TABLE 1

Amino-terminal sequence analysis of the C2 fragments

Sequences were determined as described under "Materials and Methods" and were aligned using published factor VIII sequences (1, 2).

	Cycle number						
	1	2	3	4	5	6	7
Residues 2182–2188	Ser	Lys	Ala	Ile	Ser	Asp	Ala
C2 fragment 1	Ser	Lys	Ala	Ile	Ser	Asp	Ala
pmol	10.4	17.7	14.4	14.3	8.4	6.6	7.9
Residues 2260–2264	Phe	Leu	Ile	Ser	Ser		
C2 fragment 2	Phe	Leu	Ile	Ser	Ser		
pmol	10.7	10.3	11.0	5.5	5.4		

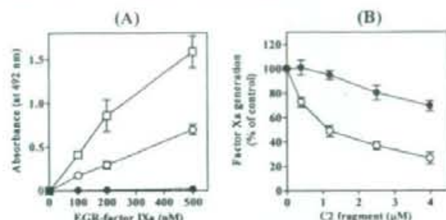

FIGURE 6. **Binding of EGR-factor IXa to C2 fragments and the inhibitory effects on factor Xa generation.** A, binding of EGR-factor IXa. Various concentrations of EGR-factor IXa were incubated with immobilized C2-(2182–2259) (600 nM; *open circles*), C2-(2260–2322) (600 nM; *closed circles*), and intact rC2 (100 nM; *open squares*) for 2 h at 37 °C in an ELISA-based assay. Absorbance values were plotted as a function of the concentration of EGR-factor IXa. *B*, inhibition of factor Xa generation in the absence of phospholipid. Various amounts of C2-(2182–2259) (*open circles*) or C2-(2260–2322) (*closed circles*) were preincubated with 5 nM factor IXa for 2 h at 37 °C, and factor Xa generation was initiated with the addition of thrombin-activated factor VIIIa (200 nM) and 1 μM factor X. The initial rate of factor Xa generated in the absence of competitor (100% level) was 1.46 ± 0.19 nM/min. Initial rates of factor Xa generation were plotted as a function of C2 fragment concentration. Experiments were performed at least three separate times, and average ± S.D. values are shown.

C2-(2260–2322) was observed. In control experiments, EGR-GDless factor IXa did not bind to either immobilized C2 fragment (data not shown). To assess the functional capacity of the two C2 fragments in factor Xase assembly, amidolytic assays were again repeated in the absence of phospholipid. Factor VIII (200 nM) was activated by thrombin and incubated with factor IXa (5 nM)/C2 fragment mixtures and factor X (1 μM). The C2-(2182–2259) competitively inhibited factor Xa generation by ~80% at the maximum concentration employed (IC_{50} = 1.2 μM; Fig. 6*B*). The effect of C2-(2260–2322) was significantly lower than that of C2-(2182–2259), however, and inhibited factor Xa generation by ~30%. Collectively, these data suggest that an interactive site(s) for the Gla domain of factor IXa was likely to be located within residues 2182–2259 of the C2 domain.

Effects of Synthetic C2 Peptides on rC2 and EGR-factor IXa Interaction and on Factor Xa Generation in the Absence of Phospholipids—On the basis of the competitive binding assays and ELISA, we focused on the 2182–2259 region in the C2 domain to further identify the potential factor IXa-interactive site. The C2-factor IXa interaction is electrostatically dependent (see Fig. 2*A*), suggesting that both interactive sites are surface-exposed. The analysis of solvent-accessible surface area was utilized, therefore, to examine regions within residues 2182–2259 exhibiting a high probability of being surface-exposed. The solvent accessibilities at the interface were calcu-

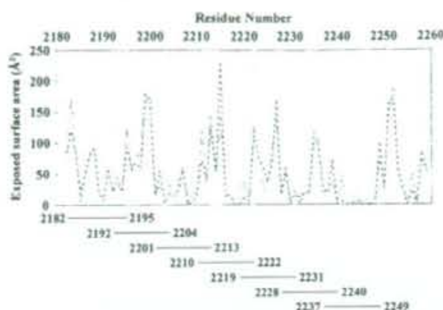

FIGURE 7. **Accessible surface area of residues 2182–2259 of the C2 domain.** The solvent accessibilities at the interface for the residues 2182–2259 of the C2 domain were calculated from the atomic coordinates in the structure of factor VIII (*solid lines*) and C2 (*dashed lines*) using Marc Gerstein's calc-surface program (see "Materials and Methods"). Accessible surface area ($Å^2$) was used to estimate the probability of a segment being exposed to the surface. Synthetic peptides corresponding to regions of high surface probability are indicated by the *horizontal bars* and are identified by residue numbers for the segments.

lated from atomic coordinates in the structures of factor VIII and C2 (Protein Data Bank code 2R7E and 1D7P, respectively), and they were similar. Using this approach, overlapping synthetic peptides encompassing the 2182–2249 region were prepared (Fig. 7). Since the C2-factor IXa interaction was not affected by anti-C2 mAb ESH8 with epitopes 2248–2285 (data not shown), the 2248–2259 region was excluded. Effects of peptides to block C2-factor IXa interaction and to inhibit factor Xa generation were examined.

The synthetic peptide corresponding to residues 2228–2240 (designated peptide 2228–2240) inhibited binding of EGR-factor IXa to rC2 by ~75% at the maximum concentrations employed (at 1 mM) (Fig. 8*A*). The IC_{50} value was ~400 μM. The other six peptides, corresponding to residues 2182–2195, 2192–2204, 2201–2213, 2210–2222, 2219–2231, and 2237–2249, demonstrated no inhibitory effects. Moreover, a control peptide (VKMTKQFDVQLWE), comprising the 2228–2240 residues in a random sequence, completely lost the ability to inhibit this interaction (data not shown). The inhibitory effects of these peptides were further studied in the factor Xa generation assay. The peptide 2228–2240, which blocked C2-factor IXa interaction, depressed factor Xa generation by ~75% at the maximum concentration employed (IC_{50} ~ 25 μM) (Fig. 8*B*). The other C2 peptides and the scrambled peptide had little effect. The ability of peptide 2228–2240 to inhibit factor Xa generation appeared to be more significant than that in the binding assay. It seemed likely, therefore, that peptide 2228–2240 not only affected interactions between the Gla domain of factor IXa and the C2 domain but also allosterically modulated other reactions. Nevertheless, the findings suggest that interactive site for the Gla domain of factor IXa was located within residues 2228–2240 of the C2 domain.

