these reasons, FVIII plays an important role in hemostasis regulation, and the increased FVIII activity during liver regeneration may indicate a pivotal role in mediating the hypercoagulability of the blood promoting abnormal thrombotic events. At the present time, however, the differential gene expression and protein activity between FVIII and all other clotting factors during liver regeneration remains to be fully elucidated.

In the present study, we induced liver regeneration in mice by performing a 2/3 partial hepatectomy, the most commonly-used experimental procedure to induce compensatory liver regeneration mode [14]. During this liver regeneration process, we investigated the gene expression profile of coagulation factors and fibrinolytic factors as well as plasma activities of these factors at different time points following hepatectomy.

Materials and methods

Animals

Wild-type C57BL/6 female mice, 10-12 weeks old, were used in this study. Experimental protocols were developed in accordance with the guidelines outlined by our institutional animal committee at Nara Medical University. Mice were placed in cages within a temperature-controlled room having a 12-h light / dark cycle (8:00 a.m. lights on / 8:00 p.m. lights off).

Two-thirds partial hepatectomy (PHx)

The liver regeneration was induced by performing a 2/3 partial hepatectomy (PHx) under the general anesthesia with isofluorane as previously described [3,14–16]. In brief, a small upper midline incision was made, and the liver was exposed allowing for the removal of the median and left lobes. This surgical procedure was performed at within a specified time window (between 8:00-10:00 a.m.) to minimize the circadian rhythm variations that may influence the speed and peak of the regenerative activity as previously reported [17]. We performed two sets of PHx experiments for the purpose of (i) evaluating liver gene expression profiles with the corresponding plasma activity of secreted coagulation factors, (ii) specific investigation of factor VIII mRNA expression changes in several mouse tissues after PHx.

Experiment 1: Comparison of liver mRNA expression levels and plasma levels of coagulation factors

C57BL/6 mice received 2/3 PHx (n = 25), and the liver lobes that were removed from each mouse at the time of PHx were treated as quiescent control liver samples. The mice were sacrificed at 1, 2, and 5 days after PHx (n = 9, 8, and 8, respectively), and the livers were harvested immediately snap-frozen in liquid nitrogen. The livers were kept at -80 °C until the extraction of RNA was performed to analyze gene expression profiling by quantitative RT-PCR. For this experiment, we examined the following gene groups: i) coagulation factors, including fibrinogen (*Fgb*), prothrombin (*F2*), factor V (*F5*), factor VII (*F7*), factor FVIII (*F8*), factor IX (*F9*), factor X (*F10*), factor XI (*F11*), factor XII (*F12*), and factor XIIIβ subunit (*F13b*); ii) coagulation-related or fibrinolytic factors, including antithrombin (*Serpinc1*), protein C (*Prosc*), protein S (*Pros1*), plasminogen (*Plg*), von Willebrand factor (*VWF*), and a disintegrin-like and metalloproteinase with thrombospondin type 1 motif 13 (*Adamts13*), and iii) factors involved in the production and degradation of FVIII, including lectin, mannose-binding 1 (*Lman1*), multiple coagulation factor deficiency 2 (*Mcfd2*), and low-density lipoprotein receptor-related protein 1 (*Lrp1*). Based on our recent experimental data in regenerating mouse livers [3], we adopted the geometric mean of the two reference genes, peptidylprolyl isomerase A (*Ppia*) and TATA box binding protein (*Tbp*), to normalize the gene expression calculated in our experiments. The gene expression profiles were correlated with the protein levels and/or activity by obtaining blood samples from the retroorbital plexus prior to the PHx and at the time of sacrifice. Samples were collected with an anticoagulant (0.1 vol 3.8% sodium citrate) and stored at -80 °C until analysis. Plasma coagulation factor activities (fibrinogen, prothrombin, factor V, VII, VIII, IX, X, XI, XII, and XIII), von Willebrand factor (VWF) antigen levels, and tissue-type plasminogen activator (t-PA) activities were measured as described below.

Experiment 2: Examination of F8 mRNA expression levels in mouse tissues during liver regeneration

C57BL/6 mice (n = 16) were either received 2/3 PHx (PHx group; n = 8) or sham-operated (non-PHx group; n = 8), and various organs (i.e., liver, lung, spleen, heart, intestine, brain, and kidney) were obtained from all of the mice 24 h after the initial procedure. This time point is known to be the peak phase in the liver regeneration process for mice [1,2]. The *F8* mRNA expression levels were analyzed in all of the extracted organs by real-time RT-PCR. To obtain a reliable gene expression profile for *F8* in the liver and other extra-hepatic organs, appropriate reference genes that are minimally affected by the PHx needed to be validated. To achieve this goal, we analyzed the expression levels of 8 commonly-used reference genes, which included: glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*); β-actin (*Actb*); hypoxanthine phosphoribosyltransferase (*Hprt1*); β-glucuronidase (*Gusb*); peptidylprolyl isomerase A (*Ppia*); TATA box binding protein (*Tbp*); transferrin receptor (*Tfrc*), and ribosomal protein L4 (*Rpl4*). The variance of the reference gene expression in all samples were assessed using two software programs, geNorm and NormFinder [18,19], which have been described elsewhere [3,20]. Using this approach, the *F8* mRNA expression were calculated following normalization with the most stable reference gene combination.

RNA isolation and quality controls

Total RNA was extracted from each tissue samples using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNase I (QIAGEN) was used to eliminate any genomic DNA contamination, and the concentration of the RNA was determined by UV spectrometry.

Reverse transcription coupled to quantitative real-time PCR (real-time RT-PCR)

Total RNA (1 μg) was reverse-transcribed using oligo $d(T)_{16}$ primers as described by the manufacturer (Omniscript RT Kit) (QIAGEN). First-strand cDNA samples were subjected to quantitative PCR amplification using a PRISM 7700 Sequence Detector (Applied Biosystems Japan, Tokyo, Japan). Coagulation factor genes, fibrinolytic factor genes, and the genes of coagulation related factors or protein as listed in Table 1 were assessed in each tissue cDNA. TaqMan probes and primers for these genes were chosen from TaqMan Gene Expression Assay (Applied Biosystems) (Table 1). The primer sets of reference genes were the same products as described in our previous report [3]. For quantification of gene expression, the cDNAs derived from total RNA extracted from pooled normal mouse livers were serially-diluted, and used to generate the reference standard curves.

Plasma analyses

Coagulation factor activities were measured by one-stage clotting assay using human plasma deficient for each coagulation factor (Sysmex, Kobe, Japan). The activities of FVIII, FIX, FXI, and FXII were measured based on aPTT using ThromboCheck APTT-SLA (Sysmex) and prothrombin, FV, FVII, and FX based on PT using ThromboCheck PT Plus (Sysmex), by a KC10A Coagulometer (Amelung, Lemgo, Germany). Plasma VWF antigen levels were assayed by sandwich ELISA using a

Table 1
Description of analyzed target gene primers used in the real-time RT-PCR assay.

Symbol	Gene Name	Assay ID	Amplicon Length (bp)
Fgb	fibrinogen, B beta polypeptide	Mm00805336_m1	154
F2	coagulation factor II (prothrombin)	Mm00438843_m1	68
F5	coagulation factor V	Mm00484202_m1	61
F7	coagulation factor VII	Mm00487329_m1	78
F8	coagulation factor VIII	Mm00433174_m1	110
F9	coagulation factor IX	Mm01308427_m1	74
F10	coagulation factor X	Mm00484177_m1	81
F11	coagulation factor XI	Mm00511167_m1	115
F12	coagulation factor XII	Mm00491349_m1	64
F13b	coagulation factor XIII, beta subunit	Mm00491938_m1	62
Serpinc1	antithrombin	Mm00446573_m1	94
Prosc	protein C	Mm00435966_m1	52
Pros1	protein S (alpha)	Mm01343426_m1	62
Plg	plasminogen	Mm00447087_m1	83
VWF	von Willebrand factor	Mm00550376_m1	63
Adamts13	a disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 13	Mm01218030_g1	77
Lman1	lectin, mannose-binding, 1	Mm00522499_m1	79
Mcfd2	multiple coagulation factor deficiency 2	Mm00454818_m1	57
Lrp1	low density lipoprotein receptor-related protein 1	Mm00464608_m1	104

capture antibody against human VWF (Dako, Glostrup, Denmark), and goat anti-human VWF-HRP antibody (Dako) as a detecting antibody. Fibrinogen levels, FXIII levels, and tPA levels were measured by the Clauss method using bovine thrombin, Berichrom FXIII chromogenic assay (Dade Behring, Marburg, Germany), and Mouse tPA Activity Assay (Oxford Biomedical Research, Oxford, MI) according to the manufacturer's instructions, respectively. For all the assessments, pooled plasma collected from 50 normal C57BL/6 mice was used as standards.

Statistical analysis

Significant differences between two groups were analyzed by 2-tailed Mann-Whitney U-test with ystat2006 software (Igakutosyosyuppan, Tokyo, Japan). $P<0.05$ was considered significant.

Results

Experiment 1: Comparison of liver mRNA expression levels and plasma levels of coagulation factors

Liver samples were analyzed for mRNA expression levels by real-time RT-PCR in both quiescent (Day 0, n = 25) and actively proliferating livers following PHx at Day 1, 2, 5 after the procedure (n = 9, 8, and 8, respectively). First, we assessed the 10 coagulation factor genes; *Fgb*, *F2*, *F5*, *F7*, *F8*, *F9*, *F10*, *F11*, *F12*, and *F13b*, which are mainly produced in the liver. Compared with values at Day 0, mRNA expression levels of all the analyzed coagulation factors were significantly reduced ($P<0.05$) (Fig. 1). All of the reduced gene expression levels gradually recovered to control (Day 0) levels by Day 5 in all except for *F7* and *F8*. The *F7* and *F8* mRNA expression levels remained significantly lower ($P<0.05$) at Day 2 and 5 compared with those at Day 0 (Fig. 1).

