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LETTER TO THE EDITOR

Higher recovery of factor VIII (FVIII) with intermediate FVIII/von Willebrand factor concentrate than with recombinant FVIII in a haemophilia A patient with an inhibitor

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The first line of therapy for acute bleeding in patients with low-responding factor VIII (FVIII) inhibitors is FVIII concentrates (1). Most inhibitors, recognizing the FVIII light chain, inhibit von Willebrand factor (VWF) and phospholipid binding to FVIII (2,3), and appears to be less active *in vitro* against plasma-derived FVIII concentrates containing VWF (pdFVIII/VWF) than VWF-free FVIII concentrates (4–7). These findings suggest that pdFVIII/VWF might be therapeutically more effective than recombinant FVIII (rFVIII) in patients with FVIII inhibitors. However, no clinical studies supporting this concept have been reported. In the present study, we have compared the recovery of FVIII activity (FVIII:C) after treatment with pdFVIII/VWF and rFVIII for massive intramuscular bleeding that occurred during regular infusion of FVIII for immune tolerance induction (ITI) therapy in a young male haemophilia A patient with an inhibitor.

Immune tolerance induction therapy was commenced in our patient at the age of 9 years (18 October 1999) with the administration of 100 U kg^{-1} of rFVIII (Recombinate^{TM}; Baxter Healthcare Corp., Westlake Village, CA, USA) daily for 3 weeks, followed by infusions three to four times a week. Inhibitor levels fluctuated for 3 years after ITI therapy were initiated (maximum inhibitor level, 152.0 BU mL^{-1}) and regular infusions of FVIII were continued. The number of bleeding episodes appeared to decline, and since January 2003, the inhibitor level has been kept constant within a low range from 0.9 to 2.1 BU mL^{-1}. He was admitted into our hospital with severe pain in his right buttock and walking difficulties on 6 January 2004. He had suffered from painful swelling in his right buttock 2 weeks before admission without improvement in spite of daily infusions of FVIII (100 U kg^{-1}). A subcutaneous haematoma (7 × 8 cm) was evident on the right buttock, with heat sensation, and impaired flexion and extension of the right hip joint. Computer tomography scanning demonstrated a massive intramuscular haematoma, measuring 10 cm in diameter, in the right gluteus maximus and gluteus medius muscles. On admission, 12 h after infusion of 4000 U (87 IU kg^{-1}) of rFVIII, the FVIII inhibitor titre was 1.7 BU mL^{-1}. As the bleeding manifestations had not responded to the infusion of rFVIII, 4000 U of activated prothrombin complex concentrate (Feiba Immuno^{TM}; Baxter Healthcare Corp.) were administered. Nevertheless, the swelling and pain in the right buttock increased. Subsequently, replacement therapy with the same dose of rFVIII (4000 IU) was administered and continued (Fig. 1). Clinical symptoms gradually improved and the patient was discharged on 30 January. Regular prophylaxis in this patient is now maintained using FVIII/VWF concentrate.

The inhibitor titre in this patient remained constant, within the range of 1.5–2.0 BU mL^{-1}, throughout the present series of investigations. Therefore, it was possible to compare the recovery

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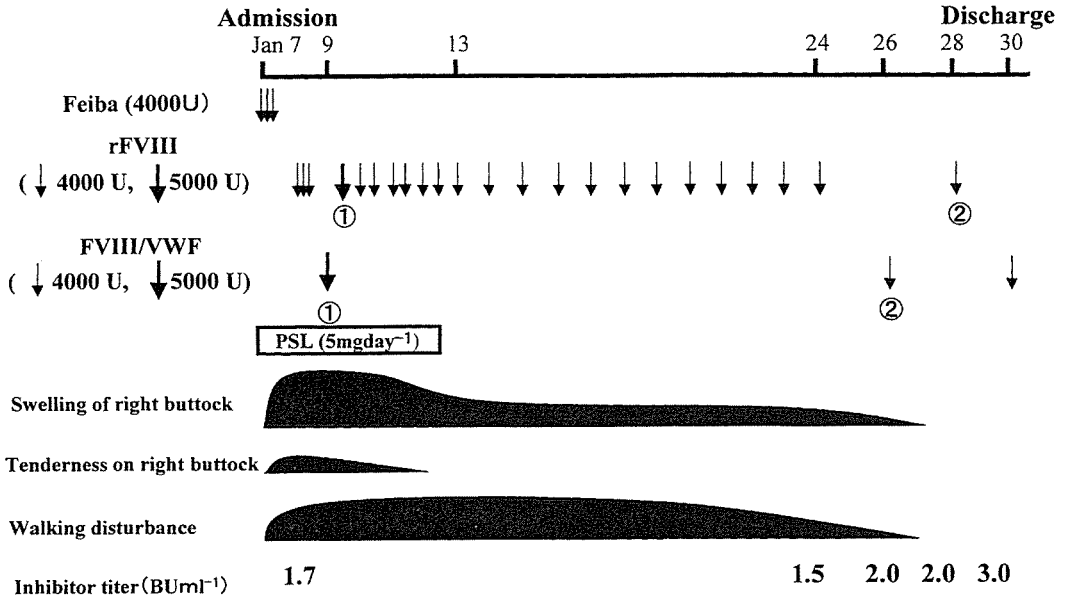

Fig. 1. Clinical course after admission and haemostatic treatment including comparative recovery tests. No. 1, the first recovery test; No. 2, the second recovery test.

of rFVIII with that of FVIII/VWF under similar conditions. For this purpose, FVIII:C recovery was assessed twice with each product during the course of replacement therapy for the intramuscular haematoma. The first comparison was performed on 9 and 11 January when the inhibitor titre was 1.7 BU mL^{-1}. The second comparison was performed on 26 and 28 January when the inhibitor titre was 2.0 BU mL^{-1}. In the first instance, 5000 U (109 IU kg^{-1}) of FVIII/VWF (Confact F^{TM}; The Chemo-Sero-Therapeutic Research Institute, Kaketsuken, Kumamoto, Japan) were compared with an identical dose of rFVIII (Recombinate^{TM}; Baxter Healthcare Corp.). On the second occasion, 4000 U (87 IU kg^{-1}) of the same products were administered. FVIII:C was measured before infusion, and at 30 min, 1 and 2 h after infusion. Levels of FVIII:C were much higher following treatment with FVIII/VWF than with rFVIII, and higher levels were maintained for at least 2 h after infusion (Fig. 2 and 3). In the second recovery test, when the inhibitor titre was slightly higher (2.0 BU mL^{-1}), initial differences were more pronounced following infusion of FVIII/VWF, although the survival time of circulating FVIII:C appeared to be diminished (Fig. 3).

These *in vivo* findings were in keeping with the suggestion that the presence of VWF or the complex formation between FVIII and VWF minimized the neutralizing activity of the inhibitor antibody. To further confirm this *in vitro*, residual FVIII:C was measured after incubation of the inhibitor with different doses of rFVIII in the absence or presence

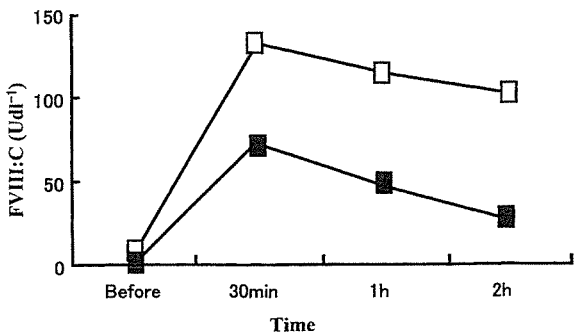

Fig. 2. The recovery of factor VIII (FVIII):C in the first test. □-□, FVIII concentrates containing von Willebrand factor complex concentrates; ■-■, recombinant FVIII concentrates.

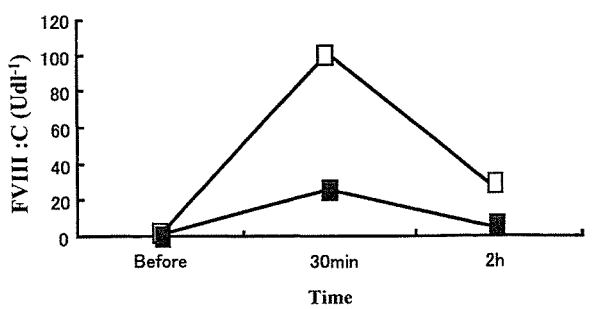

Fig. 3. The recovery of factor VIII (FVIII):C in the second test. □-□, FVIII concentrates containing von Willebrand factor complex concentrates; ■-■, recombinant FVIII concentrates.

of purified VWF. The residual FVIII:C was much higher in the presence of VWF at each dose of rFVIII (Fig. 4).

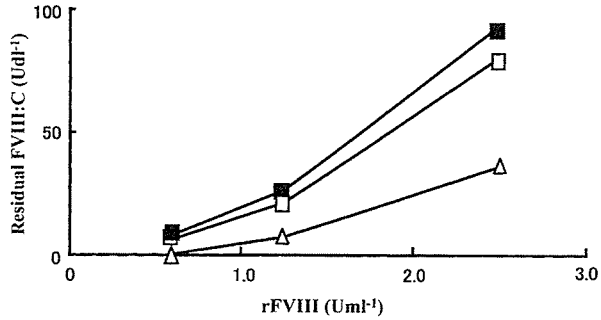

Fig. 4. The inhibitory effect of von Willebrand factor (VWF) on the anti-factor VIII:C activity of the patient's alloantibody. △-△, buffer control; □-□, in the presence of VWF at a final concentration of 5 mg mL^{-1}; ■-■, in the presence of VWF at a final concentration of 10 mg mL^{-1}.