Effects of peptide 2228–2240 on Factor Xa Generation in the Presence of Phospholipid—Intact C2 and isolated C2 fragments contain phospholipid-binding regions, and to preclude interference by these reactions, our current factor Xa generation

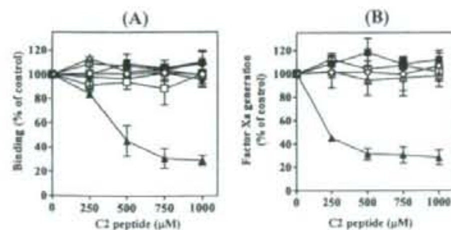

FIGURE 8. **Inhibition of synthetic C2 peptides on EGR-factor IXa binding to rC2 and on factor Xa generation in the absence of phospholipid.** *A*, EGR-factor IXa binding to rC2. Various amounts of synthetic C2 peptide were preincubated with 100 nM EGR-factor IXa for 2 h at 37 °C, prior to the addition to immobilized rC2 (200 nM) in an ELISA-based assay. Absorbance values for the EGR-factor IXa binding to rC2 in the absence of competitor represent the 100% level. The percentage of EGR-factor IXa binding was plotted as a function of peptide concentration. *B*, factor Xa generation in the absence of phospholipid. Various amounts of C2 peptide were preincubated with 5 nM factor IXa for 2 h at 37 °C, and factor Xa generation was initiated with the addition of thrombin-activated factor VIIIa (200 nM) and 1 μM factor X. The initial rate of factor Xa generated in the absence of competitor (100% level) was 0.83 ± 0.01 nM/min. Initial rates of factor Xa generation were plotted as a function of C2 peptide concentration. *Open circles*, peptide 2182–2195; *closed circles*, peptide 2192–2204; *open squares*, peptide 2201–2213; *closed squares*, peptide 2210–2222; *open triangles*, peptide 2219–2231; *closed triangles*, peptide 2228–2240; *open diamonds*, peptide 2237–2249. Experiments were performed at least three separate times, and average ± S.D. values are shown.

assays were performed in the absence of phospholipid. Crystal structure analysis has demonstrated that binding of the C2 domain to phospholipid membranes involves three hydrophobic "feet" containing residues Met^{2199}/Phe^{2200}, Val^{2223}, and Leu^{2251}/Leu^{2252} and four basic residues, Arg^{2215}, Arg^{2220}, Lys^{2227}, and Lys^{2249} (35, 36). The factor IXa-interactive site that we have identified within residues 2228–2240 appears, therefore, to be in close proximity to, but not likely to be overlapping, the phospholipid-binding region. In support of this contention, we found that binding of factor VIII and factor IXa to phosphatidylserine was not significantly inhibited by peptide 2228–2240 (data not shown). Nevertheless, to further examine the physiological role of peptide 2228–2240 binding to the Gla domain of factor IXa, factor Xa generation was measured in the presence of factor VIIIa and phospholipid. Factor VIII (30 nM) was activated by thrombin and incubated with factor IXa (0.5 nM)/peptide 2228–2240 mixtures together with various concentrations of factor X in the presence of phospholipid (20 μM). Since rC2-factor IXa interaction was optimal at relatively low concentrations of Ca^{2+} (~1 mM), under these circumstances, the V_{max} was ~20-fold lower than that previously reported (31). Nevertheless, in the presence of peptide 2228–2240, the K_m value remained unchanged, whereas the V_{max} was decreased, dependent on the concentration of the peptide (Fig. 9). Factor Xa generation was completely inhibited (>95%) in the presence of 15 μM peptide. These results suggested that peptide 2228–2240 inhibited factor Xa generation on phospholipid micelles by noncompetitive inhibitory mechanisms. Furthermore, peptide 2228–2240 did not affect factor Xa generation using GDless factor IXa in place of factor IXa in these assays (data not shown), again indicating that peptide 2228–2240 specifically bound to the Gla domain of factor IXa but did not moderate interactions between factor VIIIa and factor X.

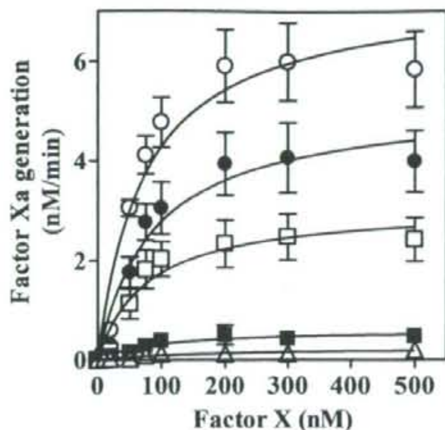

FIGURE 9. **Effect of peptide 2228–2240 on factor Xa generation in the presence of phospholipid.** Factor IXa (0.5 nM) was preincubated with 0 μM (*open circles*), 2.5 μM (*closed circles*), 5 μM (*open squares*), 10 μM (*closed squares*), and 15 μM (*open triangle*) of peptide 2228–2240 for 2 h at 37 °C. Factor Xa generation was initiated by the addition of thrombin-activated factor VIIIa (30 nM) and various concentrations of factor X (0–500 nM) in the presence of phospholipid vesicles (20 μM). Initial rates of factor Xa generation were plotted as a function of factor X concentration and fitted to the Michaelis-Menten equation by nonlinear least squares regression. Experiments were performed at least three separate times, and average ± S.D. values are shown. The V_{max} values in the presence of 0, 2.5, 5, and 10 μM peptide were 7.42 ± 0.85, 5.15 ± 0.73, 3.07 ± 0.49, and 0.61 ± 0.12 nM/min, respectively. The K_m values were 72.9 ± 25.5, 84.8 ± 34.9, 75.0 ± 36.0, and 89.6 ± 51.3 nM, respectively. The kinetic parameters in the presence of 15 μM peptide could not be determined because of very low values.

DISCUSSION

The enzyme factor IXa and its cofactor factor VIIIa are assembled on phospholipid membranes for the activation of factor X. In previous reports, factor IXa recognition sites were identified within the A2 and A3 domains of factor VIIIa. In the A2 domain, the extended surface, centered on residues 484–509 (16), 558–565 (17), and 708–717 (18), appeared to interact with the factor IXa with weak affinity (~300 nM) (15). In contrast, in the A3 domain, the light chain, including residues 1804–1818 (20), interacted with the protease with high affinity (~15 nM) (19). The structural model of factor VIIIa-factor IXa on phospholipid membranes reported by Blostein *et al.* (21) proposed that the C2 domain of factor VIIIa and the Gla domain of factor IXa bound to phospholipid would be in close proximity, suggesting that both domains might bind to each other. In the present study, we show for the first time that the residues 2228–2240 in the C2 domain and the Gla domain of factor IXa bind to each other.

This conclusion is based on several novel findings using the established models. (i) Direct binding studies demonstrated that active site-modified EGR-factor IXa bound to the C2 domain with mild affinity (~100 nM), whereas GDless EGR-factor IXa failed to bind. In addition, mAb IXa-GD, recognizing the Gla domain of factor IXa, blocked C2-factor IXa interaction. (ii) A factor Xa generation assay without phospholipid showed that rC2 and mAb IXa-GD inhibited factor IXa-medi-

ated factor X activation in the presence of factor VIIIa. (iii) A C2-(2182–2259) fragment, derived from V8 protease-cleaved rC2, directly bound to EGR-factor IXa and inhibited factor Xa generation, whereas the C2-(2260–2322) did not bind. (iv) Competitive assays, using overlapping synthetic peptides encompassing residues 2182–2259, showed that peptide 2228–2240 significantly inhibited both factor IXa binding and factor Xa generation, independently of phospholipid. These data identified amino acid residues 2228–2240 within the C2 domain as essential for factor IXa docking.