To correlate whether the protein activity in the plasma for the coagulation factors were concomitantly suppressed similar to the gene expression, blood was collected from each mouse and examined for their level of activity. As shown in Fig. 2, plasma activities were found to have differential activity levels during liver regeneration compared to the quiescent state (Day 0). Most of the coagulation factors (prothrombin, FV, FVII, FIX, FX, and FXII) were significantly lower at Day 1 compared to their corresponding values at Day 0. Among all of the factors examined, FVII showed the greatest decline in activity, whereby only ~30% of the activity remained at Day 1 compared to Day 0. Consistent with the recovery in liver mRNA expressions, plasma activities of many factors returned towards the Day 0 levels by the end of the experimental period (Day 5). Plasma activities of fibrinogen, FXI, and FXIII, however, showed no significant changes during the liver regeneration process. For several factors (prothrombin, FV, FIX, and VWF), there were substantial variances in Day 0 values from those of the reference pooled plasma. We assume this deviation might attribute to an individual variability in the level of plasma coagulation factor levels of mice.

In sharp contrast, plasma FVIII activity was significantly increased at Day 1 ($166.1 \pm 20.2\%$; $P<0.05$) compared to the quiescent state (Day 0; $110.4 \pm 5.1\%$), even though the *F8* mRNA expression was paradoxically suppressed at Day 1. In addition, we detected a significant increase by 2.1-fold ($P<0.05$) in the plasma antigen levels of VWF compared to the quiescent Day 0 state. Both the FVIII and VWF tended to gradually decline back towards quiescent (Day 0) values until Day 5 (Fig. 2).

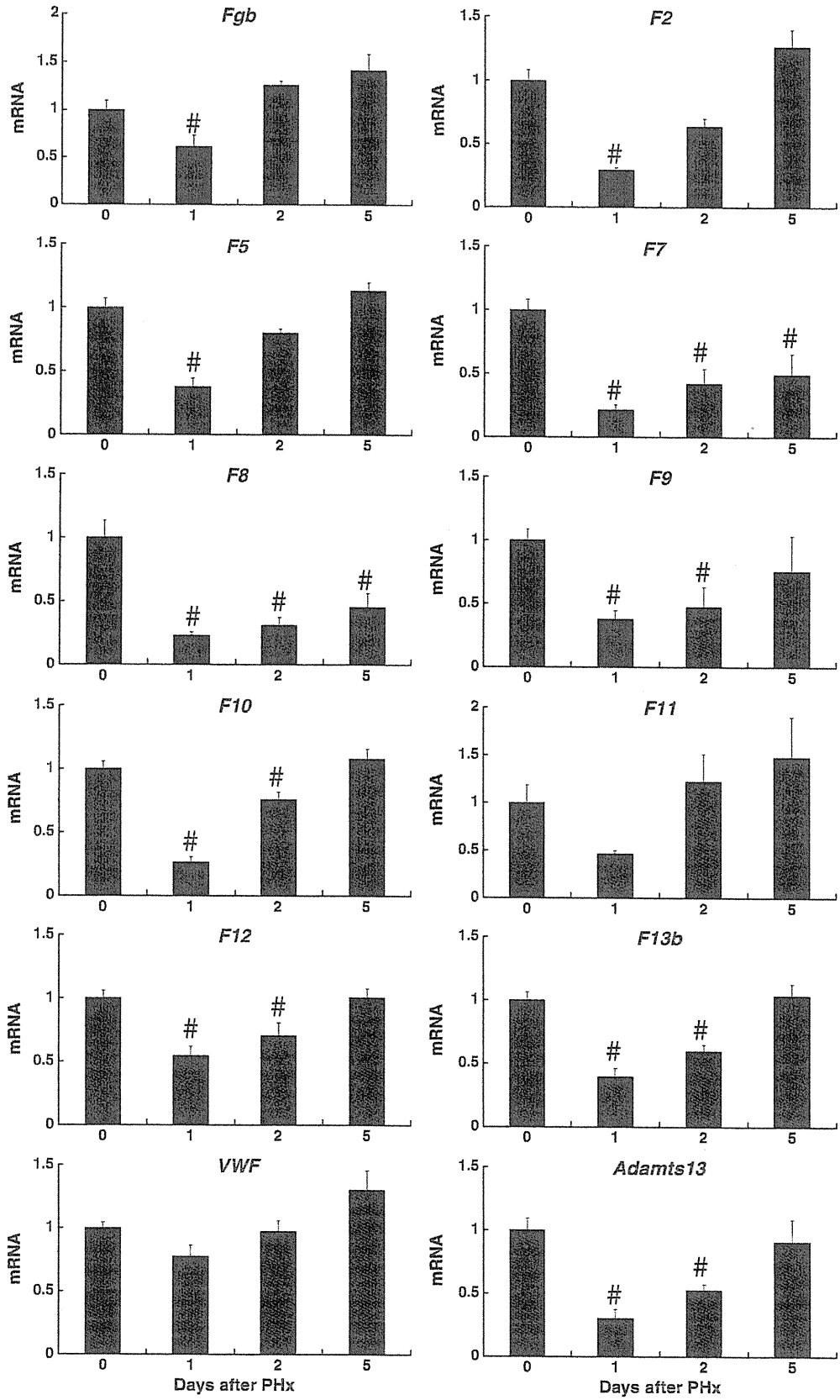
To determine whether the increased plasma FVIII activities and VWF antigen levels were associated with anesthesia and surgical stress, we performed sham-operation (i.e., abdominal incision and opening) under general anesthesia in the C57BL/6 mice (n = 10) and investigated the plasma FVIII activities and VWF antigen levels. Slight increases were observed after sham-operation in all individuals but reached no statistical significance (data not shown), which indicated that the increased FVIII and VWF in plasma were predominantly associated with liver resection and the subsequent liver regeneration process.

In addition to coagulation factors analyzed above, we also assessed the mRNA expression profiles of: i) fibrinolytic factor (*Plg*); ii) anti-coagulation factors (*Serpinc1*, *Prosc*, and *Pros1*); iii) other coagulation-related factors (*VWF* and *Adamts13*); and iv) factors related to FVIII production and degradation (*Lman1*, *Mcfd2*, and *Lrp1*). It has been documented that *Plg*, *Serpinc1*, *Prosc*, *Pros1*, and *Adamts13* are expressed mainly in the liver, and VWF is expressed by vascular endothelial cells throughout the body. *Lman1* and *Mcfd2* genes encode the 53-kDa ER-Golgi intermediate compartment protein, called ERGIC53, which plays important roles in the transfer of the primary translation products of *F5* and *F8* from the ER to the Golgi [21]. *Lrp1* is a hepatic cell-surface receptor protein which mediates the clearance of circulating FVIII [22]. As shown in Figs. 1 and 3, the mRNA levels of *Plg*, *Serpinc1*, *Prosc*, *Pros1*, and *Adamts13* were reduced at Day 1 and 2 after PHx, and the levels returned back towards the quiescent levels by Day 5. As for *Pros1*, the mRNA levels were significantly above those at Day 0 by Day 2 and 5. This reason is unclear, but it may due to the changes of some female hormones or transcription factors. The mRNA expression levels of *VWF*, *Mcfd2*, and *Lrp1* did not significantly change during the liver regeneration process, whereas *Lman1* mRNA levels were increased at Day 1, but returned to quiescent Day 0 levels by Day 2.

Experiment 2: Examination of F8 mRNA expression levels in mouse tissues during liver regeneration

In spite of the *F8* mRNA reduction in the liver as shown in Experiment 1, the plasma FVIII activity was paradoxically and significantly increased ($P<0.05$) during the liver regeneration phase. Because of this finding,

Fig. 1. Gene expression profiling of coagulation factors in quiescent and actively regenerating mouse livers. The gene expression profiles for 10 coagulation factors (*Fgb*, *F2*, *F5*, *F7*, *F8*, *F9*, *F10*, *F11*, *F12*, and *F13b*) and 2 hemostasis-related factors (*VWF* and *Adamts13*) in mouse livers under quiescent and regenerating status from Experiment 1 were assessed. The liver lobes removed at the time of hepatectomy were denoted as quiescent liver samples (Day 0, n = 25). The remainder of the actively proliferating liver lobes were harvested at Day 1, 2, or 5 after hepatectomy (n = 9, 8, and 8, respectively). The data were normalized to the geometric mean of *Ppia* and *Tbp* mRNA levels, and expressed as a comparative ratio to the Day 0 samples. All values were represented as mean ± SEM. $^{\#}P<0.05$ vs Day 0.