From these data, we predicted that VWF and/or the complex formation between VWF and FVIII blocked antibody binding to FVIII. To confirm this, we developed an enzyme-linked immunosorbent assay to assess the competitive inhibitory effect of fluid phase FVIII/VWF and rFVIII on the binding of inhibitor antibody (IgG_4) to immobilized rFVIII. The addition of rFVIII in the fluid phase competitively inhibited the IgG_4 antibody binding to the immobilized rFVIII in a dose-dependent manner. Similarly, FVIII/VWF also inhibited antibody binding in a dose-dependent manner, but the effect was less marked than that of rFVIII alone (Fig. 5). We further examined if the inhibitor antibody blocked FVIII binding to VWF using the BIAcore system (Biacore AB, Uppsala, Sweden). Recombinant FVIII bound to VWF immobilized on a BIAcore CM5 tip in a dose-dependent manner. The Kd was 0.26 nM (Fig. 6a). Preincubation of 4 nM rFVIII with antibody IgG for 1 h, inhibited binding to VWF in a dose-dependent

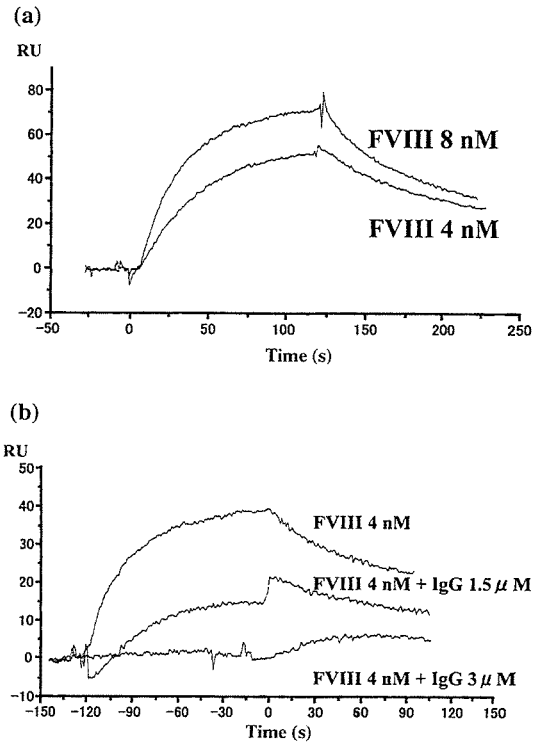

Fig. 6. The effect of the inhibitor IgG on the binding of factor VIII (FVIII) to von Willebrand factor (VWF). (a) Interaction of FVIII with VWF immobilized to BIAcore CM5 tip on surface plasmon resonance (SPR) assay. (b) The effect of the inhibitor IgG on the binding of FVIII to VWF.

manner. At a final concentration of 3 μM antibody IgG, no significant binding was observed (Fig. 6b).

The inhibitor in our present case was a typical anti-FVIII alloantibody. The major IgG subclass was IgG_4, and immunoblotting identified binding to the 80-kDa light chain of purified FVIII and the 72-kDa light chain fragment of thrombin-treated FVIII

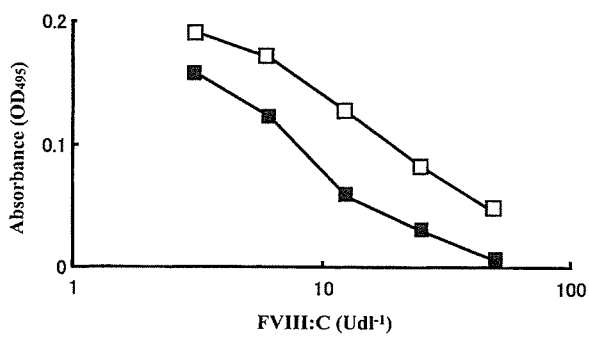

Fig. 5. The blocking effect of factor VIII concentrates containing von Willebrand factor (FVIII/VWF) or recombinant FVIII (rFVIII) on binding of the anti-FVIII IgG_4 antibody to immobilized rFVIII. □-□, FVIII/VWF complex concentrates; ■-■, rFVIII concentrates.

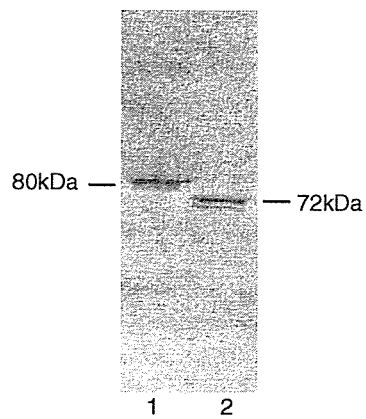

Fig. 7. Immunoblotting analysis of the inhibitor alloantibody. Lane 1, factor VIII (FVIII); lane 2, thrombin-treated FVIII.

(Fig. 7). The VWF-binding site has been found within the FVIII light chain, and most inhibitors recognizing the light chain have been shown to inhibit FVIII binding to VWF (3,8). Several studies *in vitro* have demonstrated that the reactivity of these light-chain inhibitors is weaker against FVIII/VWF than free FVIII (4–7), and Kallas and Talpsep (7) emphasized that clinical studies were warranted to determine whether haemophilia A patients, with relatively high amounts of FVIII light-chain antibodies in their plasma, might benefit from infusions of FVIII concentrates containing VWF.

We have confirmed the *in vitro* findings and shown for the first time *in vivo* that FVIII/VWF concentrates might be superior to rFVIII for the haemostatic management of patients with the low-responding inhibitor antibodies. As these studies, our patient has received regular prophylactic treatment with intermediate FVIII/VWF concentrate. He has had no bleeding episodes other than minor subcutaneous bleeds for more than 1 year and his inhibitor titre has decreased to <1.0 BU mL^{-1}. Although FVIII/VWF concentrates are generally favoured for VWD, the present findings suggest that this type of therapeutic product should also be considered for both acute haemostatic and prophylactic treatment in the haemophilia A patients with inhibitor.

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ORIGINAL ARTICLE

Naked plasmid DNA transfer to the porcine liver using rapid injection with large volume

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The naked plasmid DNA transfer method of rapid injection with large volume has been useful for gene therapy in experimental study. However, only small animals like rodents have usually been reported on. In this study, the authors attempted to transfect naked plasmid DNA to the porcine liver by modified hydrodynamic method. We decided to transfer plasmid DNA to a part of the liver using the angiocatheter to reduce the liver damage. To discern the condition of injection, naked plasmid DNA-encoding green fluorescent protein (GFP) was transferred for use as a marker gene. The GFP gene expression was markedly observed in gene-transferred pig livers. In large animals, not only the naked gene quantity, the solution volume containing the plasmid DNA and the injection speed, but also the additional treatments of the portal vein and the hepatic artery preparation were crucial. We found that the following injection condition were needed: plasmid DNA, 3 mg; the solution volume, 150 ml and the injection speed, 5 ml/s. The portal vein and the hepatic artery were clamped during gene delivery and the blood flow of the portal vein was flushed out using normal saline. Cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4-Ig) gene was used to test for secretory protein. CTLA4-Ig gene was injected with a large volume of solution via the hepatic vein to the left outer lobe of the liver selectively. CTLA4-Ig was detected in the pig blood at a maximum serum level of 161.7 ng/ml 1 day after gene transfer, and the CTLA4-Ig was detected for several weeks. Our new technique of inserting a catheter into only a selected portion of the liver reduced liver toxicity and increased gene transfer efficiency. This is the first report of successful gene transfer, using a hydrodynamic method, to the segmental liver in pigs, and achieved more than enough secretory protein for the clinically therapeutic level in pigs.
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Keywords: *gene transfer; plasmid DNA; hydrodynamic; porcine; liver; CTLA4-Ig*

Introduction

Non-viral gene transfer is a useful technique to reduce the side effects of virus vector.[1] The hydrodynamic method has shown that plasmid DNA can be injected rapidly into blood vessels with a large volume of solution, with high-level gene expression to the liver[2–4] and skeletal muscle[5–7] reported. The hydrodynamic method is an easy and useful technique for gene experiments. Also, the mechanism of hydrodynamic gene transfer was elucidated as the membrane defect or the hydropore induced by hydropressure.[8,9] However, the hydrodynamic method has a major side effect of heart failure because of the large volume of solution. Therefore, many studies have examined rodents, but only a few have been carried out on animals larger than rodents, such as rabbits.[10] One report described dogs,[11] but it concentrated mainly on rodent data and dogs were not discussed in detail. There has been no effective report discussing animals larger than rabbits, so the hydrodynamic method has been considered only as an experimental method. Recently, in an electronic journal, Herreo *et al.*[12] reported hydrodynamic gene transfer to the pig whole liver. This study was very interesting as it was the first report of hydrodynamic gene transfer to a large animal like a pig, but the efficiency of the gene transfer was three orders lower than their study in mice. Therefore, the problem of effective gene transfer to pigs using hydrodynamic gene delivery still remained.

In previous studies, we reported that we used a catheter to reduce the volume of solution in the rat hydrodynamic procedure; we transferred cytotoxic T-lymphocyte antigen 4-immunoglobulin (*CTLA4-Ig*) gene to the rat liver and showed the transplantation data;[13] and we transferred some genes to the rat limb and performed limb transplantation.[14] Furthermore, we successfully transferred the small interfering RNA to the transgenic rat liver and limbs.[15] The hydrodynamic method has been a useful technique for experimental gene transfer, but it has not been accommodated to clinical gene transfer. In this study, we tried to transfer plasmid DNA to the pig liver using the hydrodynamic method with some additional techniques.