In the present study, we utilized EGR-factor IXa, a catalytically inactive derivative of factor IXa, in direct binding experiments. Modified factor IXa prepared with EGR-ck is well known to minimize enzyme-catalyzed degradation, but conformational changes and/or steric hindrance due to incorporation of EGR-ck into the active site of factor IXa may cause difficulties. Nevertheless, Lenting *et al.* (19) reported that thrombin-cleaved factor VIII light chain bound to modified factor IXa with high affinity (~15 nM), and we also analyzed direct binding of the C2 domain using untreated factor IXa and EGR-factor IXa. Binding patterns were similar using active factor IXa and EGR-factor IXa (data not shown), suggesting that any potential effects of conformational changes and/or steric hindrance induced by EGR-ck were minimal. The results also indicated that the C2 domain does not participate in docking to the active site pocket of factor IXa.

We obtained direct evidence for a restricted factor IXa-interactive site in the C2 domain using solvent-accessible surface area analysis with overlapping peptides encompassing residues 2182–2259. The sequence 2228–2240 appeared to be specific for this interaction, and a scrambled peptide confirmed this specificity. The peptide 2228–2240 did not affect factor VIII binding to phospholipid, however, in keeping with an earlier study using similar C2 peptides (7). Our observations add significantly to understanding the nature of the factor Xase complex involving factor IXa and the C2 domain of factor VIII. Our suggestion that this region is not related to phospholipid binding by experiments using peptide can be supported by the following reasons.

First, based on the ability of synthetic peptides encompassing residues 2303–2332 in C2 to inhibit factor VIII-phospholipid binding, a major site was previously located within this region (7). In addition, earlier elegant examination of the 1.5 Å x-ray structure of the C2 domain revealed the presence of three hydrophobic "feet" (Met^{2199}/Phe^{2200}, Val^{2223}, and Leu^{2251}/Leu^{2252}) that penetrate the membrane and four basic residues (Arg^{2215}, Arg^{2220}, Lys^{2227}, and Lys^{2249}) that lie underneath the "feet" and stabilize binding by electrostatic interaction with phospholipid (35, 36). These findings show that the 2228–2240 region in C2 is in close proximity to, but is not likely to overlap, the phospholipid-binding region. Second, The interaction between factor IXa and thrombin-cleaved factor VIII light chain, lacking the acidic region of the A3 domain involved in high affinity VWF binding, was not affected by the presence of VWF (19). Our present study also showed that the C2-factor IXa interaction was not affected by VWF (data not shown). Since the C2 domain is involved in VWF binding at a site that overlaps the phospholipid-binding site (8, 9), the 2228–2240 region does not overlap this site. Last, factor VIIIa contacts with residue Phe^{25} and/or Val^{46} of the Gla domain of factor IXa but not with the membrane-binding ω loop (residues 1–11) (21). Furthermore, a naturally occurring mutation (G12R) within the Gla domain is associated with reduced activity of the factor Xase complex but does not affect phospholipid binding (37). These results are consistent with the view that interaction between the Gla domain and the C2 domain is not dependent on phospholipid binding. Taken together, our findings imply that interactions between both domains facilitate a tight ternary complex with phospholipid.

Binding of C2 to the Gla domain of factor IXa was governed by electrostatic and/or calcium-dependent interactions. This mechanism was similar to that observed between the light chain of factor VIII and factor IXa (19). Furthermore, peptide 2228–2240 significantly inhibited (>95%) factor Xa generation in the presence of phospholipid through noncompetitive inhibitory mechanisms, similar to those observed using peptide 1804–1818, previously reported as a factor IXa-binding site in A3. These data strongly indicated that the properties of both interactions were common and that both peptides inhibited the enzyme activity of factor IXa by binding at a site distinct from the substrate binding pocket. Of interest, the binding affinity of C2 for factor IXa (~100 nM) was ~7-fold lower than that of the light chain (~15 nM). In the absence of phospholipid, the inhibitory effect of peptide 2228–2240 on C2-factor IXa interaction was not significantly different from that of peptide 1804–1818 on light chain-factor IXa interaction. In the presence of phospholipid, however, peptide 2228–2240 appeared to inhibit factor Xa generation more strongly than peptide 1804–1818 (IC_{50} ~ 5 and ~600 μM, respectively). The binding affinity of the A3 domain for factor IXa is not known; nevertheless, the high affinity of the light chain appears to make an essential contribution to reactions involving not only the A3 domain but also the C2 domain. Furthermore, the data indicate that peptide 2228–2240 predominantly participates in factor IXa docking for catalyzing the activity of the factor Xase enzyme.

Recently, two groups have reported the intermediate resolution x-ray crystallographic structure of B-domainless factor VIII (38, 39). Factor IXa-interactive sites within factor VIII based on crystal structure reveal that residues 558–565 and 708–717 in A2 and 1804–1818 in A3 are located on one face of factor VIII, whereas residues 484–509 in A2 and our identified 2228–2240 in C2 are located on another face. Ngo *et al.* (39) have constructed a model of the factor VIIIa-factor IXa complex with x-ray crystal structure of human factor VIII and porcine factor IXa backbone with the following constraints. Residues 558–565, 708–717, and 1804–1818 of factor VIIIa interact with the residues 330–339, residues 301–303, and the putative binding region, including EGF domains (Tyr^{69} and Asn^{92}) and Gla domain (Phe^{25}) of factor IXa, respectively. Although this differed from our data in the binding site of C2 for the Gla domain, a factor IXa-interactive site comprising residues 2228–2240 in C2 is unlikely to contact the Gla domain simultaneously according to this model. This discrepancy may be due to conformational change of the C2 domain. Conformational changes in C2 of factor VIIIa upon removal of the NH_2 terminus of the light chain (residues 1649–1689) (40) probably

leads to enhancement of the factor VIIIa affinity for phospholipid membrane (41). This may affect the Gla domain binding. In addition, the C2 domain is relatively loosely docked to the remainder of factor VIII molecules (38, 39); consequently, the position of this domain within active form factor VIIIa on the phospholipid surface may change easily. These findings can be supported by the case of residues 484–509 in A2. The model proposed by Ngo showed that this region did not interact with factor IXa despite the factor IXa-interactive site. Bajaj *et al.* (42) also demonstrated that residues 484–509 in A2 were not in close proximity to one face consisting of residues 558–565, 708–717, and 1804–1818 and did not contact factor IXa. Furthermore, Stoilova-McPhie *et al.* (43) found that it was unable to modify the factor VIII-factor IXa binding model, including the 484–509 region. The following possibilities are raised for this reason: the conformational change in A2 upon binding of the catalytic domain of factor IXa and different A2 arrangement between unactive form factor VIII and active form factor VIIIa. Therefore, it is not so surprising that the 2228–2240 region in factor VIIIa interacts with factor IXa Gla domain.