Fgb
F2
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VWF
Adamts13
mRNA
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0
1
2
5
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we speculated that other extra-hepatic organs may be involved in producing FVIII to compensate for the loss of the liver production of this specific coagulation factor after the PHx. We assessed the *F8* mRNA expression levels from the following organs taken from mice with (n = 8) or without PHx (n = 8): liver, lung, spleen, heart, intestine, brain, and kidney. Prior to analyzing *F8* mRNA expression in the liver and extra-hepatic organs, we determined appropriate housekeeping reference genes that would have minimal variance between the examined organs under quiescent status or liver regeneration status using two different programs. As a result, it was elucidated that applying combination of *Hprt1*, *Rpl4*, and *Tbp*, provide the least fluctuation in the reference gene expression. Therefore, we decided to normalize the FVIII mRNA expression by calculating the geometric mean of these three genes. Using this approach, we found that *F8* mRNA levels in the liver were significantly suppressed ($P<0.05$) at Day 1 after PHx (Fig. 4), which was consistent with the results obtained in Experiment 1 (Fig. 1). However, the *F8* mRNA expression levels in all of the organs other than the liver showed no significant difference between PH group and non-PHx groups (Fig. 4). These results indicated that FVIII transcription *per se* from extra-hepatic organs were not affected by stimuli associated with liver regeneration, and that the elevated circulating plasma FVIII activity during the liver regeneration process was unlikely due to increased gene transcription of this protein.

Discussion

The present study demonstrated liver gene expression profiles of coagulation and anti-coagulation / fibrinolytic factors as well as plasma levels of coagulation factors during the active proliferation of the livers in mice. At the peak of the liver regenerative phase (i.e. Day 1 and 2), the expression levels for nearly all of the genes analyzed were temporally reduced and recovered back towards the normal levels as the regeneration event comes to the completion (i.e. Day 5). Consistent with the gene expression levels, plasma activities for all of the coagulation factors, except for FVIII, were temporally decreased during the same liver regenerative period.

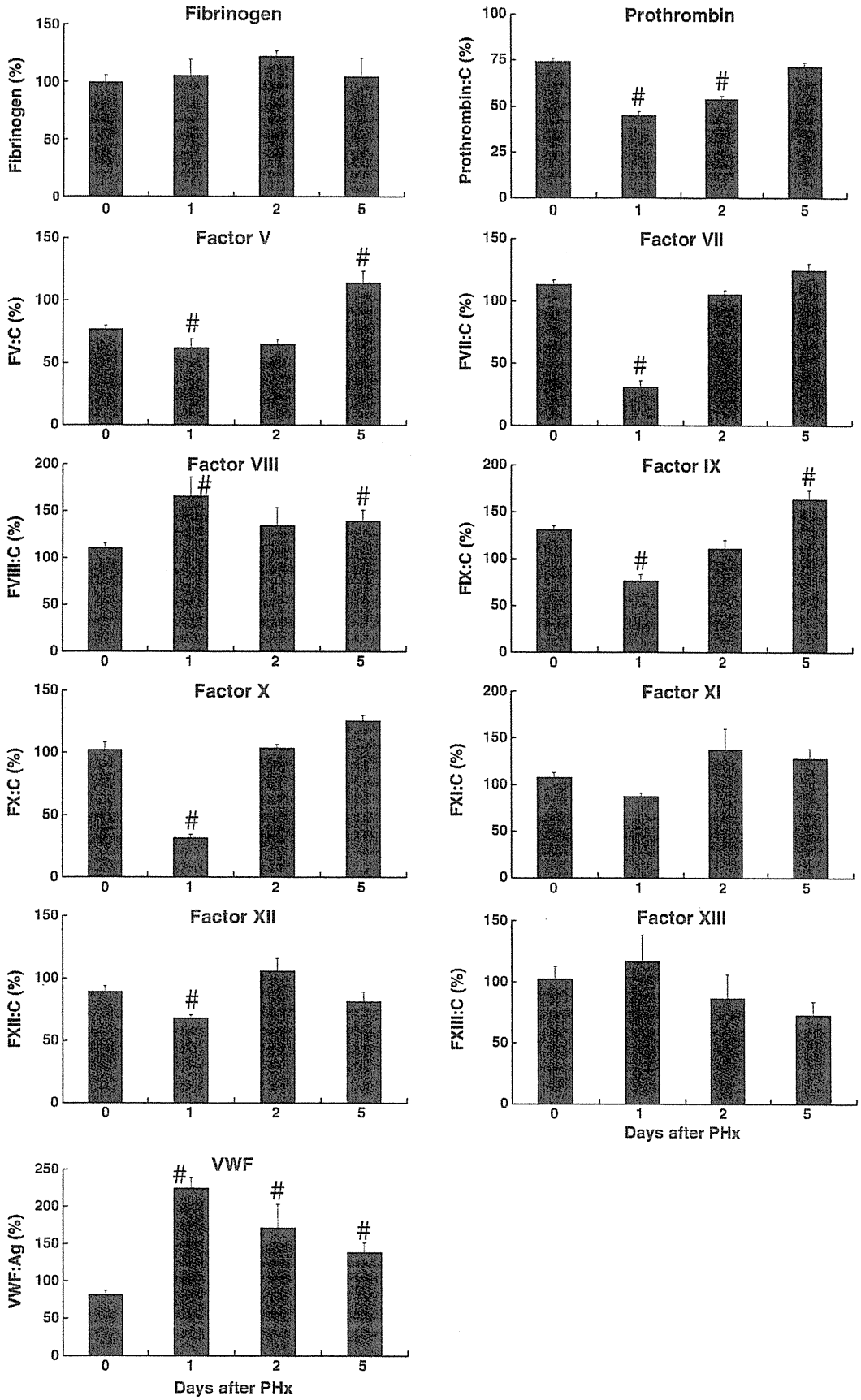
Using real-time RT-PCR, we determined that gene expression levels for the coagulation factors (*Fgb*, *F2*, *F5*, *F7*, *F8*, *F9*, *F10*, *F11*, F12, and *F13b*), a fibrinolytic factor (*Plg*), and anti-coagulation factors (*Serpinc1*, *Prosc*, and *Pros1*) decreased at the peak of the liver regenerative state (Day 1 and 2) following PHx, and this was followed by a rebound increase back towards to pre-hepatectomy levels at Day 5. The results are consistent with previous studies in which gene expression levels of many liver-specific proteins are temporally suppressed during the process of liver regeneration [3,23]. The time line for the changes in gene expression for the coagulation factors appears to have an inverse relationship to the DNA synthetic phase. It is known that the first 24 h period following PHx, hepatocyte DNA synthesis is initiated and begins to synchronize and peak by 48 h. Subsequently, the DNA synthesis begins to slow and returns towards the quiescent levels at Day 5 [1,2]. Our findings would suggests that the regenerating liver sacrifices the production of liver-specific proteins, which are predominantly represented as plasma proteins, to promote the cell division and growth [24]. This speculation is strengthened by our previous report in which the liver gene expressions of coagulation-related factors were significantly reduced during the progression of direct hyperplasia-mediated liver regeneration in mouse model [25].

Interestingly, the corresponding plasma activities for FVIII and other coagulation proteins, fibrinogen and FXIII, were paradoxically increased or not decreased during the same stages of liver regeneration after PHx, even though all of the mRNA expression profiles and all other coagulation factor activities were decreased. As for fibrinogen and FXIII, the half-life time of both factors are relatively long (4 to 7 days and 9 to 14 days, respectively) among all coagulation factors. This might be the reason why these two factors showed no significant decrease in plasma activities while the gene expression levels in the liver were temporally suppressed. Plasma FXIII is composed of two types of subunits, α and β, each of which is produced in different tissues (bone marrow and liver, respectively). The mRNA levels assessed in this study were FXIII β (*F13b*) and plasma activity measured mainly reflects the FXIII α function. This difference may also account for the sustained FXIII activity in plasma. In contrast, the half-life time of FVII is the shortest (1.5 - 5 h) among all coagulation factors, and this could have been associated with the drastic drop of the plasma FVII activity observed at Day 1 after PHx (27.5 % of non-PHx levels). The reason why plasma FVII levels recovered by Day 5 in spite of suppressed mRNA levels at the time point is unclear, but it is reasonable to postulate that it might represent the trauma-induced FVII activation and overestimation by the presence of FVIIa. On the other hand, the mechanism for the increased activity levels for FVIII remains to be well understood. As possible mechanisms for this paradoxical FVIII activity, we postulated the following reasons; (i) increased extrahepatic FVIII production, (ii) delayed inactivation or clearance of circulating FVIII in plasma, (iii) excessive releases of pooled FVIII from storage sites, and (iv) an alteration of intracellular trafficking pathway of FVIII.

First, the liver has been shown to be the major site of FVIII production. A previous study transplanted a normal functioning liver into a hemophilia A patient to completely correct the bleeding diathesis [26]. There is also documentation that FVIII production may be assisted by extra-hepatic tissues, such as the lung, kidney, and/or spleen [27,28]. Consistent with these previous reports, we detected *F8* mRNA in various organs, but detected no PHx-associated changes for all of the extra-hepatic organs studied. Hollestelle et al. observed the sustained plasma FVIII levels in anhepatic pig models, and they also detected no increase of *F8* mRNA in spleen, kidney, and lung [29]. Although their data as well as our data cannot totally exclude the possibility of FVIII production from organs not analyzed in the studies, it is unlikely that the induction of extrahepatic FVIII production is involved in mediating the increased FVIII activity during liver regeneration.

