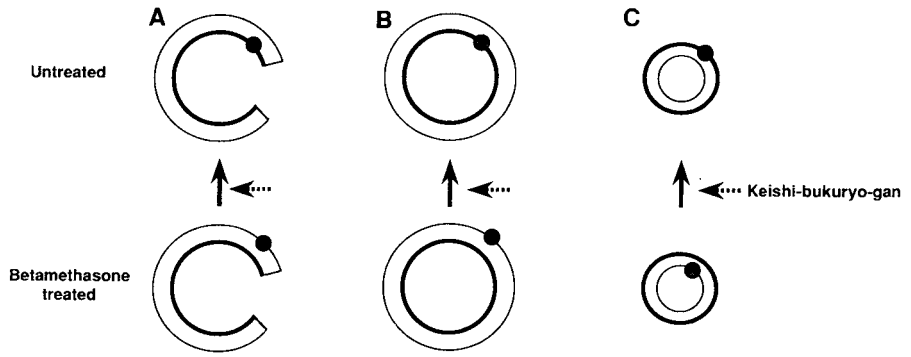

Fig. 4 Hypothetic structure models of erythrocyte membrane preparations, unsealed white ghost (A), resealed ghost (B) and sealed inside-out vesicle (C), and localization of sialidase activity in the membrane with or without betamethasone and Keishi-bukuryo-gan treatments.
●, sialidase activity ; ——,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

membrane was increased from 5 days after first administration (data not shown). These results suggest that the sialic acid content on erythrocyte membrane changes depending on the administration periods of betamethasone. On short administration, increased sialidase activity on erythrocyte surface may hydrolyze endogenous substrate on erythrocyte membrane. The result on long administration also gives the possibility that sialylation of glycoconjugates on erythrocyte membrane may be stimulated by betamethasone through unknown mechanism.

Acknowledgement

We are grateful to Mr. H. Tatai for his technical assistance.

和文抄録

マウスを用いた *in vivo* の実験系で糖質コルチコイドの赤血球膜シアリダーゼ活性上昇作用，並びにこれに対する桂枝茯苓丸の抑制効果の作用機序について検討を行なった。C3H/HeN 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，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に活性が上昇し，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。

マウスにベタメタゾンを 1.6 mg/kg の用量で1回筋肉内投与すると，6時間後に赤血球膜（ホワイトゴースト）のシアリダーゼ活性の上昇が認められた。これに対し，ベタメタゾンと同時に桂枝茯苓丸を 2 g/kg の用量で1回経口投与すると，6時間後に赤血球膜のシアリダーゼ活性の上昇が認められたが，18時間後にはベタメタゾン投与群と比べ，シアリダーゼ活性の有意な低下が認められた。また，ベタメタゾン投与群では投与6時間後から赤血球膜 *N*-グリコリルノイラミン酸含量の低下が認められたが，桂枝茯苓丸投与群ではこの低下は認められなかった。これらの結果より，糖質コルチコイドの赤血球膜シアリダーゼ活性上昇作用は筋肉内投与6時間後から現われ，桂枝茯苓丸の糖質コルチコイドによる赤血球膜シアリダーゼ活性上昇抑制効果は経口投与18時間後から発現することが明らかとなった。

References

1) Reutter, W., Koettgen, E., Bauer, C. and Gerok, W. : Biological significance of sialic acids. *Cell Biol. Monogr.* **10**, 263-305, 1982.
2) Rosenberg, A. and Schengrund, C.-L. : Circulating sialyl compounds. In "Biological roles of sialic acid" (Eds. by Rosenberg, A. and Schengrund, C.-L.), Plenum Press, New York, pp. 275-294, 1976.
3) Greenwalt, T.J. and Steane, E.A. : Quantitative hemagglutination : V. Influence of *in vivo* aging and neuraminidase treatment on the M and N antigens of human red cells. *Br. J. Haematol.* **25**, 217-226, 1973.
4) Durocher, J.R., Payne, R.C. and Conrad, M.E. : Roles of sialic acid in erythrocyte survival. *Blood* **53**, 923-934, 1975.
5) Neufeld, E.F. and Ashwell, G. : Carbohydrate recognition systems for receptor-mediated pinocytosis. In "The biochemistry of glycoproteins and proteoglycans" (Ed. by Lennarz, W.J.), Plenum Press, New York, pp. 241-266, 1980.
6) Riggs, M.G. and Ingram, V.M. : Differences in erythrocyte membrane proteins and glycoproteins in sickle cell disease. *Biochem. Biophys. Res. Commun.* **74**, 191-198, 1977.
7) Kahane, I., Polliack, A., Rachmilewitz, E.A., Bayer, E.A. and Skutelsky, E. : Distribution of sialic acids on the red blood cell membrane in β-thalassaemia. *Nature* **271**, 674-675, 1978.
8) Balduini, C.L., Ricevuti, G., Sosso, M.C., Ascari, E., Brovelli, A. and Balduini, C. : *In vivo* behavior of neuraminidase-treated rabbit erythrocytes and reticulocytes. *Acta Haematol.* **57**, 178-187, 1977.
9) 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.
10) 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.
11) 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.
12) Funder, J. and Wieth, J.O. : Chloride transport in human erythrocytes and ghosts : a quantitative comparison. *J. Physiol. (London)* **262**, 679-698, 1976.
13) 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.
14) Hara, S., Takemori, Y., Yamaguchi, M., Nakamura, M. and Ohkuma, 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.

15) Tani, T., Iwanaga, M., Ohno, T. and Arichi, S. : Effect of crude drugs and their prescriptions on the blood rheology affected by glucocorticoid treatment (1) : Effect of betamethasone on rat blood rheology. *Med. J. Kinki Univ.* **9**, 229-238, 1984.

16) 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.

17) 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.

18) Shiga, T., Maeda, N. and Kon, K. : Erythrocyte rheology. *Crit. Rev. Oncol. Hematol.* **10**, 9-48, 1990.

19) Maeda, N. : Rheology of blood. *Jap. J. Clin. Pathol.* **891**, 82-91, 1991.

20) Abe, H. : The relations between Oketsu and abnormal microcirculations. *Biomed. Therapeut.* **10** supple., 26-30, 1983.

21) 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.

22) 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.