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Results

The conditions of injection

The green fluorescent protein (GFP) gene was used as a reporter gene to confirm the conditions for the hydrodynamic method in pigs. The gene expression of the hydrodynamic gene transfer method depended on the gene quantity, the solution volume and the injection speeds.[4,9] In our rat study, the best conditions for the quantity of naked gene was 125 μg with solution (2.5% of the body weight) per rat.[13] However, the partial liver (half of the left outer lobe) in pigs was 8–12 times heavier than the whole liver in rats; therefore, for the same condition in pigs the quantity of naked gene was about 1–1.5 mg and the volume of solution was 60–90 ml per pig. However, in the pig experiment, balloon occlusion was not stanched completely; therefore, the solution and the naked gene were expanded about two times, so that we used 150 ml solution and 3 mg plasmid DNA per pig, respectively, in this study. One hundred and fifty milliliters of normal saline containing 3 mg GFP-expressing gene was injected rapidly by hand or with a power injector. We occluded vessels such as the hepatic artery and the portal vein. Additionally, we infused 200 ml of normal saline to flush out the liver blood. These occlusion of the hepatic artery and the portal vein and the flushing out of the blood from the liver were important techniques and are talked about in detail in the next section.

In injecting the solution by hand within a maximum of 60–80 s, only a little GFP expression was observed. Therefore, the power injector was an essential tool. Injection speeds of 20, 15, 10, 5 and 3 ml/s were tested, and the injection times were 10, 16, 17.5, 32.5 and 52.5 s, respectively, because in the first 5 s we set the 'rising time' so that the injection speed gradually increased. At 20 ml/s, the pig liver burst upon injection. At 15 and 10 ml/s, the livers were broken, with bleeding under the liver capsula and detachment of the vascular endothelium, respectively. At 3 ml/s, GFP expression was not found. However, at 5 ml/s, GFP expression was observed. Therefore, the best injection speed for pig liver was 5 ml/s.

In hydrodynamic gene delivery, a suitable gene quantity was present. With less than the suitable gene quantity, we found little or no gene expression. With more than the suitable gene quantity, gene expression was almost identical. We compared the delivery of 3 mg of plasmid DNA and 1 mg of plasmid DNA. In the 3 mg study, GFP expression was well observed but in the 1 mg study, only limited GFP expression was observed. And also, we compared the delivery of more than 3 mg of plasmid DNA, but gene expression was almost identical to that in the 3 mg study. Therefore, the gene quantity we decided to use was 3 mg.

The solution volume was also important in hydrodynamic gene transfer. We tried 100 and 150 ml solutions containing 3 mg plasmid DNA of GFP, respectively. In the examination of 100 ml solution, little GFP was observed, but in the 150 ml solution, GFP expression was well defined. We could not try a higher volume of solution because our power injector was limited to a maximum of 150 ml.

We decided that the condition for the hydrodynamic method in pigs was 3 mg plasmid DNA containing 150 ml normal saline and 5 ml/s high-speed injection with a power injector. Additionally, occlusion of the hepatic artery and the portal vein and flushing out of the blood from the liver were performed in this experiment. The GFP gene expression was good and identically observed in almost all of the targeted area of the pig liver lobe (Figure 1).

Surgical preparation of the pig

In rodent studies, only plasmid DNA and solution were injected via the tail vein or using a catheter with the hydrodynamic method. However, in the pig study, blood flow occlusion was necessary. We tried to inject plasmid DNA using a catheter-based hydrodynamic method similar to that in rat[13] or rabbit,[10] but no GFP expression was observed in the pig liver.

In the pig hydrodynamic gene transfer method, the blood flow of the liver was crucial. Therefore, we tested to occlude or wash out the blood of the liver. We divided the four groups as follows: 'only the portal vein clamp', 'only the left hepatic artery clamp', 'portal vein and left hepatic artery clamp' and 'both vessels clamp and wash out blood flow by 200 ml lactate linger solution from the portal vein' (Table 1). Also, the conditions of the hydrodynamic gene transfer to the pig liver were 3 mg of plasmid DNA, 5 ml/s speed injection and 150 ml solution volume. 'Only the portal vein clamp' group and 'only the hepatic artery clamp' group showed no GFP gene expression, but in the group of 'both vessels clamp' GFP gene expression was observed. However, in this group, which did not wash out the portal blood flow, GFP gene expression was limited to being around central veins (Figure 2a). And in the 'wash out the portal blood flow and clamp both vessels' group, GFP expression was observed in a greater quantity and area (Figure 2b). Therefore, the best conditions for hydrodynamic gene transfer to the pig liver were that the portal flow was occluded and the portal blood was washed out, and the left hepatic artery was also clamped (Figure 3).

Wedge pressure of the portal vein

In hydrodynamic gene transfer to the liver method, it was well recognized that the pressure of the hepatic vein was important,[9,13] so we examined to monitor the venous pressure. However, in this study, the hepatic vein of the outer left branch was not measured directly because of the intravessel turbulent flow, so we monitored the wedge pressure of the portal vein of the left outer branch as the approximate pressure (Figure 4). At 3 ml/s of injection speed, the peak pressure was 44 mm Hg (Figure 5a), and at 5 ml/s, the peak pressure was 58 mm Hg (Figure 5b).

CTLA4Ig expression in pigs

Next, we examined the serum level of gene expression using pCAG-CTLA4Ig. The conditions for hydrodynamic injection were the same as in the experiment of GFP gene transfer, the solution volume was 150 ml, CTLA4Ig gene was 3 mg and injection speed was 5 ml/s (32.5 s), and with clamping the hepatic artery and the portal vein and washing out the liver blood. The serum CTLA4Ig level in the pig peripheral blood obtained on days 0, 1, 2, 4, 7, 14 and 21 thereafter was measured by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 6, in No. 1 a remarkably high level of CTLA4Ig

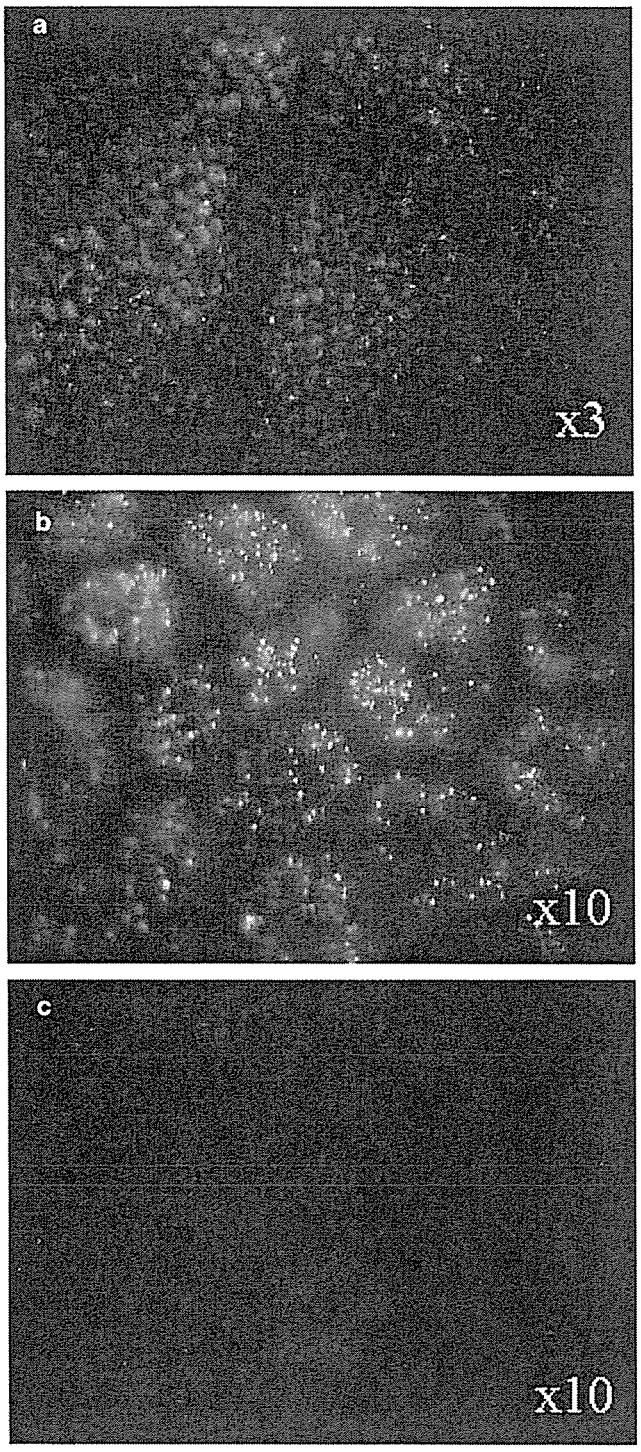

Figure 1 GFP expression in the pig liver after hydrodynamic injection (plasmid DNA: 3 mg; solution: 150 ml normal saline; and injection speed: 5 ml/s, with the hepatic artery and the portal vein occluded and the liver blood flushed out). GFP expression was shown in almost all areas of the target liver (left outer lobe of the pig liver) in (**a**) and (**b**). No GFP expression was shown in the non-hydrodynamic gene-transferred liver in (**c**). Magnification: (**a**) × 3; (**b**) × 10 and (**c**) × 10.

was detected (161.7 ng/ml) in the serum on 1 day after gene transfer and also during the next 4 days, in No. 2 a not so high serum level of CTLA4Ig was detected (98.1 ng/ml on day 4) and in No. 3 a lower serum level

Table 1 Relationship between gene expression and the preparation of the vessels in the pig liver

PV wash out	*PV clamp*	*HA clamp*	*Gene expression*
−	+	−	No
−	−	+	No
−	+	+	Good
+	+	+	Very good

Abbreviations: clamp, a temporary clamp using the vessel clamp during hydrodynamic injection; good, GFP expression was limitedly observed; HA, hepatic artery; no, no GFP expression was observed; PV, portal vein; very good, GFP expression was widely observed; wash out, PV blood flow was washed out using 200 ml lactate linger solution.
GFP expression was observed 2 days after hydrodynamic gene transfer.