Second, several factors are known to be involved in delaying the loss of plasma FVIII activity. VWF acts as FVIII carrier protein and protects FVIII from proteolysis in the circulation. Increased plasma VWF may lead to enhanced binding and protection of FVIII during the liver regenerative phase and promote increased activity (Fig. 2). Mechanistically, the elevated plasma VWF levels may involve the tight regulation by ADAMTS13. In line with the recent clinical report [30], we have observed that regenerating liver temporally suppressed ADAMTS13 expression (Fig. 1), and so the reduction of ADAMTS13 may be involved in mediating the elevated plasma VWF. Alternatively, the clearance of circulating FVIII is normally mediated by low-density lipoprotein receptor-related protein 1 (*Lrp1*), a hepatic clearance receptor protein [22]. Suppression of the interaction between FVIII and Lrp1 could thus theoretically prolong the plasma FVIII half-life time. The present study showed that the *Lrp1* mRNA expression levels were constant regardless of the regeneration status, suggesting that total Lrp1 production levels from the liver had been dropped during the liver regeneration phase because of PHx-induced liver mass reduction. Taken together, the observed plasma FVIII increase could be attributed to the enhanced protection from proteolysis by higher

Fig. 2. Changes in plasma activity of circulating coagulation factors during liver regeneration. Plasma activity levels of 10 coagulation factors (fibrinogen, prothrombin, factor V, VII, VIII, IX, X, XI, XII, and XIII) and plasma VWF antigen levels were determined from mice that either had quiescent livers or were undergoing active liver proliferation from Experiment 1. Levels of prothrombin, factor V, VII, VIII, IX, X, XI, and XII were measured by one-stage clotting assay, fibrinogen levels were measured by the Clauss method, factor XIII levels were measured by chromogenic assay, and VWF antigen levels were determined by specific ELISA. The plasma samples obtained before hepatectomy were used as Day 0 samples to determine plasma activities during liver quiescence (n = 25), and the plasma samples obtained 1, 2, or 5 days after hepatectomy were used as samples to assess active proliferation at various states of liver regeneration (n = 9, 8, and 8, respectively). The data were described as percentage of pooled normal plasma, and represented as the mean ± SEM. $^{\#}P<0.05$ vs day 0.


Fibrinogen
Fibrinogen (%)
Prothrombin
Prothrombin:C (%)
Factor V
FV:C (%)
Factor VII
FVII:C (%)
Factor VIII
FVIII:C (%)
Factor IX
FIX:C (%)
Factor X
FX:C (%)
Factor XI
FXI:C (%)
Factor XII
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Factor XIII
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VWF
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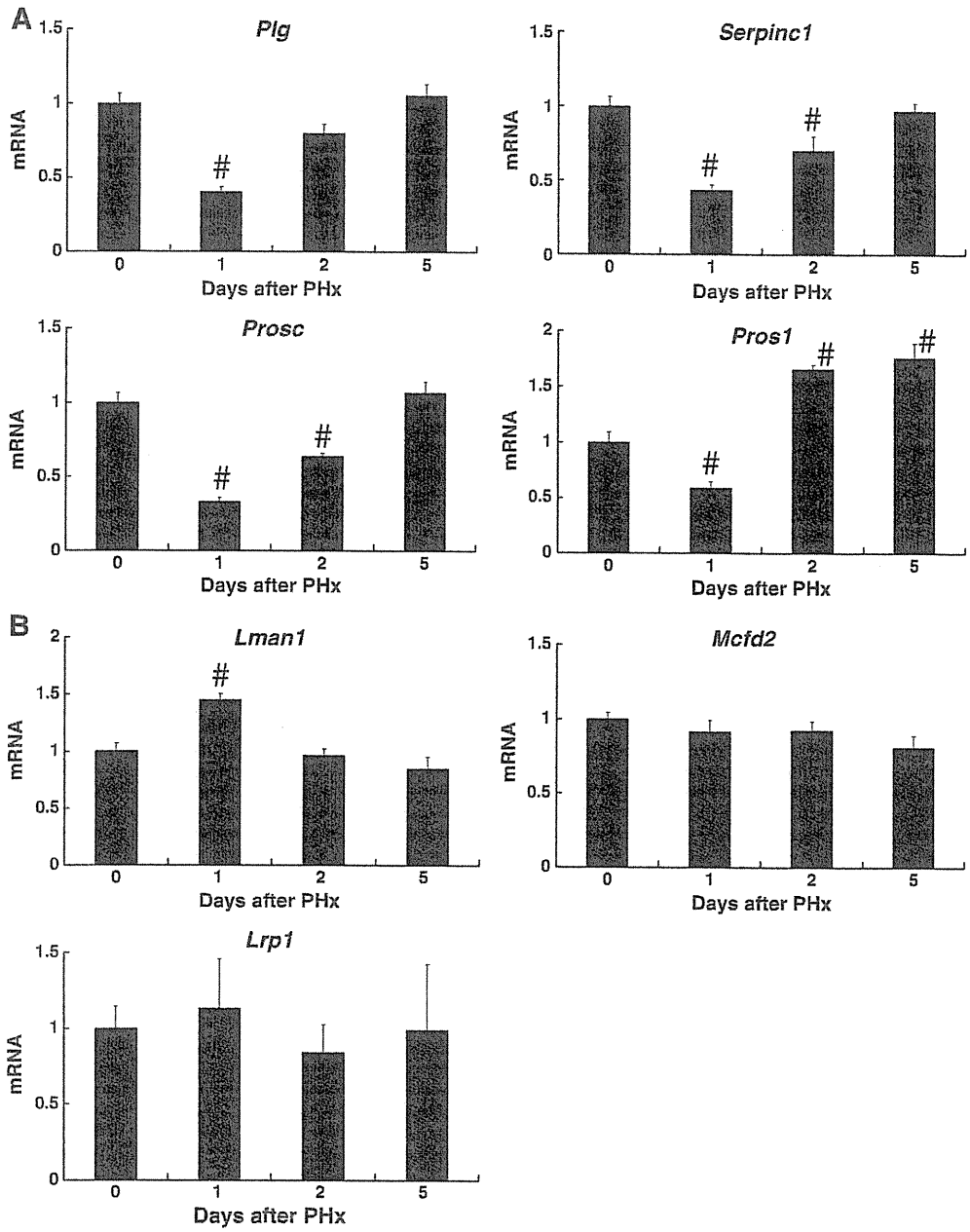

Fig. 3. The gene expression profiling of fibrinolytic and anti-coagulation factors (A), and several important factors related to factor VIII production and degradation (B) in quiescent and actively regenerating mouse livers. The gene expression levels of fibrinolytic factor (*Plg*), anti-coagulation factors (*Serpinc1*, *Prosc*, and *Pros1*), and several important factors relating to factor VIII production and degradation (*Lman1*, *Mcfd2*, and *Lrp1*) were examined from quiescent and actively regenerating mouse livers as described in Experiment 1 by quantitative RT-PCR. The same liver samples used in Fig. 1 were used for this analysis. The data were normalized to the geometric mean of *Ppia* and *Tbp* mRNA levels, and expressed as a comparative ratio to the Day 0 samples. #$P<0.05$ between groups.

levels of VWF and/or the reduced protein clearance by Lrp1. These speculations were supported by the previous findings by Hollestelle et al., in which the prolonged half-life time of administered FVIII was associated with the increased plasma levels of VWF in anhepatic pig models [29]. However, it is less likely that only the delayed inactivation or clearance is the major contributing factor for the observed plasma FVIII increase by more than 150% under the condition of markedly suppressed FVIII production in the liver (70% down-sizing and additional 70% suppress of gene expression in the remnant liver).

Thus, we investigated further two mechanisms responsible for the increased plasma FVIII. One possible mechanism is the excessive release of FVIII from the storage sites. Although the precise site(s) of FVIII production remains controversial [6,7,31], it has been assumed that synthesized FVIII is trafficked with VWF and partially becomes pooled into granules found within endothelial cells known as Weibel-Palade bodies [32], and the pooled contents of the granules are released by various stimuli [33]. The Weibel-Palade bodies also contain several other substances including IL-8, tPA and P-selectin, and both tPA and VWF are coordinately released in response to an agonist stimulation [33]. In this present study, the plasma levels of tPA activity were significantly increased in a similar manner of VWF and FVIII at Day 1 compared to the quiescent Day 0 period (191.2 ± 9.8 vs 100 ± 13.6 %). Another possible mechanism is the change of intracellular processing in the FVIII producing cells. The efficient FVIII secretion into the bloodstream requires the intracellular trafficking of the FVIII protein, including *Lman1* and *Mcfd2* genes, which encode proteins that forms a

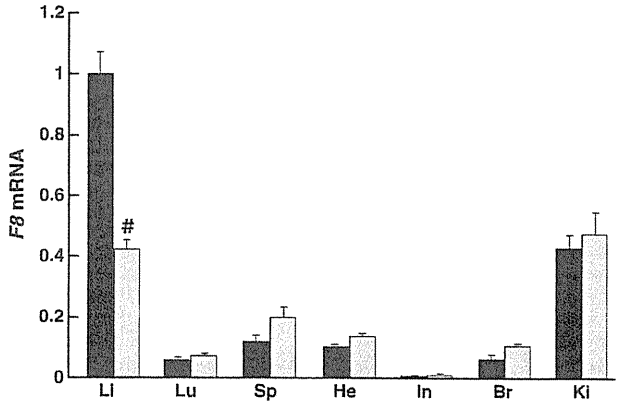

Fig. 4. Extra-hepatic gene expression of *F8* mRNA following hepatectomy in mice. Liver (Li), lung (Lu), spleen (Sp), heart (He), intestine (In), brain (Br), and kidney (Ki) were obtained from mice with hepatectomy (PHx group) or without hepatectomy (non-PHx group) at 24 h time point (n = 8 in each group) in Experiment 2. *F8* mRNA expression levels were normalized to the geometric mean of three housekeeping genes (*Hprt1*, *Rpl4*, and *Tbp*), which were validated as the most stable reference genes in this experimental condition by geNorm and NormFinder. Normalized data were expressed as a comparative ratio to the non-PHx liver samples. Black bars = non-PHx mouse group; gray bars = PHx-group mice. $^{\#}P<0.05$ vs non-PHx group.

complex that mediates FVIII transport from the endoplasmic reticulum to the Golgi apparatus [21]. During the liver regeneration phase, the present data showed that *Lman1* mRNA expression was significantly increased, and we speculate that the increase of *Lman1* expression might result in elevated levels of FVIII activity.