An earlier report by Nogami *et al.* (11) demonstrated that residues 2253–2270 within the C2 domain of factor VIII contribute to a unique factor Xa-interactive site within the light chain that promotes factor Xa docking during cofactor activation and cleavage of the light chain at Arg^{1689}. Binding of factor Xa to the C2 domain was independent of binding to phospholipid or VWF, indicative of a distinct factor Xa-binding site in the C2 domain. This binding was remarkably similar to that of the C2-factor IXa interaction observed in this study. In addition, interaction between the light chain of factor VIIIa and factor IXa was not inhibited by active site-modified factor Xa (19). However, the C2-factor IXa interaction was not inhibited by anti-C2 mAb ESH8 (data not shown), which recognizes residues 2248–2285 and inhibits the factor VIII-factor Xa interaction (11). These findings suggest that the factor IXa-interactive site in the C2 domain does not overlap the factor VIII-Xa interactive site.

Comparisons of amino acid sequences among human, porcine, murine, and canine factor VIII molecules indicate that residues 2228–2240 within the C2 domain are well conserved, in keeping with the suggestion that this region could be fundamental for interaction with the Gla domain of factor IXa (44–46). This region appears to be unique, and the specific sequence of residues is distinct from those of the factor IXa-interactive sites within the A2 and A3 domains of factor VIIIa (16–18, 20). Naturally occurring mutations of residues 2228–2240 (W2229C, W2229S, Q2231H, V2232A/E, and M2238V) have been reported in the hemophilia A data base (HAMSTeRS), and are seen in mild/moderate hemophilia A. It is tempting to speculate that the pathogenic mechanism for these point mutations might be associated with dysfunctional blood coagulation by moderating interactions between the C2 domain of factor VIIIa and the Gla domain of factor IXa. Furthermore, substitutions at Trp^{2229} to Cys and Val^{2232} to Ala are related to the development of inhibitors (47, 48), consistent with our suggestion that the 2228–2240 region in C2 is surface-exposed and influences antigenicity.

In conclusion, we provide the first evidence for an essential role of the association between the 2228–2240 region of the C2 domain and the Gla domain of factor IXa in the factor Xase complex. Further studies using site-directed mutagenesis are warranted to further clarify the functional role of residues 2228–2240 in the C2 domain.

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Therapeutic Effects of Hepatocyte Transplantation on Hemophilia B

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Hepatocyte transplantation offers an alternative therapeutic approach in the treatment of liver-related diseases. Hemophilia B is a bleeding disorder lacking factor IX (FIX) production in the liver, and achieving more than 1% coagulation activity results in significant improvement in the quality of life of the patients. The aim of this study was to investigate the efficacy of hepatocyte transplantation in the mouse model of hemophilia B. We transplanted isolated normal mouse hepatocytes into the liver of FIX knock-out mice. In some recipient mice, additional hepatocyte transplantations were performed 15 days after the first transplant. The recipient plasma FIX activities increased at 1% to 2% and persisted throughout the experimental period. An additional increase was achieved by the repeated transplantation. Close correlation between FIX messenger RNA levels of the liver and plasma FIX activity levels was observed. These results demonstrate that hepatocyte transplantation can provide therapeutic benefits in the treatment of hemophilia B.

Keywords: Hepatocyte transplantation, Hemophilia B, Coagulation factor IX, Experimental transplantation.

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Hemophilia B, a recessive X-chromosome linked congenital bleeding disorder, is caused by a failure in the production of coagulation factor IX (FIX) (*1*). The only treatments that are currently available are the replacement therapy with FIX concentrates from plasma-derived or recombinant protein sources (*2*). This treatment modality is inefficient and expensive, because of the requirement of life-long and frequent intravenous infusion of FIX concentrates. Although the gene therapy has been actively studied over the past decade to establish a novel therapy that could provide longer acting and safer production of FIX (*3*), recent clinical trials have yet to conclusively shown long-term therapeutic benefits (*4, 5*). One potential approach that may provide the FIX producing ability in hemophilia B is a whole liver transplantation, because FIX is predominantly produced in the liver (*6–8*). However, the establishment of organ transplantation as a common therapy is hampered by a worldwide shortage of donor livers. Provided that some portion of the donated liver can be used for the isolation of individual hepatocytes, this donor shortage would no longer be a major issue. This is an important point, because the cell type responsible for synthesizing coagulation FIX is the hepatocyte (*9*). Therefore, a cell-based therapy using isolated hepatocytes could provide a therapeutic approach in the treatment of hemophilia B. Hepatocyte transplantation has been recently performed in several countries for various inherited disorders of hepatic metabolism and acute liver failure (*10, 11*). In bleeding disorder, hepatocyte transplantation was applied in the clinics by Dhawan et al. (*12*), who described therapeutic benefits in two patients suffering with congenital factor VII deficiency. Our group has recently shown applying a tissue engineering approach using primary hepatocytes could successfully provide therapeutic effects in hemophilia A mice (*13*). However, the effect of hepatocyte transplantation to treat hemophilia B has yet to be experimentally documented in animals or in the clinics to the best of our knowledge. For this reason, this study was designed to investigate the efficacy of hepatocyte transplantation on hemophilia B.

Hepatocytes were isolated from C57Bl/6 wild-type mice using a collagenase perfusion method as previously described (*13–16*). The recipient FIX knock-out (FIX-KO) mice, syngeneic to donor mice (*17*), were transplanted with the isolated hepatocytes (1.5×10^6 cells in 200 μL) into the liver through the inferior pole of the spleen (n=25). As an experimental control, several FIX-KO mice received sham operation (n=7). To avoid excessive surgical procedure-related bleeding, all FIX-KO mice received intraperitoneal injection of 0.5 mL pooled normal mouse plasma 30 min before abdominal surgery (*18*). All procedures were successfully carried out without any issues related to bleeding and all of the mice survived throughout the experimental period. At days 5, 10, and 15, some of the mice were killed for histologic and messenger RNA (mRNA) analyses (n=7, 5, and 4, at each time point, respectively). All sham-operated mice were killed at day 15.