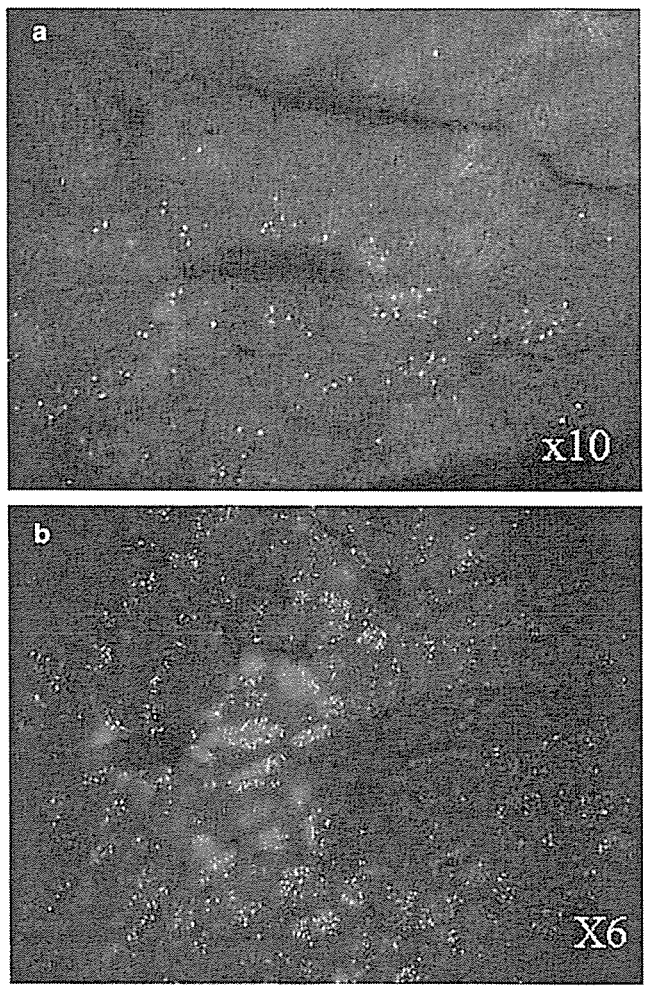

Figure 2 The GFP expression of the pig liver under the blood condition. The portal vein and the left hepatic artery were clamped, and the GFP gene was injected into the left hepatic vein (3 mg of p-DNA containing with 150 ml of normal saline and injection speed was 5 ml/s) (**a**). After occlusion of the hepatic inflow, 200 ml of the lactate linger solution were injected from the portal vein (**b**). The GFP expression of hydrodynamically gene delivered pig liver was limited to an area near the hepatic vein (**a**). Both vessels were clamped and the liver blood was washed out from the portal vein using the lactate linger solution. Gene expression was observed in almost all areas of the target liver (**b**).

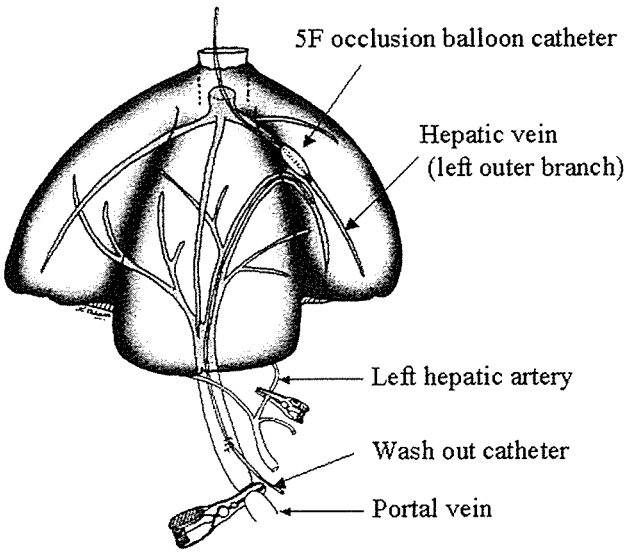

Figure 3 Schema of the catheterization for the left outer lobe of the pig liver. The left outer lobe of the pig liver was the target of gene delivery. The occlusion balloon catheter was inserted from the right outer jugular vein to the left outer branch of the hepatic vein. And the left hepatic artery and the main portal vein were isolated and clamped using vessel clamps. A catheter was inserted from the main portal vein to the left outer blanch of the hepatic vein in order to wash out the blood.

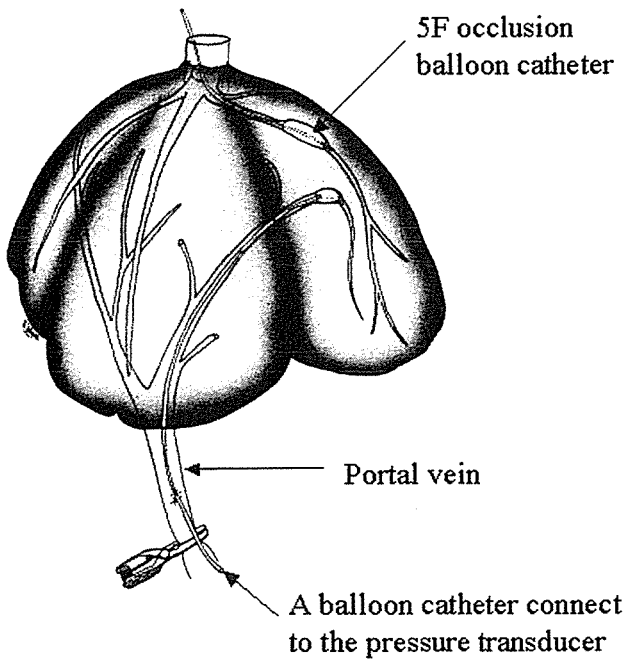

Figure 4 Schema of the portal vein wedge pressure measurement. A balloon catheter was inserted from the main portal vein to the left outer branch of the liver instead of the washing catheter so as to monitor the portal vein wedge pressure as being approximately equal to the pressure of the left outer branch of the hepatic vein pressure.

was detected. The peak of serum levels were seen on 1 day after gene transfer in No. 1 and No. 3, but on 4 days after gene transfer in No. 2, in spite of the same conditions.

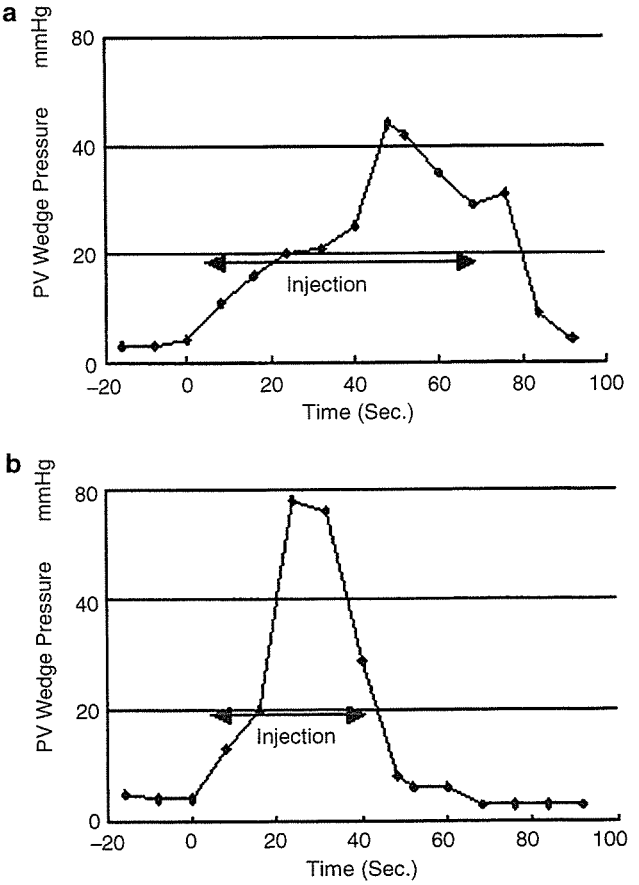

Figure 5 Measurement of the portal vein wedge pressure during hydrodynamic injection. The wedge pressure of the left outer branch of the pig liver during hydrodynamic injection was monitored. (**a**) Portal vein wedge pressure in 3 ml/s speed injection. Maximum pressure was 44 mm Hg. (**b**) Pressure in 5 ml/s. Maximum pressure was 58 mm Hg.

The toxicity of the pig liver in hydrodynamic gene transfer

In the CTLA4-Ig experiment, the liver injury induced by the hydrodynamic gene transfer was studied by AST (aspartate aminotransferase) and ALT (alkaline aminotransferase) in the following phases: pre-operation, 1 day, and 2, 4 and 7 days after gene delivery ($n=3$). AST increased on the first day after hydrodynamic gene transfer (maximum 501.3 IU/l) and decreased on the second day, returning to a normal level on the fourth day after gene delivery (Figure 7).