In conclusion, the current study showed that the process of liver regeneration involves a general reduction of gene expression for many of the coagulation cascade proteins, but there are paradoxical increases in plasma levels of FVIII and VWF. Detailed investigation revealed that this increase of plasma FVIII activity may be associated with a delay in the inactivation or clearance coupled to the release from the storage sites in endothelial cells and the alteration of *Lman1* associated intracellular trafficking pathway during liver regeneration. This finding for the increased activity of circulating FVIII may suggest that this particular coagulation factor may play an important role in maintaining the blood coagulation balance during liver regeneration, and possibly exacerbate the potential for a thrombotic episode. In addition, our observation that gene expressions of coagulation-related proteins are generally reduced in dividing cells gives an important insight to the field of hepatocyte-directed stem cell research, especially for coagulation factor production.

Conflict of interest statement

All authors have no conflict of interest to declare.

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ORIGINAL ARTICLE *Inhibitors*

An analysis of factors affecting the incidence of inhibitor formation in patients with congenital haemophilia in Japan

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Summary. Studies conducted in European and North American countries have demonstrated that various factors including races affect the frequency of inhibitor formation in haemophilia patients. The present study was undertaken to analyse factors affecting the incidence of inhibitor formation in Japanese haemophilia A and B patients. Analytical data were retrospectively collected from haemophilia A and B patients born after 1988, the year when monoclonal antibody-purified factor VIII products were first marketed in Japan. Various data were collected from 184 patients (153 cases of haemophilia A; 31 cases of haemophilia B). The sample size of haemophilia B cases was too small to reveal any significant differences between the inhibitor formation group and the inhibitor-free group in any of background variables. For patients with haemophilia A, on the other hand, univariate analysis identified the severity of haemophilia and a positive family history of inhibitor development as risk factors for the formation of inhibitors. In analyses of the clotting factor products used, the incidence of inhibitor formation did not differ significantly between the group treated with plasma-derived products (29.7%) and the group treated with recombinant products (25.0%). When background variables were compared, age was higher in the group treated with plasma-derived products but none of the other background variables differed between the two groups. These results suggest that in Japanese haemophilia patients, the type of clotting factor preparations used for therapy has not influenced the incidence of inhibitor formation.

Keywords: coagulation products, haemophilia, incidence, inhibitor, risk factor

Introduction

Haemostatic treatment for patients with haemophilia has advanced considerably in the past two decades. Safe clotting factor concentrates with high haemostatic activity have become available, and increasing clinical evidence has been accumulated to confirm that regular prophylactic infusions of these products help to prevent the onset and progression of haemophilic arthropathy arising from repeated intra-articular bleeding. It is also evident, however, that the infusion of clotting factor products induce the formation of allo-antibodies (inhibitors), which inactivate factor VIII (or factor IX) in 20–30% of patients with severe haemophilia A and 3–5% of patients with severe haemophilia B. Conventional treatment protocols involving infusion of deficient clotting factors in these patients with inhibitors are poorly effective, and haemostatic control is often difficult in these circumstances, especially in patients with high-responding inhibitors. Moreover, quality of life (QOL) indices are reduced markedly in these patients [1].

Studies conducted in European and North American countries have demonstrated that various genetic factors affect the frequency of inhibitor formation. The most detailed analyses have examined the type and location of

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factor VIII gene mutations, and have revealed that the incidence of inhibitor formation was highest in haemophilia A patients with large deletions, nonsense mutations or inversions [2–9]. A higher incidence of inhibitor formation is known for Black patients [10], and a recent report suggested that differences in factor VIII haplotype contributed to this feature [11]. It is also known that within the same family some patients with haemophilia develop inhibitors whilst others do not, possibly reflecting the polymorphism of genes encoding immunomodulating cytokines [12–14] or indicating the influence of acquired factors [15–20]. With regard to the type of product used for therapy, close attention has been paid to the relationship between the incidence of inhibitor formation and the use of recombinant products or plasma-derived products [21–28].

The present study was undertaken to assess the incidence of inhibitors in patients born after 1988, the year when monoclonal antibody (mAb)-purified factor VIII products were first marketed in Japan. The analyses was restricted to patients that had been treated with clotting factor products for at least 2-years, with the goal of identifying background variables and focusing on whether or not the type of clotting factor products affected the incidence of inhibitor formation.

Methods

Selection of treatment centres included in the survey

Medical centres experienced in the treatment of haemophilia and with comprehensive records enabling a detailed investigation of the history of treatment in individual patients were invited to participate in this study.

Inclusion criteria

Patients with congenital haemophilia A or B satisfying the all following criteria were eligible to study:

1. Born between 1 January 1988 and 31 December 2006.
2. Data on the history of treatment with factor VIII (IX) products available for at least 2 years after the first infusion of clotting factor products.
3. Data on inhibitor assays available for at least 2 years after the first infusion.
4. Informed consent available in writing from the guardian (and also the patient aged 16 years and older).

Investigations

1. Background variables: name initials, gender, birth date, time (year and month) of diagnosis, factor VIII (IX) activity and blood group.
2. Complications: presence/absence of haemophilic arthropathy, hepatitis B, hepatitis C, HIV infection, haemorrhagic disease other than haemophilia and any other severe disease.
3. Neonatal history: manner of delivery and feeding method.
4. Family history: presence/absence of family history of haemophilia with or without inhibitor.
5. Treatment methods: age at the time of first infusion, and administration method of clotting factor products (on demand or regular prophylaxis).
6. Clotting factor products: the name of the clotting factor products and the number of days used during the 2-year period after the first infusion or before detection of inhibitor in patients showing inhibitor formation.
7. Severe bleeding episodes and invasive surgery: presence/absence and time of episode/surgery and the site of bleeding during the 2-year period after the first infusion.
8. Presence/absence of inhibitors: in patients showing inhibitor formation, the inhibitor level on detection, the date of measurement, peak inhibitor level and latest inhibitor level [presence/absence of immune tolerance induction (ITI) therapy if inhibitor had disappeared].

Survey period

The survey was conducted between 1 January 2008 and 31 December 2009.

Statistical analysis

The *t*-test and analysis of variance were used on numerical variables and chi-squared test was employed for nominal variables. In addition, multivariate analysis was conducted by means of logistic regression.

Ethical considerations

The study was conducted in accordance with the Japanese Ethical Guidelines on Epidemiological Studies after approval by the Nara Medical University Ethics Committee. In addition where necessary, the approval of the ethics committee of individual participating medical centres was also obtained.

Results

Analytical data were collected from 184 patients (153 cases of haemophilia A; 31 cases of haemophilia B). Of the 153 patients with haemophilia A, 41 (26.8%) developed inhibitors. In 29 of these 41 patients (70.7%), including four patients who had not received ITI, inhibitors had disappeared by the time of last evaluation. On the other hand inhibitors were persistent

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in 12 patients including 10 patients who had received ITI.

In univariate analyses, the background variables found to differ significantly between the inhibitor formation group and the inhibitor-free group were: the severity of haemophilia, family history of haemophilia patients with inhibitor (Table 1), age at the time of the first infusion of clotting factor products (Fig. 1) and factor VIII activity (Fig. 2). No other background variable differed significantly between the two groups (Table 1), including blood group and presence/absence of hepatitis A, hepatitis B and HIV infection. Logistic regression analyses of these variables demonstrated that only the family history of haemophilia patients with inhibitor had a significant influence on inhibitor formation ($P = 0.002$).

One hundred and fifty patients, excluding three haemophilia A patients who had received treatment with factor IX products, were classified into three groups according to the type of clotting factor product used during the 2-year period after the first infusion, or before the detection of inhibitor: (i) patients treated with plasma-derived products alone ($n = 37$), (ii) patients treated with recombinant products alone ($n = 104$), and (iii) patients treated with both plasma-derived products and recombinant products ($n = 9$). The incidence of inhibitor formation did not differ significantly among these three groups (29.7%, 25.0% and 22.2% respectively). Of the 46 patients who received plasma-derived products, nine received only factor VIII products containing von Willebrand factor. Among these nine patients, inhibitor formation was seen in four patients, but the incidence for this group did not differ significantly from that for the other groups. We also compared the peak inhibitor level, the status of inhibitor formation at the time of last observation and the response rate to ITI between the group treated with plasma-derived products alone and the group treated with recombinant products alone. These comparisons revealed no inter-group differences (Table 2). Furthermore, other background variables were compared in these two groups i.e. age, severity of haemophilia, family history of inhibitor, treatment method (on demand or regular prophylaxis) during the 2-year period after the first infusion, the presence/absence of intracranial haemorrhage, other severe bleeding episodes and invasive surgery. The average age of the patients at the end of the survey (31 December 2009) was significantly higher in the group treated with plasma-derived products alone than in the group treated with recombinant products alone, but no other background variable differed significantly between the two groups (Table 3).

Among the 31 patients with haemophilia B, six (19.4%) developed inhibitors. As in the haemophilia A patients, background variables in those haemophilia B patients who developed inhibitors were compared with those that remained inhibitor-free. Univariate analyses indicated that there were no significant inter-group differences in any of the background variables, possibly attributable to some extent to the small sample size.

Table 1. Background variables compared between inhibitor formation cases and inhibitor-free cases with haemophilia A.