Blood samples were periodically obtained from retroorbital plexus of the experimental mice. After anticoagulated with 0.1 volume of 3.8% sodium citrate, blood samples were centrifuged, and plasma samples were stored at −80°C until being analyzed. The plasma FIX activity (FIX:C) was quanti-

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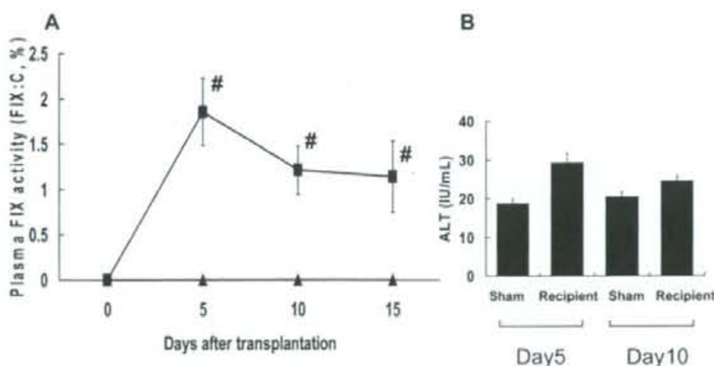

FIGURE 1. Plasma FIX activity (FIX:C) and alanine aminotransferase (ALT) levels of FIX-KO mice after hepatocyte transplantation. (A) FIX:C levels in plasma obtained from FIX-KO mice after hepatocyte transplantation (1.5×10^6 cells/mouse) into the liver (■; n=25, 18, and 13 at day 5, 10, and 15, respectively) or sham operation (▲; n=7 at all time points). Pooled normal mouse plasma was used as a standard. #*P* less than 0.05 between groups. (B) Plasma ALT levels of FIX-KO mice following hepatocyte transplantation (n=25 and 18) or sham operation (n=7) at day 5 and 10 of the experiment.

fied by 1-stage clotting assay based on the activated partial thromboplastin time using human FIX-deficient plasma. Normal mouse plasma was used as FIX:C standard. Each measurement was reported after subtraction of the preoperational baseline FIX:C levels. As a result, FIX:C of recipient mice increased to more than 1% and were stably maintained throughout the experimental period (Fig. 1A). The FIX:C levels were significantly higher in the recipient mice when compared with the levels in the sham-operated mice at every time point examined. At day 5, recipient mice showed a small, but insignificant increase in plasma alanine aminotransferase after the transplantation (n=25) compared with the sham-operated mice (n=7). The slight increase in the alanine aminotransferase levels were found to be declined back toward baseline levels at day 10 (Fig. 1B). These results indicated that hepatocyte transplantation into hemophilia B mice could provide a therapeutic effect by producing FIX from the engrafted donor hepatocytes without significant liver injuries.

Histologic detection of transplanted and engrafted hepatocytes was performed by fluorescence in situ hybridization analysis using mouse Y-chromosome specific probe on sections of female FIX-KO recipient liver that received male hepatocytes. The presence of hepatocytes with Y-chromosome signals were confirmed, indicating the transplanted hepatocytes engrafted into the liver parenchyma (figure not shown). It is also important to note that any cell fusion events were not observed.

To enhance the therapeutic production of FIX in the recipient mice, a repeat transplantation of isolated hepatocytes was performed 15 days after the initial procedure in some recipients by infusing 1.2×10^6 hepatocytes into the upper pole of the spleen (n=4). The other remaining recipients (n=5) were examined with only a single transplantation procedure. As shown in Figure 2, the FIX:C values of the FIX-KO mice at day 25 (10 days after the second transplantation) were 0.94%±0.05% and 1.85%±0.09% in the single- and double-transplanted recipient mice, respectively ($P=0.038$). Similar increases in FIX:C were also observed at day 35 (20 days after the second transplantation) in the double-transplanted group. These data clearly demonstrated that increasing therapeutic effects could be obtained with a repeated transplantation.

We also examined whether the engrafted hepatocytes were capable of transcribing FIX mRNA in the recipient mouse livers. Because shunting of the hepatocytes into the

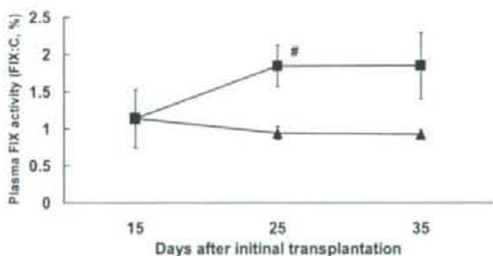

FIGURE 2. Effect of repeated hepatocyte transplantation on plasma FIX:C levels in hemophilia B mice. At day 15, some recipient FIX-KO mice received the second transplantation procedure using 1.2×10^6 hepatocytes (■; n=4), whereas remaining five recipient mice did not receive the second procedure (initial transplantation only, ▲; n=5). #*P* less than 0.05 between groups.

lung had been described in the previous experimental studies (*19*), we also investigated FIX mRNA levels in the lung. Total RNA was extracted from liver, lung, and spleen. Total RNA (1 μg) was reverse transcribed, and the first-strand complementary DNA samples were subjected to quantitative real-time polymerase chain reaction amplification for mouse FIX gene and β-actin gene. Serial dilutions of complementary DNAs of normal mouse liver were used to generate the standard amplification curves. As shown in Figure 3(A), an abundant level of FIX mRNA was detected in the liver, with even higher mRNA expression detected in the livers manipulated with the repeated transplantation. No FIX mRNA signal was detected in the lungs in any of the mouse groups. Incremental expression of FIX was detected in the spleen of single and double hepatocyte transplanted mice, but the levels were markedly lower compared with the livers. We examined the relationship between the FIX:C levels and the liver FIX mRNA levels, and found a direct positive correlation between the two parameters ($R^2=0.7214$) (Fig. 3B).

Furthermore, we assessed the development of neutralizing antibodies against FIX (FIX inhibitor) by Bethesda method using plasma obtained at killing (*20*). Detectable levels (>0.5 Bethesda U/mL) of FIX inhibitor was not measured

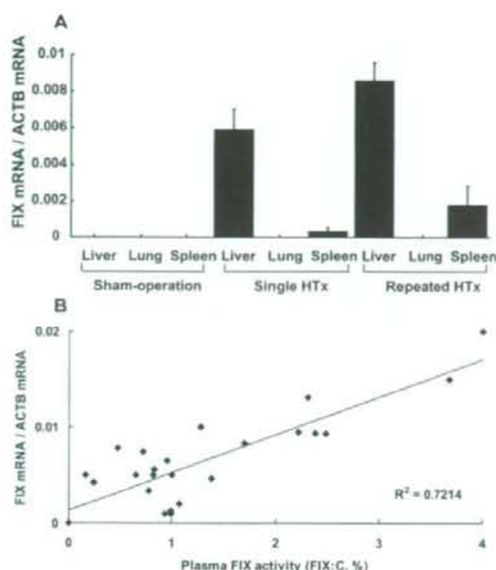

FIGURE 3. Functional engraftment of hepatocytes determined by FIX mRNA expression in the recipient mice. (A) Expression levels of FIX mRNA were determined by quantitative real-time reverse-transcriptase polymerase chain reaction in the liver, spleen, and lung from three experimentally manipulated groups: (1) single hepatocyte transplantation (n=21); (2) repeated hepatocyte transplantation (n=4); and (3) sham-operation (control) (n=7). Each of the FIX mRNA expression values were normalized to a housekeeping gene, β-actin. (B) Relationship between plasma FIX:C levels and FIX mRNA expression levels in the liver of recipient mice. The FIX:C levels of plasma obtained on the day of animal sacrifice were found to correlate with the relative FIX mRNA levels determined in (A) (R^2=0.7214).

in any of recipient mice. This demonstrates that bioengineered FIX produced from the transplanted hepatocytes does not associate with the development of FIX inhibitors.