Discussion

In this study, we found that the hydrodynamic gene transfer method is not only a technique for small animals, such as rodents and rabbits, but also pigs. A paper that reported gene transfer to the dog liver has been published.[11] However, the report described mainly rodent data, and the dog data were not clear. Moreover, the method in that report was not highly efficient because it used 10–30 mg plasmid DNA. A paper mentioned naked gene transfer using the hydrodynamic method to the large animals, but no data were shown.[16] There was only one report that transferred naked DNA

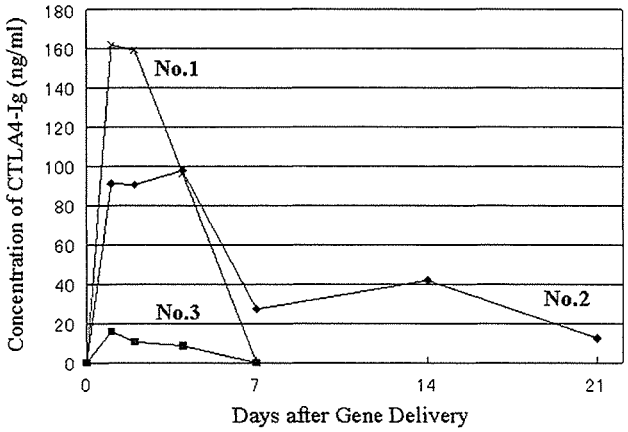

Figure 6 Concentration of the CTLA4-Ig in the serum of gene delivered pig using a hydrodynamic method. Hydrodynamic gene transfer was performed on all pigs (Nos. 1–3) under the same conditions (plasmid DNA: 3 mg; solution volume: 150 ml and injection speed: 5 ml/s). Even under the same conditions, a comparison of No. 1, No. 2 and No. 3 shows that levels of gene expression were quite varied.

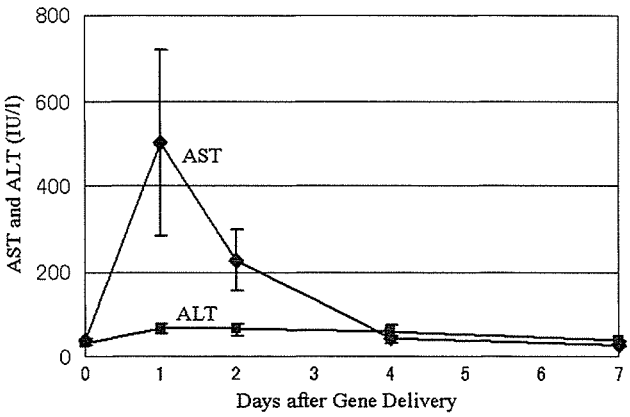

Figure 7 Toxicity of the pig liver in hydrodynamic gene transfer. AST (aspartate aminotransferase) and ALT (alkaline aminotransferase) level were measured in the serum of the CTLA4-Ig-transferred pigs using a hydrodynamic method in pre-operation, 1 day, and 2, 4 and 7 days after gene delivery ($n=3$).

to the pig liver using a hydrodynamic method.[12] The investigators transferred human α-1 antitrypsin (hAAT) to the whole liver of the pig using a catheter. This paper was very impressive and important because it was the first report of hydrodynamic gene transfer to the pig liver, and the toxicity of the liver was limited (maximum of AST level was 123 U/l). The conditions for injection were 2 mg of naked DNA with 100 ml of solution and 7.5 ml/s injection speed. These conditions were similar to our conditions but not the same, because their target organ was the whole liver and our target was a part of the liver. Moreover, their report said the gene transfer was not so efficient, so a more effective method was still necessary. We found that the hydrodynamic method needs additional techniques when used in pigs.

First, the hydrodynamic gene transfer in pigs required 3 mg of pDNA containing 150 ml of normal saline. We tried 1 mg of pDNA (GFP) and although gene expression was detectable, it was much less than 3 mg. We also tried 100 ml of solution, but GFP expression was not detectable. The solution volume was much larger in the pig than the rodent compared to the liver weight. The reason was the occlusion balloon. In this study, we used the 'Dilatation balloon'. The solution containing plasmid DNA leaked easily, so a greater volume of solution might be required in the pig study. A special balloon should be developed for the pig hydrodynamic method for better gene transfer efficiency. In this study, we operated in open surgery to fix the balloon to the best position and to prevent the leakage of solution, but the operative procedure may be performable using laparoscopic surgery to reduce surgical invasion.

Second, the blood flow of the pig liver was important in this study. It was necessary to occlude the portal vein and the hepatic artery. Moreover, effective gene transfer required washing out the liver blood. A paper suggested that the DNAase reduced the plasmid DNA contacting the blood,[17] and this study may support the hypothesis.

In the GFP experiments, GFP expression was identical in almost all of the area that was controlled by the hepatic vein with the balloon catheter clamped. GFP expression was quite effective; however, in the CTLA4-Ig study, the serum level of CTLA4-Ig was not as effective as in the rodent studies. Because in the pig study, the injected area of hydrodynamic gene transfer was $\frac{1}{16}$ to $\frac{1}{18}$ of the whole liver, the production was lower than in whole liver transfer studies (like rodent experiments). Moreover, the hydrodynamic gene transfer method leaves room for improvement in efficiency, such as the use of a specific occlusion catheter to reduce the leakage of the solution containing plasmid DNA.

In the hydrodynamic method, the transferred gene expression was not controllable in spite of using the same procedure and same surgeon. In our study, the CTLA4-Ig expression also suggested uncontrollability. Liver damage and the uncontrollable gene expression were demerits of the hydrodynamic method, but the liver damage improved in a few days and the gene expression level was high enough to meet the goal of objective experiments, such as CTLA4-Ig level in organ transplantation.[18]

Gene therapy for the liver is a strategy for various situations, such as an assist therapy for the immunosuppression in organ transplantation, the treatment of patients with metabolic disorders and DNA vaccination for treatment of fulminate hepatitis.[19] Moreover, the non-viral gene transfer method is beneficial for recipients inducing immunosuppressive agents, in whom the clinical application of virus vector may be limited because of cytotoxicity and immune responses. Delivery of naked DNA using an intravascular route results in effective gene transfer to hepatocytes because the vascular system accesses every cell. Furthermore, the immunosuppression-limited graft organ using gene therapy has great potential in solid organ transplantation and also in cell transplantation. We will investigate the hydrodynamic method for the combination therapy of gene transfer and transplantation.

Materials and methods

Plasmid constructs

The expressing construct for enhanced type of mutant in the GFP gene, *ph-GFP-105-c1*, driven under a cyto-

megalovirus promoter, was kindly donated by Dr T Osumi (Himeji Institute of Technology, Hyougo, Japan). The GFP and the human CTLA4-Ig expression plasmid *pCAG-CTLA4-Ig* were amplified in the DH5 α strain of *Escherichia coli*, and large-scale preparation of plasmid DNA was performed by the alkaline lysis method. Closed circular plasmid DNA was then purified twice by equilibrium centrifugation in CsCl-ethidium-bromide gradients. DNA concentrations were measured by ultraviolet absorption at 260 nm.[13,14]

Animals

Mail miniature pigs (Mexican hairless pig), weighing 14–27 kg, obtained from the Ibaragi Firm (Ibaragi, Japan), were used. Pigs were kept at our animal center. All experiments were performed in accordance with the 'Jichi Medical University Guide for Laboratory Animals'.

Anesthesia and surgical preparation

As preparation for surgery, the miniature pigs received an intramuscular injection of ketamine (10 mg/kg) and atropine sulfate (0.5 mg/each animal). The pigs were intubated and anesthetized generally by isoflurane.[20] The occlusion balloon catheter, Ultra-thin Diamond Balloon Dilatation catheter, 5F, 75 cm, balloon 5 mm × 4 cm (Boston Scientific, Boston, MA, USA), was inserted from the right external jugular vein with the 8 French angiocatheter sheath, and into the left hepatic vein, navigated under fluoroscopic control. The pigs were operated with inverted T skin incision. The hepatic artery and the portal vein were identified and clamped with vessel clips. The injection catheter was inserted into the portal vein, and 200 ml normal saline was injected for washing out the liver blood. After that, the hepatic vein was occluded with the balloon catheter and the surgeon held the left outer lobe of the liver with the hands to fix the liver and the occlusion balloon and to prevent leakage of the solution.

Hydrodynamic injection

The plasmid DNA was rapidly injected into the hepatic vein with 150 ml cold normal saline using the power injector (Mark five Pro Vis injection system, Medrad Inc., Pittsburgh, PA, USA). The balloon occlusion was continued during rapid injection and 30 s after the injection. After that, the occlusion balloon was deleted, the portal vein and hepatic artery clamps were removed, and the abdominal and cervical wounds were sutured. The pigs were kept in the special cage for the after-operation pigs at our animal center.

The measurement of the portal vein wedge pressure

A balloon catheter was inserted from the main portal vein, and advanced into the left outer branch of the portal vein. The balloon was expanded during the rapid injection and after 30 s. The portal vein wedge pressure was continuously monitored on Pressure Transducer (NEC, Tokyo, Japan).

Detection of marker gene

Expression of the introduced GFP gene was visualized macroscopically under 489-nm wavelength excitation light as specified in previous reports.[21,22] The pigs were killed 2 days after gene transfer to evaluate the technical procedure.