Background variable (number of patients)	Inhibitor-free (%)	Inhibitor positive (%)	*P*-value
Severity ($n = 153$)			
Mild	17/112 (15.2)	0/41 (0)	0.0243
Moderate	13/112 (11.6)	4/41 (9.8)	
Severe	82/112 (73.2)	37/41 (90.2)	
Haemophilic arthropathy ($n = 153$)			
Present	25/112 (22.3)	14/41 (34.1)	0.2044
Family history of haemophilia ($n = 148$)			
Positive	46/108 (42.6)	21/40 (52.5)	0.3736
Family history of inhibitor formation ($n = 122$)			
Positive	3/91 (3.3)	9/31 (29.0)	0.0001
Treatment after first infusion ($n = 151$)*			
On demand	78/111 (70.3)	30/40 (75.0)	0.7255
Regular prophylaxis	33/111 (29.7)	10/40 (25.0)	
History of intracranial haemorrhage ($n = 149$)*			
Positive	11/108 (10.2)	8/41 (19.5)	0.2160
History of other severe bleeding ($n = 147$)*			
Positive	8/106 (7.5)	1/41 (2.4)	0.4384
History of invasive surgery ($n = 148$)*			
Positive	9/107 (8.4)	1/41 (2.4)	0.3577
Manner of child delivery ($n = 109$)			
Transvaginal	67/79 (84.8)	27/30 (90.0)	0.7198
Caesarean section	12/79 (15.2)	3/30 (10.0)	
Feeding method ($n = 94$)			
Breast-feeding	26/66 (39.4)	15/28 (53.6)	0.4470
Bottle-feeding	15/66 (22.7)	5/28 (17.9)	
Mixed	25/66 (37.9)	8/28 (28.6)	

*Before detection of inhibitor in inhibitor formation cases and for a 2-year period after the first infusion in inhibitor-free cases.

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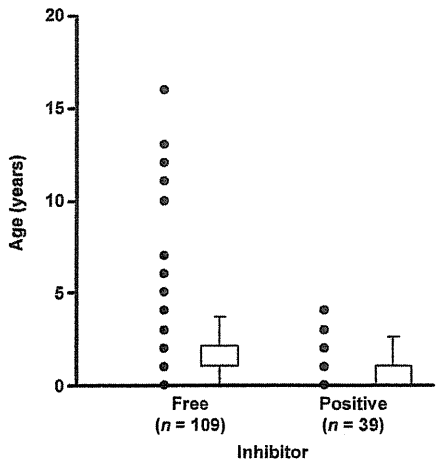

Fig. 1. Age at the time of first administration of clotting factor product (haemophilia A). Age at the time of the first infusion of clotting factor VIII products was significantly earlier in the inhibitor formation group than in the inhibitor-free group ($P < 0.001$).

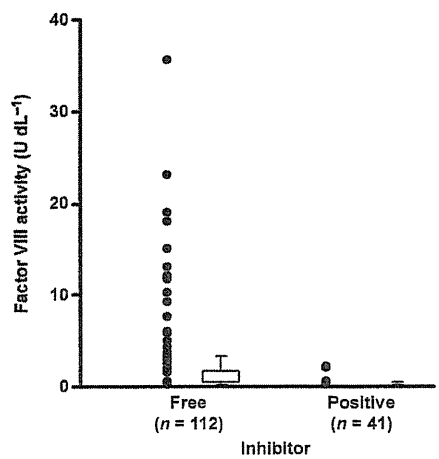

Fig. 2. Factor VIII activity. Factor VIII activity was significantly higher in the inhibitor-free group than in the inhibitor formation group ($P < 0.001$).

Discussion

Of the possible factors affecting the incidence of inhibitor formation in haemophilia patients, the type of factor VIII and IX products used for therapy has been attracting the closest attention. In particular, a retrospective study in France suggested that the incidence of inhibitor formation was lower in haemophilia A patients treated with single plasma-derived products than in patients treated with recombinant products [23]. Subsequently, however, the CANAL study (Concerted Action on Neutralizing Antibodies in severe haemophilia A) indicated that the incidence of inhibitor formation did not differ between patients treated with plasma-derived products (including products containing von Willebrand factor) and patients treated with recombinant products [26], and this result was consistent with a report published at about the same time in the United Kingdom [28]. All of these findings were derived from studies conducted in European and North American countries, primarily involving caucasians. It is known, however, that the formation of inhibitors following treatment with clotting factor products varies between different ethnic populations [10]. The present investigation was conducted, therefore, in Japanese haemophilia patients. The study was retrospective and

Table 2. Status of inhibitor formation in relation to the type of clotting factor products used during the 2-year period after the first infusion in inhibitor-free cases, or before detection of inhibitor in inhibitor formation cases.

Status of inhibitor formation	Plasma-derived products alone n = 37 (%)	Recombinant products alone n = 104 (%)	P-value
Number of inhibitor formation cases	11 (29.7)	26 (25.0)	0.7308
Peak inhibitor level			
≤5 BU mL^{-1}	2 (18.2)	7 (26.9)	0.8829
>5 BU mL^{-1}	9 (81.8)	19 (73.1)	
Status at the time of survey			
Present	4 (36.4)	7 (26.9)	0.8565
Disappeared	7 (63.6)	19 (73.1)	
Outcome of ITI			
Successful	6 (66.7)	17 (73.9)	0.9194
Ongoing	2 (22.2)	4 (23.5)	
Failure	1 (11.1)	2 (8.6)	
Cases not having received ITI among inhibitor disappearing cases	1 (14.3)	2 (10.5)	–

Table 3. Background variables in relation to the type of clotting factor products used during the 2-year period after the first infusion in inhibitor-free cases, or before detection of inhibitor in inhibitor formation cases.

Background variable	Plasma-derived products alone n = 37 (%)	Recombinant products alone n = 104 (%)	P-value
Age (years)*	14.0 ± 5.6	9.7 ± 4.3	0.0491
Severity			
Mild	4 (10.8)	12 (11.5)	0.3342
Moderate	2 (5.4)	15 (14.4)	
Severe	31 (83.8)	77 (74.0)	
Family history of haemophilia†			
Positive	20 (55.6)	42 (42.0)	0.2281
Negative	16 (44.4)	58 (58.0)	
Family history of inhibitor formation†			
Positive	1 (4.2)	8 (9.1)	0.7166
Negative	23 (95.8)	80 (90.9)	
Treatment during the 2-year period after first infusion†			
On demand	29 (78.4)	71 (68.9)	0.3795
Regular prophylaxis	8 (21.6)	32 (31.1)	
Intracranial haemorrhage†			
Present	7 (19.4)	11 (10.9)	0.3091
Absent	29 (80.6)	90 (89.1)	
Other severe bleeding†			
Present	1 (2.9)	6 (6.0)	0.7804
Absent	34 (97.1)	94 (94.0)	
Invasive surgery†			
Present	1 (2.8)	8 (8.0)	0.4903
Absent	35 (97.2)	92 (92.0)	

*Age as of 31 December 2009.
†Excluding cases where data are unavailable.

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was designed to include only those patients in whom inhibitor status could be checked for at least 2 years after the first infusion of clotting factor product. In spite of this limitation, the results on our relatively large number of patients born after 1988 (the year when mAb-purified factor VIII products were first marketed in Japan) were consistent with those of the CANAL studies. No differences in the incidence of inhibitor formation were revealed between the Japanese patients treated with plasma-derived products alone and those treated with recombinant products alone. Genetic analyses of haemophilia patients are available at some specialized facilities in Japan, but data of this nature was not comprehensively available for the present study and was not included. The incidence of inhibitor formation appeared to be very high, however, in patients from pedigrees where different family members were inhibitor positive compared to pedigrees where there was no family history of an inhibitor. This finding might have reflected an influence of gene mutation.

In the CANAL study, intensive treatment with clotting factor products at an early age appeared to be closely associated with the incidence of inhibitor formation [20], and we attempted to investigate the influence of high-dose therapy on inhibitor formation by analysing the incidence of inhibitors in patients treated for intracranial haemorrhage, other severe bleeding episodes and invasive surgery within the 2-year period prior to the detection of inhibitor. In these analyses, previous treatment for intracranial haemorrhage had been recorded approximately twice as often in the inhibitor group than in the inhibitor-free cases, although the differences were not statistically significant. In addition, the incidence of inhibitor formation did not differ between the on-demand-treated group and the group receiving regular prophylactic administration of clotting factors.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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LETTERS TO THE EDITORS

Factor VIII haplotypes of Japanese population show similarity to those of Caucasian populations

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Haemophilia A is the most common inherited bleeding disorder which is X-linked recessive. This disease is caused by a quantitative or qualitative abnormality of plasma factor VIII (FVIII), which is affected by a genetic mutation located in the coagulation FVIII gene. Diagnosis and replacement therapy strategies in haemophilia A patients are well established; however, some issues remain to be addressed.

The most important issue concerning replacement therapy in haemophilia A patients is the development of inhibitors (alloantibody) against FVIII. This development leads to marked attenuation in the effectiveness of replacement therapy, leading to a substantial deterioration in the quality of life of patients. In general, the incidence of inhibitor development in treatment-naive patients with haemophilia A is estimated to be 20–30% [1]. However, it has been shown that its incidence in the Black population is markedly higher, about twice as high as in other racial groups [2]. The question of why this inhibitor develops with such high incidence in the Black population remains undetermined.