To investigate the long-term engraftment of hepatocytes, we performed an another set of single transplantation experiment for 12 weeks (n=6), and confirmed long-term persistency of the increased FIX activities at 0.92%±0.22%, 0.78%±0.22%, 0.78%±0.22%, and 0.83%±0.17% at week 2, 4, 8, and 12, respectively.

The present study confirmed the proof-concept feasibility of hepatocyte transplantation as an alternative therapy to treat hemophilia B. The functional engraftment of transplanted hepatocytes within the recipient livers was confirmed by fluorescence in situ hybridization analyses, FIX mRNA expression, and the secretion of functional FIX into the blood circulation. To acquire the proper hemostatic activity, synthesized coagulation FIX requires several posttranscriptional modification steps within the hepatocytes, including cleavage and removal the prepro leader sequence of 46 amino-acids, and γ-carboxylation of the first 12 glutamic acid residues (*21*). For this reason, primary hepatocytes would be more appropriate for transplantation to produce coagulation factors in hemophilia B than other possible types of genetically modified cells expressing FIX.

Previous studies have shown that engrafted hepatocytes within the livers are able to proliferate in response to the regeneration signals occurred by surgical hepatectomy or chronic liver injuries (*22, 23*). Using primary hepatocytes, our group has developed several innovative approaches to create a functional liver system under the kidney capsule or in subcutaneous locations (*13, 15, 16, 24, 25*), and we have clearly demonstrated that these ectopically engrafted hepatocytes also possess the ability for proliferation (*13, 16, 26*). This would be a significant benefit in the use of these hepatocytes, because most of the adult hemophilia B patients presented with chronic hepatitis B and/or C viral infection as a result of treatments with blood-borne contaminated plasma-derived FIX concentrates. Although portion of the transplanted hepatocytes would be infected with hepatitis viruses in the mean time, it would be reasonable to speculate that engrafted hepatocytes will proliferate and expand, which would further increase the therapeutic effects.

In conclusion, the present studies described the feasibility and safety of hepatocyte transplantation as a treatment modality for hemophilia B. Current therapies to treat hemophilia have been confounded with problems, and the present findings represent an important step toward establishing an alternative therapeutic approach for the treatment of not only hemophilia, but other similar genetic disorders affecting the liver.

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Successful *in vivo* propagation of factor IX-producing hepatocytes in mice: Potential for cell-based therapy in haemophilia B

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Summary

Cell-based therapies using isolated hepatocytes have been proposed to be an attractive application in the treatment of haemophilia B due to the normal production of coagulation factor IX (FIX) in these particular cells. Current cell culture technologies have largely failed to provide adequate isolated hepatocytes, so the present studies were designed to examine a new approach to efficiently proliferate hepatocytes that can retain normal biological function, including the ability to synthesize coagulation factors like FIX. Canine or human primary hepatocytes were transplanted into urokinase-type plasminogen activator-severe combined immunodeficiency (uPA/SCID) transgenic mice. Both donor hepatocytes from canines and humans were found to progressively proliferate in the recipient mouse livers as evidenced by a sharp increase in the circulating blood levels of species-specific albumin, which was correlated with the production and release of canine and human FIX antigen levels into the plasma. Histological examination confirmed that the transplanted canine and human hepatocytes were able to proliferate and occupy >80% of the host livers. In addition, the transplanted hepatocytes demonstrated strong cytoplasmic staining for human FIX, and the secreted coagulation factor IX was found to be haemostatically competent using specific procoagulant assays. In all, the results from the present study indicated that developments based on this technology could provide sufficient FIX-producing hepatocytes for cell-based therapy for haemophilia B.

Keywords

Haemophilia A/B, haemophilia therapy, coagulation factors, hepatology

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Introduction

Haemophilia B is a rare X-chromosome-linked recessive bleeding disorder, caused by a failure in the production of functional coagulation factor IX (FIX), and this disease affects ~1 in 30,000 males (1, 2). The main clinical manifestation of this disease is similar to haemophilia A (factor VIII deficiency), and under severe conditions the affected patient can be found to have unpredictable, recurrent, spontaneous bleeding in various areas, including soft tissues, major joints and occasionally in internal organs. In these circumstances, the onset and progression of chronic haemoarthropathy leads to a marked disruption in the physical and social aspects of the affected patients. Standard treatment for haemophilia B is either on-demand or prophylactic therapy with plasma-derived or recombinant human FIX concentrates. This type of treatment requires frequent intravenous infusion, which can be a potential biohazard from blood-borne viral infections to the patient if the infusate is derived from a heterogeneous population of human blood. In addition, the high cost of commercial concentrates and the life-long requirement for replacement therapy can have a significant impact on economic resources. In an attempt to resolve these difficulties, longer acting

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and safer therapeutic strategies have been investigated. For example, gene therapy using viral vectors has been extensively studied in the past decade (3), and although therapeutic and long-term efficacy has been demonstrated in animal models (4–12), clinical trials have not conclusively shown long-term therapeutic benefit (13, 14). It seems likely, therefore, that alternate therapeutic options will need to be developed.

Recent clinical success with liver transplantation in haemophilia has encouraged further investigation into cell-based therapies (15–17). In haemophilia B patients, elevations in biologically active FIX levels from <1.0% to >1.0%, can alter the phenotype from severe to moderate resulting in a marked improvement in the symptomology and quality of life (1). Coagulation FIX is synthesized in hepatocytes (18), and so cell-based therapies using isolated hepatocytes could provide therapeutic potential. Hepatocytes also produce other coagulation factors, such as factors VII and VIII (19–24), and it may be that this type of treatment could have broader applications to not only haemophlia B, but other coagulation deficiencies. Recently, we have adopted several approaches to bioengineer functional liver tissue *in vivo* (25–30). We have demonstrated that isolated hepatocytes transplanted under the kidney capsule in haemophilia A mice produced therapeutic plasma FVIII activity and corrected the phenotypic defect (28). Dhawan et al. (31) also recently described the therapeutic benefits of hepatocyte transplantation in congenital factor VII deficiency, and the relative technical simplicity of cell-based therapy may offer a significant and technological advantage.

One of the major hurdles in establishing this type of therapy is the limited availability of biologically functional hepatocytes. At present, the number of donor livers remains severely restricted and even if they are available, these livers are frequently of marginal quality (32). Current procedures for the culture of primary hepatocytes do not appear to support extensive cell proliferation (33), so methods to circumvent this problem have recently been studied, but their role to treat haemophilia were not examined. Isolated hepatocytes were genetically modified via transfection with an immortalizing gene, such as simian virus 40 large T antigen, to promote long-term survival (34), but FIX gene expression and production was not investigated. Although the genetic manipulation of hepatocytes can be achieved following isolation *in vitro*, this type of approach to promote hepatocyte proliferation is not a trivial matter *in vivo*. Towards this end, methods to provide proliferative stimuli has been studied *in vivo*, such as a reduction in existing liver mass, or alternatively in a condition where there is likely to be a selective advantage for transplanted cells to proliferate (26, 28). Due to these limitations, we investigated a different method to isolate and proliferate hepatocytes that can retain the hepatic machinery to sustain the synthesis of coagulation factors, such as FIX. In the present study, we studied whether transplantation of canine or human primary hepatocytes into urokinase-type plasminogen activator-severe combined immunodeficiency (uPA/SCID) transgenic mice could enhance the production of coagulation factor IX. The uPA/SCID mouse has been previously shown to have hepatic parenchymal cell damage, which results in the continuous release of regenerative stimuli (35), so we believed that the hepatic environment may be more conducive to the engraftment of *in vitro* isolated hepatocytes. The functionality of the transplanted hepatocytes was assessed in terms of FIX mRNA and protein production and biological activity as a means to treat haemophilia B.