Detection of serum CTLA4-Ig level

CTLA4-Ig level in serum were assayed by ELISA as specified in a previous report.[13]

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血友病A患者に認められた第VIII因子A1ドメイン内のミスセンス変異Asp116Asnの分子病態

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【要旨】 血友病Aは第VIII因子 (FVIII) の異常による出血性疾患である。血友病AにはFVIII抗原が認められるがFVIII活性の低い病型があり、Cross-Reacting Material-positive (CRM$^+$) と呼ぶ。CRM$^+$血友病A患者に見出されたミスセンス変異Asp116Asn (D116N) の分子異常を明らかにするために発現実験を行った。ワンステップのsite-directed mutagenesisキットにて作製した変異FVIIIをHEK293細胞にトランスフェクションし培養上清中のFVIII活性、抗原を測定した。変異FVIIIの活性、抗原はそれぞれ野生型に対して、1.8±0.7%、120.9±25.5%であり、D116NはCRM$^+$であることを確認した。活性低下の原因として、新しい糖鎖が付加されている可能性について検討した。イムノブロット解析では、抗A2抗体を用いた間接証明であるがD116Nでは糖鎖付加による分子量の増大に伴う易動度の変化は無いと考えられた。N-Glycanaseにより糖鎖を切断することでFVIII活性の上昇が得られるかどうかを確認したが、活性は上昇しなかった。これらの結果から、D116Nの活性低下要因は新しい糖鎖付加によるものではなく、構造変化に伴うカルシウム結合性の低下によることが示唆された。

はじめに

血友病Aは第VIII因子 (FVIII) の量的あるいは質的異常が病因の出血性疾患である。X染色体性劣性遺伝形式である本症の発生頻度は男児出生1万人に1人の頻度である。第VIII因子遺伝子 (F8) はX染色体長腕上の末端側Xq28に存在し、全長約186 kbで26のエクソンと25のイントロンにより構成される。1984年にFVIII cDNAのクローニングが成功して以来、血友病AとF8異常に関する多くの知見が蓄積されてきた。血友病Aの重症度は血漿FVIII活性 (FVIII: C) と概ね相関しており、1%未満を重症型、1～5%を中等症型、5%以上を軽症型に分類する[1)2)]。またFVIII抗原量 (FVIII: Ag) がFVIII: Cと一致しない分子異常のFVIIIを有する血友病Aの変異病型があり、正常量 (≧50%) のFVIII: Agを有する病型をCross-Reacting Material (CRM)−positive (CRM$^+$) と呼び、その頻度は全体の約10%に認められる。それに対し、FVIII: Agが正常量の≦50%でFVIII: Cがそれよりも低下しているものをCRM-reduced、FVIII: AgもFVIII: Cも同様に低下しているものをCRM-negative (CRM$^-$) と呼ぶ[3)4)]。中等症、軽症型にはミスセンス変異による分子異常が原因と考えられるCRM-positiveやreduced症例が多く報告されている[5)]。

FVIIIは分子量330 kDaの糖タンパク質で、アミノ酸配列の相同性からA1-A2-B-A3-C1-C2のドメイン構造をとる[6)]。2351残基のFVIII前駆体は主に肝臓で

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キーワード: 血友病A、ミスセンス変異、第VIII因子
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合成される一本鎖糖タンパク質で小胞体内腔に移行する際に種々のタンパク翻訳後修飾が、小胞体内腔でタンパクの折り畳みや糖付加が行われる。ゴルジ体に移行したFVIIIは更に糖鎖付加、修飾、硫酸化が行われ、N末端由来200 kDaの重鎖（A1-A2-B）とC末端由来80 kDaの軽鎖（A3-C1-C2）に分解されてヘテロダイマーとなり、成熟したFVIIIとして分泌される[7-9]。トロンビンによる活性化に伴い重鎖は50 kDaのA1と43 kDaのA2フラグメントに、軽鎖は73 kDaのA3-C1-C2のフラグメントに分解される[10]。

Aドメインは第V因子（FV）や銅結合タンパクのセルロプラスミンと相同性があり、FVIII 1分子内には1つの銅イオンの存在が同定されている[11]。またFVIIIの重鎖と軽鎖はマンガンやカルシウムなどの2価陽イオンにて再構成されることが報告されており、FVIII活性の出現のためには、Aドメインと陽イオンの何らかの会合が必要であると考えられている[12]。

本研究では、CRM^+血友病A患者に見出されたA1ドメイン内のミスセンス変異Asp116Asn（D116N）のFVIII分子異常を明らかにするために、タンパク質発現実験を行った。

研究材料および方法

1. 変異FVIIIプラスミドの作製

フルレングスFVIII cDNAの入ったFVIII合成プラスミド（pMT2 VIII）[13]はRandal J Kaufman（Biological Chemistry, University of Michigan, Ann Arbor, MI, USA）より提供を受けた。pMT2 VIIIを鋳型として、QuickChange® Site-Directed Mutagenesis Kit（Stratagene®, LA Jolla, CA, USA）を用いて、FVIII D116Nプラスミド変異体の作製を行った[14)15)]。FVIII合成プラスミドのアスパラギン酸116のコドン配列（gat）からアスパラギン（aat）に点突然変異を発生させる為に、5′-gctgaatatgataatcagaccagtcaa-3′、5′-ttgactggtctgattatcatattcagc-3′のミュータジェニックプライマーを設計した。*PfuTurbo*® DNA polymeraseを用いて、pMT2 VIIIプラスミド全体を一周増幅させるPCRを行った。PCR条件は、95°C 30秒の後、95°C 30秒、55°C 1分、68°C 20分を12サイクル行った。PCR産物は*Dpn1*で37°C 1時間の制限酵素処理反応を行い、処理後のPCR産物1 μlを、Competent high DH5α（TOYOBO）にトランスフォーメーションした。LB寒天培地（Invitrogen）に発育したコロニーを採取し、コロニーのPCR産物をテンプレートとし、Thermo Sequenase® core sequencing kit（Amersham）を用いてジデオキシ法によるダイレクトシークエンスを行った。解析にはHITACHI SQ5500Eシークエンサーを用いて点変異を確認した。変異が確認できたコロニーは、QIAprep® Maxi Kit（Qiagen）にて変異プラスミド精製を行った。精製した変異体プラスミドは、さらにダイレクトシークエンスにより変異の確認を行った。

2. リコンビナントFVIIIの合成

リポフェクタミン試薬（Invitrogen）を用いて、野生型（Wild Type, WT）のpMT2 VIIIプラスミドと、作製したD116N変異体をHEK293細胞に一過性発現させた。トランスフェクション48時間後にそれぞれの培養上清（Conditioned Medium, CM）を採取し、測定までは−80°Cに凍結保存した。

3. FVIII活性（FVIII：C）と抗原量（FVIII：Ag）の測定

FVIII：Cは、自動血液凝固能測定装置（ACL9000, Instrumentation Laboratory）に、APTT試薬（IL-test™, Instrumentation Laboratory）とFVIII欠乏血漿（George King）を用いて凝固一段法で測定した。FVIII：Agはポリクローナル抗体を用いたサンドイッチEIA法（ASSERACROM® FVIII：Ag（Roche Diagnostica Stago））を使用して測定した。

4. 免疫沈降法とSDS-PAGE、イムノブロット法によるFVIIIタンパクの解析

CM中のリコンビナントFVIIIを抗ヒトFVIIIポリクローナル抗体と反応後、FVIII−抗体複合体をプロテインAセファロースで免疫沈降させた。終濃度1単位/mlのα-トロンビンを添加し37°C 30分で処理したリコンビナントFVIIIを2/15ポリアクリルアミドゲル（PAGミニ「第一」、第一化学薬品）でSDS-PAGE電気泳動した。電気泳動したゲルはPVDF膜（Immobilon-P, MILLIPORE）にブロットし、抗FVIII A2ドメインモノクローナル抗体を反応させ、リコンビナントFVIIIの性状を観察した。HRP基質はコニカイムノステインを用いて発色した[9]。

5. 変異FVIIIのN-Glycanase® 処理

WTと変異体リコンビナントFVIIIにそれぞれ等量のFVIII欠乏血漿を混和することで、生理的状態に近づけた上で、N-Glycanase®（Glyko）を反応させた。N-Glycanase®と反応後、経時的にFVIII：Cの変化を凝固一段法で測定した[16)17)]。

結　果

FVIII 変異体の作製

QuickChange® Site-Directed Mutagenesis Kit を用いて血友病 A 遺伝子変異である、血液凝固第 VIII 因子の D116N 変異遺伝子を含むプラスミドを作製した。Fig. 1 に示す DNA のダイレクトシークエンスの結果、目的の遺伝子変異 (gat → aat) が正しく導入されていることが確認された。

変異 FVIII の発現型

FVIII 変異体 D116N についてトランスフェクション 48 時間後の CM の FVIII : C、FVIII : Ag を測定した。3 回の異なるトランスフェクションにおける結果を Fig. 2 に示す。WT に対して、D116N は FVIII : C が 1.8±0.7%、FVIII : Ag が 120.9±25.5% であった。D116N はほぼ正常の抗原量であるが、活性は著しく低下しており CRM^+ であることが確認された。

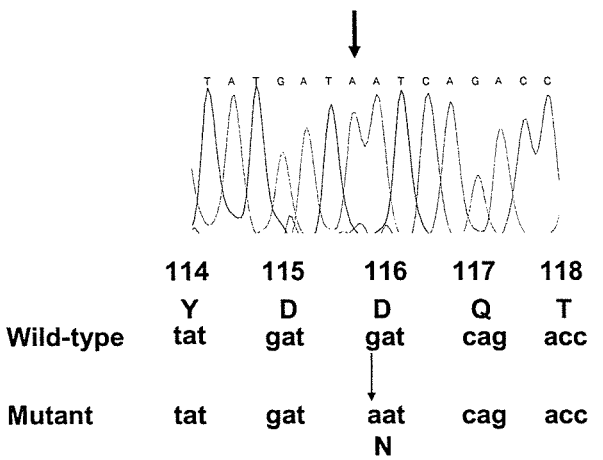

Fig. 1 DNA sequencing electropherogram showing the pMT2-FVIII-D116N mutant.
After the pMT2-FVIII-D116N mutant was constructed using QuickChange® Site-Directed Mutagenesis Kit, nucleotide sequence analysis identified g to a mutation, which causes the FVIII-D116 (gat) to N116 (aat) substitution.