Recently, Viel *et al.* reported that six wild-type FVIII proteins, the H1–H6 haplotypes, had different prevalence rates among racial groups [3]. They speculated that a mismatch of the FVIII haplotype between the FVIII concentrate and its recipients, particularly in immunodominant epitopes, caused an increase in the frequency of inhibitor development. In particular, they focused on four different amino acid polymorphisms (R484H, R776G, D1241E and M2238V), based on a non-synonymous single nucleotide polymorphism. By using a combination of those amino acids, they classified the FVIII protein into six haplotypes, namely, H1: RRDM, H2: RREM, H3: RREV, H4: HREM, H5: RRDV and H6: RGEM. It is presumed that amino acid positions 484 and 2238, located in the A2 and C2 domains, respectively, are components of the immunodominant epitope of FVIII. In Caucasian participants, positions 484 and 2238 have been observed as 'R' and 'M', respectively, and have only shown the haplotypes of H1 and H2. However, Viel *et al.* confirmed the presence of 'H' in place of 'R' at position 484, or 'V' in place of 'M' at position 2238, in approximately 25% of Black participants. Thus, the haplotype frequencies in Black participants (H1: 0.354; H2: 0.374; H3: 0.222; H4: 0.040 and H5: 0.010) are different from those in Caucasian participants. Furthermore, they also analysed haplotypes in ethnic Chinese participants, and observed the H6 haplotype, but not the H3, H4 or H5 haplotype.

We set out to analyse the haplotypes of FVIII proteins among 106 unrelated Japanese subjects at our institution (63 with haemophilia A and 43 with haemophilia B as a control group), as shown in Table 1. The study was approved by the Ethics Committee of Tokyo Medical University and written informed consent was obtained from each patient. The studies were carried out in accordance with the principles of the Declaration of Helsinki. Among the Japanese participants, only the H1 and H2 haplotypes (H1: 0.896 and H2: 0.104) were found at frequencies not significantly different from the Caucasian frequencies (H1: 0.926 and H2: 0.074). Despite the Chinese being geographical neighbours of the Japanese, the H6 haplotype which was observed in approximately 8% of Chinese was not detected in any Japanese.

Table 1. Factor VIII haplotypes in Japanese participants.

Haplotype	Hemophilia A (frequency)	Hemophilia B (frequency)	Total (frequency)
H1	57 (0.905)	38 (0.884)	95 (0.896)
H2	6 (0.095)	5 (0.116)	11 (0.104)
H3–H6	0 (0.000)	0 (0.000)	0 (0.000)

Currently in Japan, approximately 80% of haemophilia A patients receive replacement therapy using the recombinant FVIII concentrates Kogenate (Bayer) or Advate (Baxter). Data regarding the incidence of inhibitor development in Japanese patients with severe haemophilia A were previously presented as poster presentations on post-authorisation safety studies for Kogenate or Advate at the 22nd Congress of the International Society on Thrombosis and Hemostasis (ISTH) (2009) and the Hemophilia World Congress (World Federation of Hemophilia) (2010) by Fukutake *et al.*, who reported that the incidence was similar to that in Caucasians.

Although the role of the haplotypes of FVIII proteins as a risk factor for inhibitor development is not yet determined, the similarities of haplotype incidence may partially explain the similarities in inhibitor incidence between Japanese patients and Caucasian patients. Further studies are needed to clarify the role and characteristics of FVIII haplotypes in Japanese population.

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Disclosures

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血栓止血誌 22(5) : 259～265, 2011

◆特集：東日本大震災と日本血栓止血学会◆

東日本大震災で被災された血友病患者への支援

Support for patients with hemophilia affected by the Great East Japan Earthquake

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Key words: earthquake, hemophilia, network system, consultation service

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はじめに

平成23年3月11日に発生した東日本大震災は東北地方を中心に未曾有の被害をもたらし，被災された方々は死者・行方不明者をあわせて2万人を超えるとされています[1]（8月9日現在，警察庁まとめ）．さらに，今なお避難・転居を余儀なくされている方々は9万人を超えるとされています[2]（7月26日現在，内閣府まとめ）．血液凝固異常症全国調査平成22年度報告書（財団法人エイズ予防財団）[3]によると，青森，岩手，宮城，秋田，山形，福島の東北ブロックで血友病A 345名，血友病B 72名の方々が登録されており，これらの方々の中には深刻な被害に遭われた方もいるものと推測されます．これら被災された血友病患者の方々を血友病部会で支援するようにと震災から8日後の3月19日に池田康夫理事長ならびに坂田洋一学術標準化委員会委員長からご指示をいただきました．それに応える形で血友病部会としていくつかの支援策を行ってきましたので，この誌面をお借りしてご報告させていただきます．現実に，被災地でお困りの血友病患者の方々に対して何ができたのか，また，何ができなかったのかを検証したいと思います．

1．情報の収集と発信

震災直後は，被災地の情報，特に医療関係の情報が全く入らず，混乱の状態が続きましたが，しばらくすると，今度は様々な情報が入り乱れ錯綜するようになりました．テレビや新聞からの情報だけでなく，今日ではインターネットやメーリングリスト，ツイッター，フェイスブックなど情報源は多岐に渡ります．実際，血友病関連のメーリングリストなどでは連日のように現地の情報が流されていましたが，それらの多くは整理されることなく，一方的に情報が流されている状態でした．このような時には情報をいかにコントロールし得るかが極めて重要になりますが，血友病関連でいち早く情報を整理し発信されたのが，日本小児血液学会血友病委員会（嶋緑倫委員長）の血友病診療ネットワークでした．このネットワークは血友病診療を行っている，およそ480の施設が登録されており，主として小児科医で構成されています．震災から4日後の3月15日には相模原協同病院小児科の中舘尚也先生（現，国立成育医療センター総合診療部）が中心となってネットワークの管理を行い，数多くの情報を収集し整理した上で必要な情報をメーリングリストで流すという作業を開始されました．血友病部会でもこれに呼応する形で，3月18日には未登録の部会員に血友病診療ネットワークへの参加を呼びかけ，さらに3月22日には日本血栓止血学会の代議員にも広く参加を呼びかけました．これは，被災された血友病患者の救援・支援という共通の目的のもと，今回の大災害を機に学会や診療科の垣根を越えて実現した画期的な出来事だと思います．実際，このネットワークを利用して様々な有用な情報が発信されました．例えば，保険証や特定疾患の受給者証がなくても診療が受けられるといった厚生労働省からの通達事項[4,5]や血友病治療製剤の供給体制，在庫状況などの情報が発信されました．また，被災患者の受入れの申し出も全国の施設から寄せられたほか，被災地である宮城県や福島県の病院の状況，特に血友病診療の実態などが報告されました．このメーリングリストによって，震災後1か月の間に血友病関連の情報として診療情報が20回，医薬品情報が17回にわたって発信されました．

ここで，実際にネットワークを利用した事例を紹介したいと思います．患者は被災地にお住まいの血友病Aの小児で，普段から定期補充療法が行われていました．震災後，着の身着のままで首都圏の親戚宅に避難され，どこの病院にかかればいいか困っているということで，3月17日に被災地の主治医からネットワークを通じて緊急支援依頼がありました．実際には聖マリアンナ医科大学横浜市西部病院を通じて東京医科大学病院を紹介され継続して治療を受けることができました．この間，幸いにも大きな出血はなく，本例はネットワークが機能し，専門医間の連携によりうまく対応できたケースでした．

2．相談窓口の開設

血友病部会では上記のネットワークへの参加と

資料1　被災された血友病患者の方々への相談窓口開設の御案内

血友病部会では被災地でお困りの患者・家族の方々を対象にEメールでの相談窓口を設けました．Eメールでの相談が困難な場合はお電話でも受付けます．相談内容に応じて，血友病専門医師もしくは専門看護師が回答させていただきますが，内容によっては回答までにある程度お時間をいただくことがありますのでご了承ください．また，本相談窓口は緊急時には対応しておりませんので，ご理解いただきますようお願い申し上げます．なお，相談内容はプライバシー保護に十分配慮してお取り扱いさせていただきます．ご相談いただく前に，「血友病被災患者・家族の方々へQ&A」もご一読いただければ幸いです．（日本血栓止血学会学術標準化委員会血友病部会）

■Eメールでのご相談：日本血栓止血学会血友病部会事務局（ketuyubyou@gmail.com）
■お電話でのご相談*：東京医科大学病院（代表 03-3342-6111）
＜電話交換手に「血友病相談窓口への電話」とお伝え下さい．＞
平日9時～17時（臨床検査医学科外来まで）
時間外，土日祝祭日（臨床検査医学科当直医まで）
自治医科大学附属病院（代表 0285-44-2111）
平日9時～17時，土日祝祭日除く（分子病態研究部・止血血栓まで）
聖マリアンナ医科大学病院（代表 044-977-8111）
平日9時～17時，土日祝祭日除く（看護部　吉川まで）
奈良県立医科大学附属病院（代表 0744-22-3051）
平日9時～17時，土日祝祭日除く（小児科研究室まで）