Materials and methods

Animals

Normal beagles were purchased from Oriental BioService, Inc. (Kyoto, Japan). C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). uPA/SCID mice were generated at Hiroshima Prefectural Institute of Industrial Science and Technology (Higashihiroshima, Hiroshima, Japan) as described previously (35). Genotyping for the presence of the uPA transgene in the SCID mice was confirmed by polymerase chain reaction (PCR) assay of isolated genomic DNA as described previously (35, 36). Experimental protocols were developed in accordance with the guidelines of the local animal committees located at both Hiroshima Prefectural Institute of Industrial Science and Technology and Nara Medical University.

Hepatocyte isolation

Canine hepatocytes were isolated from livers (~100 g piece) harvested from two normal beagles (Dog 1: 7-year-old male and Dog 2: 1-year-old female) by a two-step perfusion method using 0.05% collagenase (Collagenase S1, Nitta Gelatin, Osaka, Japan) as described previously (25, 27). Cells were then filtered and hepatocytes were separated from non-parenchymal cells by sequential low speed centrifugation at 50 x g followed by Percoll (Percoll™, Amersham Biosciences, Uppsala, Sweden) isodensity centrifugation. The viabilities of the isolated canine hepatocytes were 96.5% and 98.0% as determined by the trypan blue exclusion test. Hepatocytes were kept at 4°C until transplantation. Human hepatocytes, isolated from a one-year-old white male and a six-year-old Afro-American female, were purchased from In Vitro Technologies (Baltimore, MD, USA). The cryopreserved hepatocytes were thawed and suspended in transplant medium (35, 37). The viabilities of thawed human hepatocytes were determined to be 64.4% and 49.2%, respectively.

Transplantation of hepatocytes for the creation of canine- or human-chimeric mice

One day prior to transplantation and one week after transplantation, the uPA/SCID mice, 20 to 30 days old, received intraperitoneal injections of 0.1 mg of anti-asialo GM1 rabbit serum (Wako Pure Chemical Industries Ltd., Osaka, Japan) to inhibit recipient natural killer cell activity against the transplanted hepatocytes. Viable canine- (1.0×10^6) or human- (0.75×10^6) hepatocytes were transplanted using an infusion technique into the inferior splenic pole in which the transplanted cells flow from the spleen into the liver via the portal system. After transplantation, the uPA/SCID mice were treated with nafamostat mesilate to inhibit complement factors activated by canine or human hepatocytes as previously described (35).

Measurement of plasma levels of albumin, FIX antigen and FIX activity

Periodically, retroorbital bleeding was performed in recipient mice, and the blood was collected in a tube containing 0.1 vol

3.8% sodium citrate. Plasma samples were stored at −80°C until analyzed. To assess the proliferating status of transplanted canine hepatocytes, we determined the plasma levels of canine albumin in the recipient plasma by ELISA using primary goat anti-dog albumin and secondary HRP-conjugated goat anti-dog albumin antibodies (Bethyl Laboratories Inc., Montgomery, TX, USA), respectively. For the assessment of proliferation in transplanted human hepatocytes, we similarly measured the blood levels of human albumin by ELISA (Human Albumin ELISA Quantitation kit, Bethyl Laboratories Inc.). The proportion of proliferating donor hepatocytes in the recipient liver (repopulation rate) was determined based on blood albumin levels (35, 38). Human and canine FIX antigen (FIX:Ag) were measured in recipient plasma by ELISA (Asserachrom IX:Ag, Diagnostica Stago, Asnières, France). Human FIX:Ag levels were measured according to the instructions provided by the manufacturer, and canine FIX:Ag levels were quantified by elongating the enzymatic color reaction step. No cross-reactivity with pooled mouse plasma was observed in this ELISA. FIX activity (FIX:C) was measured by one-stage clotting assay based on the activated partial thromboplastin time using human FIX-deficient plasma (bioMerieux Inc., Durham, NC, USA). Pooled canine plasma collected from 75 normal dogs, and normal human plasma (Verify 1, bioMerieux Inc.) were used as reference standards.

Immunohistochemistry for albumin and FIX

Formalin-fixed, paraffin-embedded liver sections from mice transplanted with canine hepatocytes were sectioned and incubated with a primary goat antibody against canine albumin (Bethyl Laboratories Inc.) at a dilution of 1:1,000. The bound antibody was detected by the avidin-biotin complex immunoperoxidase technique using an ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) followed by developing with DAB (3, 3'-diaminobenzine tetrahydrochloride). Expression of human FIX in recipient mice was determined by immunofluorescent staining of frozen liver sections embedded in O.C.T compound