変異 FVIII のイムノブロット解析

リコンビナント FVIII のイムノブロット解析を Fig. 3 に示す。抗 A1 モノクローナル抗体では、FVIII の構造変化により A1 フラグメントを認識することができないと考えられるため、抗 A2 モノクローナル抗体を用いて評価を行った。90 kDa の A1-A2 フラグメントと 43 kDa の A2 フラグメントのどちらも同時に検出することができるように、トロンビンによる FVIII 処理を不十分な条件で行った。WT も変異体も同様に 90 kDa の A1-A2、43 kDa の A2 フラグメントが検出された。よって、変異体の A1 ドメインも電気泳動上の易動度に差は無いと考えられた。

変異 FVIII の N-Glycanase® 処理

アスパラギン酸 116 がアスパラギンに置換されると、116 番残基には新しく N-linked glycosylation (糖鎖) が生ずる可能性がある。この変異において新しい糖鎖が出現し、かつ FVIII : C の低下に影響しているかどうかを検討するために、糖鎖を切断する酵素である N-Glycanase® にて変異体を処理した後、経時的に FVIII : C を測定した (Fig. 4)。WT では N-Glycanase® 処理 30 分後に、約 2 倍の活性の上昇を認めたが、その後、徐々に活性は低下した。D116N でも同様に N-Glycanase® 処理後に軽度の活性上昇を認めた

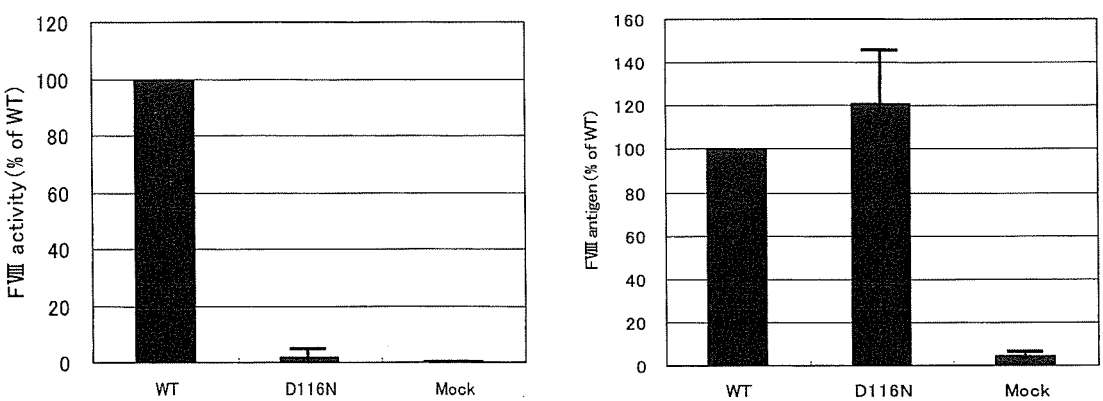

Fig. 2 The activity and antigen of recombinant FVIII.
Conditioned medium (CM) of wild type (WT) and D116N mutant FVIII were harvested at 48 hours posttransfection for FVIII assay. The activity and antigen in the CM were measured by one stage clotting assay and ELISA utilizing anti-FVIII light chain monoclonal antibody, respectively. Data are expressed as percentages of WT. Bars indicate means±standard deviation of three independent transfection experiments. The mock column indicates cells that did not receive plasmid DNA.

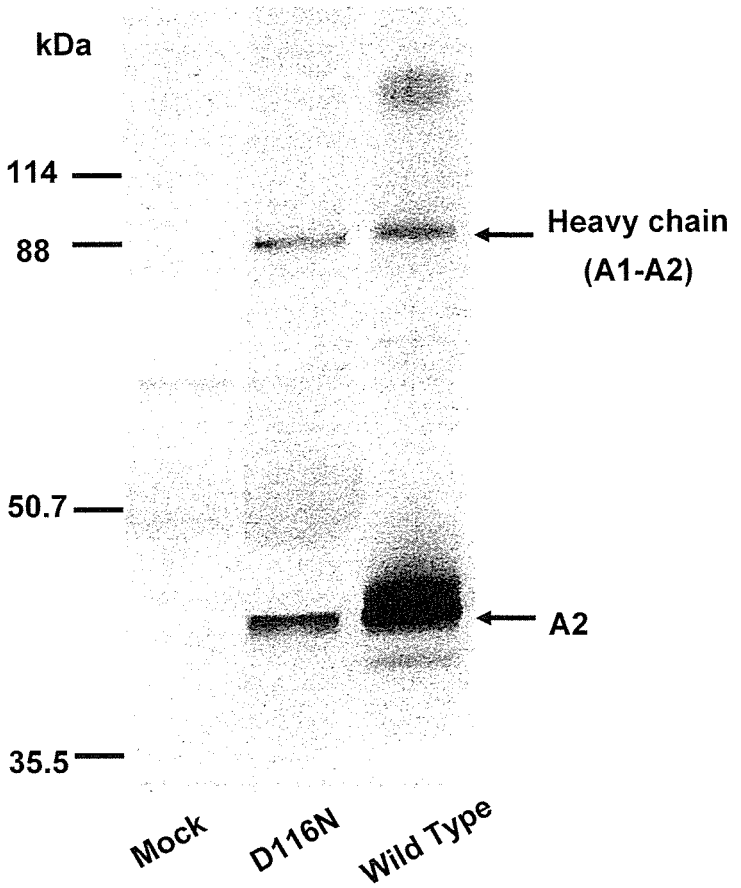

Fig. 3 Immunoblotting analysis of recombinant FVIII.
WT and D116N recombinant FVIII immunoprecipitated by anti-FVIII polyclonal antibody from conditioned medium were analyzed by SDS-PAGE after adequate thrombin digestion. Protein was transferred to PVDF membrane by Western blotting. The membrane was reacted with anti-FVIII A2 monoclonal antibody. The mock column indicates cells that did not receive plasmid DNA. Molecular weight size markers are shown on the left. A1-A2 and A2 fragments are indicated on the right.

が、徐々に活性の低下が観察された。

考　　察

血友病Aは先天性凝固障害症の中では最も頻度が高く、出血症状も重篤であり、古くより多くの研究者や臨床家の研究の対象となってきた。しかし血漿含有量が200～300 ng/mlと非常に少なく、不安定なFVIIIタンパクの精製や生化学的解析は1980年代前半まで難航していた。cDNAが明らかにされて以来、リコンビナント技術を用いたFVIII発現実験における研究成果は、より安全な血友病Aの治療薬開発にも大きく貢献することとなった。遺伝子解析において明らかにされている血友病A患者の変異FVIIIの合成と解析により、その遺伝子型と発現型との関係の確認が可能となったことで、より確実な病因遺伝子の特定が行えるようになった。さらに、そこから機能上重要な役割を持っている部位を推定し、その部位に人工的に変異を作り出すことによって、FVIIIの構造と機能の精密な解析が行われている[18]。

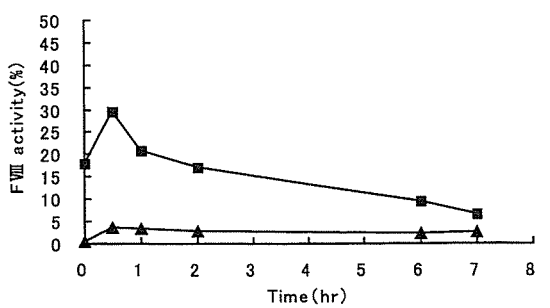

Fig. 4 Effect of N-Glycanase for D116N mutant FVIII.
FVIII (WT: ■, D116N: ▲) in conditioned medium with an equal volume of FVIII deficient plasma was incubated with N-glycanase. FVIII activity after increasing times (0, 0.5, 1, 2, 6, 7 hours) was determined by one stage clotting assay.

いままでの変異体作製技術では、目的とする変異部位を制限酵素にてとりだし、ミスマッチPCRにより

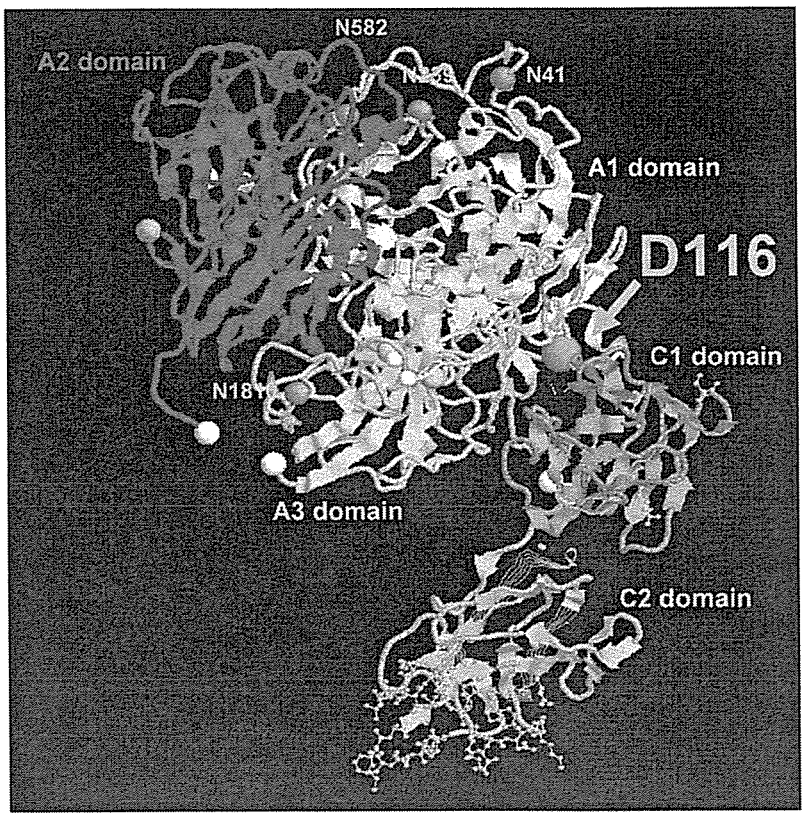

Fig. 5 Location of D116 with FVIII 3-dimensional structure model.
The FVIII molecule model was constructed based on the crystal structure of FIXa, the membrane-bound FVIII structure, and the FIXa/FVIIIa binding sites by Stoilova-McPhie et al.[21]. The FVIII molecule model is illustrated using RasMol. The lower part of the figure shows a phospholipid membrane. The mutation site D116 is represented with a large red spacefill. A1, A2, A3, C1 and C2 domains are shown as pink, purple, orange, brown and violet, respectively. Asparagine residues known to be glycosylated (N41, N239, N582, N1810, and N2118) are represented with sky-blue spacefills.