*一般診療の業務と重なった場合は対応にお時間をいただくことがあります．

ともにさらなる支援策を検討しました．ネットワークでの情報発信は主として血友病診療を行う医師を対象にしたものであり，それとは別に直接患者さんを支援するような活動ができないものかと考えました．この頃には震災直後の混乱も一段落し，初期の救命・救援活動から，長期に渡る被災者へのケアが求められるようになっていました．血友病患者の方々についても，長期の転居・避難所生活により発生する様々なトラブルや不安にいかに対応するべきかが問われる時期でした．そこで，掛り付けの病院に行きたくても行けない状況に置かれている患者の方々にとって気軽に専門の医師や看護師に直接相談できるような窓口があればいいのではないかということになり，相談窓口開設の準備に入りました．ただ，一口に相談窓口と言っても，普段の診療が多忙な中，どの施設の誰がどのような形態で相談業務を行うのかなど詰めなければいけない課題が数多くありました．しかし，これらの問題も部会員ならびにその施設のスタッフのご協力により，一つずつ解決され，4月6日には学会ホームページ上に相談窓口を開設することができました[6]（**資料1**）．そこではメールでの相談受付を行うとともに東京医科大学，自治医科大学，聖マリアンナ医科大学，奈良県立医科大学の4施設に電話相談の窓口を設けて対応することになりました．また，相談窓口開設と同時に患者の方々からの想定される質問をQ&A形式で掲載しました[7]（**資料2**）．このQ&Aの中には，製剤の室温での保管がどの程度可能かといった情報や製剤の販売元の問い合わせ先情報などが含まれています．さらに，4月22日には製剤が入手可能な被災地の施設リストも追加掲載しました[8]（**資料3**）．

3．広報活動

いかに有用な情報でも被災された患者の方々に届かなければ意味がありません．いかに多くの方々にこれらの情報をお知らせするのかが次の大きな課題でした．実際，患者の方々の手元に相談窓口開設の情報をお届けするために，様々なルートを通じて広報活動を行いました．具体的には岩手，宮城，福島県の各災害対策室や厚生労働省にご案内したほか，患者会を通じて可能な限り患者の方々にも情報を伝えていただきました．また，製剤関連メーカーを通じて被災地の病院や施設にも広報していただきました．さらに，インターネット上では，厚生労働省や宮城県災害保健医療支援室のウェブサイトのほか，患者会やその支援組織，日本赤十字社，そして製薬企業の患者向けサイト

資料2　血友病被災患者・家族の方々へ Q&A

Q1. 自分の使っている製剤についての情報を知りたい.

A1. 以下の各メーカーのお問い合わせ窓口をご利用ください.

各メーカーのお問い合わせ窓口				
血友病A治療製剤	クロスエイトM®	日本赤十字社	03-3437-7579 090-4380-3895	日中時間帯 その他時間
	コージネイト®FS バイオセット	バイエル薬品	0120-106-398 090-9090-8945	平日 9：30 ～ 17：30 時間外
	アドベイト®	バクスター	03-6204-3800	平日 10：00 ～ 17：00 土日祝祭日除く
	コンファクト®F	化血研／アステラス	0120-345-724	平日 8：30 ～ 17：30 土日祝祭日除く
血友病B治療製剤	ノバクト®M	化血研／アステラス	0120-345-724	平日 8：30 ～ 17：30 土日祝祭日除く
	ベネフィクス®	ファイザー	0120-664-467 0120-168-365	平日 9：00 ～ 17：30 土日祝祭日除く 24時間対応（溶解操作関連）
インヒビター治療製剤	ファイバ®	バクスター	03-6204-3800	平日 10：00 ～ 17：00 土日祝祭日除く
	ノボセブン®HI	ノボ ノルディスク ファーマ	0120-180-363 0120-359-516	平日 9：00 ～ 18：00 夜間，土日祝日，会社休日
フォンヴィレブランド病治療製剤	コンファクト®F	化血研／アステラス	0120-345-724	平日 8：30 ～ 17：30 土日祝祭日除く

Q2. 製剤は室温でも保管できるのですか.

A2. 室温安定性は製剤によって以下の表のように異なります. 詳しくはA1. のメーカー窓口にお問い合わせください. なお，いずれの製剤も凍結は避けて保管してください.

各製剤の室温安定性			
血友病A治療製剤	クロスエイトM®	日本赤十字社	室温保存可（1 ～ 30℃）
	コージネイト®FS バイオセット	バイエル薬品	3か月まで室温保存可（25℃以下）
	アドベイト®	バクスター	通常，2 ～ 8℃で保存 ただし，6か月まで室温保存可（1 ～ 30℃） 室温保管後は再度冷蔵庫に戻さないこと
	コンファクト®F	化血研／アステラス	10℃以下保存
血友病B治療製剤	ノバクト®M	化血研／アステラス	室温保存可（30℃以下）
	ベネフィクス®	ファイザー	6か月まで室温保存可（30℃以下）
インヒビター治療製剤	ファイバ®	バクスター	2 ～ 8℃で保存
	ノボセブン®HI	ノボ ノルディスク ファーマ	室温保存可（1 ～ 30℃）
フォンヴィレブランド病治療製剤	コンファクト®F	化血研／アステラス	10℃以下保存

Q3. 手元にいつも使っている製剤がないのですが，他の製剤に変更できますか.

A3. 緊急の場合は変更可能です. どの製剤も止血効果および安全性に大きな差はありません. また，製剤を変更することによってインヒビターが発生する可能性は極めて低いと考えられます. 具体的には，血友病Aではクロスエイト M®（日本赤十字社），コージネイト®FS バイオセット（バイエル薬品），アドベイト®（バクスター），コンファクト®F（化血研／アステラス）が使用可能です. 一方，血友病Bではノバクト®M（化血研／アステラス），ベネフィクス®（ファイザー），クリスマシン®-M（ベネシス／田辺三菱），PPSB®-HT（日本製薬）が使用可能です.

Q4. 今回の震災で全国的に製剤が不足しているのですか.

A4. 各製剤とも国内の在庫は全く問題ありません. 被災地の病院への配送も徐々に回復しているようですが，一部地域では依然として配送が困難な所もあるようです（4月1日現在の情報）. 詳しくはA1. にある各メーカーの窓口にお問い合わせください.

Q5. 震災で泥まみれになった製剤は使用できますか.

A5. 容器が破損している恐れもありますので，そういった製剤は使用しないで下さい.

Q6. 定期補充療法をしていたのですが，急にやめても大丈夫ですか．

A6. 被災状況によっては，出血時補充療法に切り替えざるを得ない方々もおられると思います．定期補充療法に比べて出血の機会が増えることが予想されますが，可能な限り出血後早期の治療を心がけるようにしていただけたらと思います．

Q7. 大きな出血をおこしたら，どうしたらいいですか．

A7. 可能であれば製剤を自己注射していただいた上で，最寄りの医師の診察を受けるようにして下さい．緊急の場合は救急車の出動を要請して下さい．

Q8. インヒビターが陽性で現在，免疫寛容導入療法を始めたところです．途中でやめてもいいのですか．

A8. 状況が落ち着いて普段通りになるまでは仕方がないと思います．免疫寛容導入療法を中断した場合，インヒビターがどうなるかは個人によって違いますので一概には予測できません．

Q9. 県外の避難先で診てもらえる病院はありますか．

A9. 日本小児血液学会の血友病診療ネットワークでは被災された患者さんの受入れ支援を全国の施設から名乗り出ていただいています．Eメール（ketuyubyou@gmail.com）でお問い合わせいただければ，ご紹介することは可能です．

Q10. 避難先の県でも医療費はかからないのですか．

A10. 厚生労働省から，「公費負担医療を受けている被災者が，医療機関において手帳，患者票等の提出ができない場合においても，受診が可能である」という通知が各都道府県に出されています．また，「緊急の場合は，小児慢性特定疾患治療研究事業や特定疾患治療研究事業の受託契約を結んだ医療機関以外の医療機関でも受診できるものとする」とされています．詳しくはおかかりの病院のソーシャルワーカーにお問い合わせください．

などに相談窓口へのリンクを貼っていただきました（**資料4**）．ただ，現時点では被災者の方々からのご相談はほとんどなく，実際にお困りになられている方々が幸いにも少ないのか，現地の方々まで情報が届いていないのか，窓口開設のタイミングが遅かったのか，患者の方々にとって窓口の敷居が高いのかなど今後の広報活動のあり方について課題を残した形になっています．

4．今後の対応

今回の大震災の経験を踏まえ，痛切に感じたのは災害に対する事前の備えの重要性でした．地震や津波以外にも災害はいつ，いかなる場所にも前触れなしに起こるものです．そのため，今回用意したような災害時の医療情報や医薬品情報などを前もって整理しておくとともに，製剤の入手可能な施設のリストを全国各地で作成しておく必要があると感じました．これは広く他の関連学会にも呼びかけて進めていきたいと考えています．また，現在，血友病部会で進めている血友病センター構想もより重要になってきます．センター構想ではセンターを中心として各地域の診療ネットワークを構築し，さらには全国レベルのネットワークに発展させることを目指していますが，それが実現すると血友病関連の様々な情報共有，診療協力が可能になります．また，各センターで疾患登録を行い，全国的なデータベースを構築することによって，患者情報の共有，安否確認などがよりスムーズになり，災害時に役立つのではないかと考えています．

おわりに

今回の大震災は原発問題も相まっていまだ収束の目処が立たない状況であり，長期の避難所生活を余儀なくされておられる被災者の方々のご苦労は察するに余りあります．ただ，テレビや新聞報道で被災地での復興を目指して頑張っておられる地元の方々，また，被災地を応援するために日本中，世界中から寄せられた数々の支援を見るにつけ，勇気と希望が与えられる思いがします．われわれ医療従事者は今回の震災を教訓として学ぶとともに，次に起こる可能性のある災害に備えることが重要ではないかと考えます．決して他人事でなく，また，はるか先のことでなく，真摯にそして着実に対応していくことが求められています．

今回の大震災で被災された皆様にはあらためて心よりお見舞い申し上げますとともに一日も早い復興を祈念して止みません．

謝　辞

今回の原稿執筆にあたり，快く貴重な資料を提供