変異を作成した後、元のプラスミドにライゲーションする必要があった。しかし、従来のこの方法では変異部位をとりだすための制限酵素は、そのプラスミドにおいて1ケ所のみを切断するものを選択する必要があり、変異を作製する部位によっては、制限酵素の選択が困難になる場合がある。これに比較すると本研究で用いたQuickChange® Site-Directed Mutagenesis Kitは、変異部位のミュータジェニックプライマーを作製するだけで、ワンステップで変異体を作製することができる簡便な方法である。しかし、本法ではプラスミドを全周にわたってPCRすることができなければ変異体を作製できない。FVIIIのcDNAは全長で7.2 kbと長く、それを含むプラスミド自体は12 kbにも及ぶため、本法にて変異体を作製することが可能かどうかは不明であり、今までに報告は無かった。本研究においてフルレングスのFVIIIでも本法で変異体を作製できることが確認できた。タンパク質の機能解析の一方法として、多部位のアミノ酸をアラニンに変異させ、多種類の変異体を作製して比較検討するアラニンスキャンニング法がある。このように多くの変異体を作製する時には、このワンステップの方法は大変有意義である。今後、FVIIIにおいても制限酵素選択にしばられること無く、本法を用いて多くの変異体の作製、検討が行えるようになると考えられる。

今回作製したD116N変異は、柴田ら[19]の報告した重症血友病A患者に見出された遺伝子変異である。患者血漿のFVIII：Cは1%未満、FVIII：Agは50%と報告されている。本研究において、リコンビナントFVIIIのD116N変異体も患者血漿同様にCRM$^+$の表現型を示したことから、このD116N変異が本患者に血友病Aを惹き起こしている病因遺伝子として確認された。2006年1月現在、血友病遺伝子変異の世界的データベース[20]には他にD116Nの報告は無く、同部位のグリシンへの変異が1例報告されているのみである。

D116Nではアスパラギン酸116がアスパラギンへ

置換されることで、Asn－X－Thr のアミノ酸配列となり、新たに N-glycosylation 部位が出現する。今までに Ile566Thr と Met1772Thr の2症例で、SDS-PAGE における易動度の違いや N-Glycanase® 処理による FVIII 活性の上昇から、この余剰糖鎖の付加が確認されている[16]。本研究では、イムノブロット解析において A1 フラグメントを直接確認することはできなかった。しかし、A1-A2 フラグメントと A2 フラグメントにおいて WT と差が無かったことから、間接的な証明であるが D116N では糖鎖付加による分子量の増大に伴う易動度の変化は無いと考えられた。そこで、さらなる確認のために N-Glycanase® 処理による FVIII 活性の変化により推測することとした。酵素処理直後にごく軽度の FVIII 活性上昇が認められたが、これは WT においても認められたもので、N-Glycanase® による非特異的な反応であり、また糖鎖処理が充分行われた為と考えられる。N-Glycanase® 処理により明らかな FVIII 活性の上昇は得られなかったことから、D116N には新しい糖鎖は付加されていないことが示唆された。前述の2症例では、実際に糖鎖結合部位となるアスパラギンにはアミノ酸置換は無く、ともに2残基C末端側のスレオニンへの置換により新たな糖鎖結合が生じている。D116N では糖鎖結合部位の条件は満たしているが、結合部位となるアスパラギン自体が新たに出現したものであることが、糖鎖結合を引き起こさない原因かもしれない。上記のアミノ酸配列以外に、糖鎖結合を制御するメカニズムの存在が疑える。

柴田らは患者血漿の解析において、抗 A1 抗体を用いた ELISA 法では FVIII 抗原量を測定できず、WB 法では抗 A1 抗体、抗 A2 抗体ともに FVIII 重鎖を認識できないため、本変異は A2 ドメインの立体構造に変化を及ぼしていると報告している[19]。本研究で用いた抗 A2 モノクローナル抗体では FVIII 重鎖を認識することができた。これは、抗体のエピトープ部位の差異のためと考えられる。我々が使用した抗体では異なる結果であったが、D116N は A1 ドメイン内の変異でも抗 A2 ドメイン抗体の反応性に影響を及ぼすような立体構造の変化をもたらしている可能性は残される。FVIII の立体構造モデルを Fig. 5 に示す[21]。モデル上、D116 は軽鎖の A3 や C1 ドメインと近傍で、A2 ドメインとは比較的離れた位置にある。近年、FV のカルシウム結合領域と相同性のある FVIII　A1 ドメイン内 110-126 残基領域は FVIII のカルシウム結合部位であることが提唱された[22][23]。D116N は離れた部位である A2 ドメインの立体構造をも変化させる変異であると考えられることから、より近傍である軽鎖とのカルシウム結合に大きく影響を与えていることが示唆される。このカルシウム結合への影響が、FVIII 活性の低下をもたらしていることが推測される。

カルシウム結合がどのように阻害されるのかはまだ明らかにされていない、さらに詳細な検討によってその分子病態が明らかにされることが、今後より高活性で安定性のある新しい FVIII 治療薬の開発に寄与することを期待する。

結　論

血友病 A 患者に認められた第 VIII 因子重鎖内のミスセンス変異 Asp116Asn が発現実験により、病因遺伝子異常であることを確認した。本変異の活性低下要因は新しい糖鎖付加によるものではなく、構造変化に伴うカルシウム結合性の低下によることが示唆された。

謝　辞

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The molecular basis for hemophilia A due to missense mutation (Asp116Asn) within the A1-domain of factor VIII

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Abstract

Factor VIII (FVIII) is the protein that is defective in the bleeding disorder hemophilia A. Some patients have normal amounts of a dysfunctional FVIII protein and are termed cross-reacting material (CRM)-positive. To investigate the molecular basis of the genetic defects within the A-domain, we constructed an Asp116Asn mutation of FVIII gene that was previously reported in a patient. Mutant was made by a site-directed mutagenesis kit. Wild-type (WT) and mutant FVIII molecules were transiently expressed in HEK293 cells. Conditioned medium (CM) was assayed for clotting activity and antigen levels (ELISA utilizing anti-FVIII light chain monoclonal antibody). FVIII activity and the antigen of this mutant (% of WT) were 1.8±0.7%, 120.9±25.5%, respectively. The phenotype of Asp116Asn was confirmed as CRM-positive. To test whether this mutant would make a new glycosylation site, SDS-PAGE analysis was performed. A2 fragment and the 90 kDa A1-A2 heavy chain of Asp116Asn were detected in the same way as WT by immunoblotting using anti-FVIII A2 domain monoclonal antibody, indicating the mobility of A1 fragment was not reduced. We speculate that there is no additional glycosylation in this mutant FVIII. We then analyzed the FVIII clotting activity after incubation with N-glycanase, to identify whether removal of additional glycosylation would increase FVIII activity of this mutant. N-glycanase digestion did not increase the activity, suggesting no new N-glycosylation in this mutant. These results suggest that the defect in the Asp116Asn is not due to a formation of new N-glycosylation, but may be caused by altering the conformation of the recently proposed calcium binding site.

〈**Key words**〉 Hemophilia A, Missense mutation, Factor VIII

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◆原　著◆

日本の血友病類縁疾患患者の入院医療コストの集計：多施設共同研究

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8施設の血友病患者の入院医療コストを集計し，その特徴と診断群分類（DPC）点数表の導入に対する問題点を考察した．2002年4月から2004年3月までの8施設における患者の各入院に対し，血友病の種類，体重，インヒビターの有無，手術の有無，入院日数，請求点数などを集計した．さらにそれらのデータからそれぞれの因子により請求点数を解析し，どの因子が請求点数に関与しているか統計学的検定を行った．入院日数，請求点数は施設間にばらつきがみられ，施設別1日あたりの平均医療コストは最少が124,110円，最大が222,080円であった．体重別では25kg未満127,280円，25－50kg 210,720円，50-75kg 280,861円，75kg以上526,958円で有意差がみられた．インヒビターの有無（524,416円，147,534円）でも有意差がみられた．2004年度の血友病類縁疾患のDPC導入案と比較すると，全施設で病院側の赤字になることが分かった．以上より，血友病患者の入院医療コストは患者の体重や状態により多種多様で，安易にDPCを導入することは問題と思われた．

Key words: hospital charges for hemophiliacs, Diagnosis Procedure Combination (DPC), comprehensive costing, inhibitor, total infusion dose

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