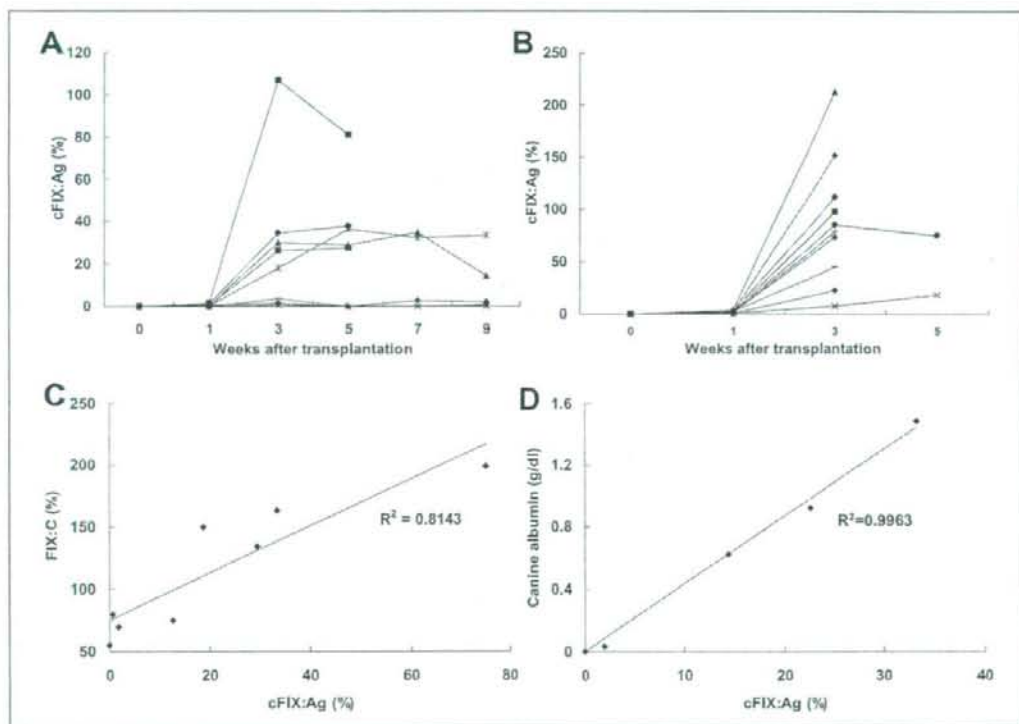

Figure 1: Proliferation of transplanted canine hepatocytes in uPA/SCID mouse livers assessed by recipient plasma analyses. A, B) Plasma canine factor IX (FIX) antigen (cFIX:Ag) levels in uPA/SCID mice after transplantation of hepatocytes isolated from a seven-year-old dog (A) and a one-year-old dog (B) (n=8, 10 in A and B, respectively) (% of pooled normal canine plasma). C) Relationship between total plasma FIX coagulation activity (FIX:C; reflecting both murine and canine FIX activities) (% of normal human plasma) and plasma cFIX:Ag levels of uPA/SCID mice transplanted with canine hepatocytes. D) Relationship between plasma canine albumin concentrations and plasma cFIX:Ag levels of uPA/SCID mice transplanted with canine hepatocytes.

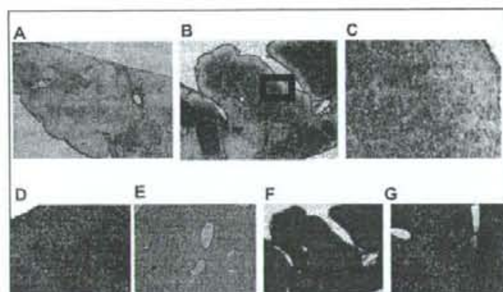

Figure 2: Mouse liver chimerism with proliferated canine hepatocytes. A-E) Immunohistochemical staining of canine albumin in liver sections of uPA/SCID mice transplanted with canine hepatocytes. Representative photomicrographs from a recipient mouse with low plasma cFIX:Ag (2.0% of normal canine plasma) (A) and a mouse with high plasma cFIX:Ag (33.2% of normal canine plasma) (B). C) Higher magnification view of the area outlined in (B). Canine albumin staining of positive control (normal dog liver) (D) and negative control (non-transplanted uPA/SCID mouse liver) (E) indicate the antibody used is specific for canine albumin. F, G) Hematoxylin and Eosin staining on the serial sections of mouse liver from (B). Ca, transplanted canine hepatocytes; Mu, recipient murine liver tissue. Original magnifications, ×40 (A, B, F), ×100 (D,E), and ×200 (C, G).

(Sakura Finetek, Torrance, CA, USA). The sections were incubated overnight at 4°C with the goat anti-human FIX antibody (Affinity Biologicals, Hamilton, ON, Canada) followed by Alexa Fluor 555 rabbit anti-goat IgG (Molecular Probes, Carlsbad, CA, USA) for 60 minutes. Stained sections were subsequently imaged using an Olympus BX51 microscope (Tokyo, Japan) and photographed using an Olympus DP70 digital camera with DP controller and DP manager computer software.

Quantitative real-time PCR

Total RNA was extracted from the liver of all recipient mice, and normal human and canine liver samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Normal human liver tissue portions were obtained from surgical specimens at liver surgery for metastatic liver tumours after acquiring written informed consent for the experimental use of harvested liver samples. Extracted RNA (1 μg) was reverse transcribed using oligo $d(T)_{16}$ primers and Omniscript RT Kit (Qiagen). First-strand cDNA samples were subsequently subjected to PCR amplification using the PRISM 7700 Sequence Detector (Applied Biosystems Japan Ltd., Tokyo, Japan). Canine glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and canine FIX sequences were detected using the following primers. The PCR primers for canine GAPDH sequence were forward, 5'CCCCACCCCCAATGTATCA3', reverse, 5'GTCGTCATATTTGGCAGCTTTCT3', and probe, 5'TGTGGATCTGACCTGCCGCCTG3'.

The primers for canine FIX sequence were forward, 5'GTTGTTGGTGGAAAAGATGCC3', reverse, 5'TGCATCAACTTTCCCATTCAAA3', probe, 'CCAGGTCAATTCCCTTGGCAGGTCC3'. TaqMan probes and primers for human sequences were Hs99999905_m1 (GAPDH) and Hs00609168_m1 (FIX) (TaqMan Gene Expression Assay, Applied Biosystems). The relative RNA copy numbers of canine FIX and human FIX in each transplanted mouse were calculated in terms of canine FIX / canine GAPDH or human FIX / human GAPDH expression ratio, respectively. RNA expression of murine FIX and murine GAPDH, combined with cDNA synthesis and real-time PCR using TaqMan probes, Mm99999915_g1 (murine GAPDH) and Mm01308427_m1 (murine FIX) (Applied Biosystems), were similarly assessed in hepatectomy experiments (see below).

Hepatectomy experiment

For the purpose of investigating the FIX mRNA expression during liver regeneration, liver proliferation stimuli was induced by performing a 70% partial hepatectomy on C57BL6 wild-type mice (n=6) as described previously (39). The resected liver lobes were used as our control for a liver sample under quiescence while the remnant liver lobes removed two days after hepatectomy were used as our proliferating samples. Mouse FIX mRNA and mouse GAPDH mRNA expression was assessed on both quiescent and proliferating liver samples as described above.

Statistical analysis

Significant differences were tested by the Wilcoxon t-test between paired groups and by the Mann-Whitney U-test between unpaired groups. Differences between three or more groups were tested by the Kruskal Wallis H-test. If the probability (p) value was less than 0.05, the Mann-Whitney U-test with Bonferroni correction was used to compare each individual group with the appropriate control. All statistical analyses were performed using Excel (Microsoft) with ystat2006 software (Igakutosyo-syuppan, Tokyo, Japan). $P<0.05$ was considered significant.

Results

Proliferation of FIX-producing canine hepatocytes in uPA/SCID mouse livers

Canine hepatocytes isolated from a seven-year-old and a one-year-old beagle were transplanted into uPA/SCID mice (n=8 and 10, respectively). Canine FIX:Ag was detected in the plasma of five out of eight mice three weeks after transplantation with the isolated hepatocytes from the seven-year-old beagle. In four out of the five mice, the FIX:Ag levels reached between 20–40% of normal canine plasma levels for FIX:Ag (Fig. 1A). One transplanted mouse was detected to have nearly 100% of normal canine plasma FIX:Ag levels. In general, the uPA/SCID mice that received hepatocytes from the one-year-old beagle demonstrated a greater rise in the circulating canine FIX:Ag, and 70% of the mice (7 out of 10) showed levels greater than 50% of normal levels three weeks after transplantation (median: 81.8%; Fig. 1B).

Plasma FIX:C was measured using a one-stage clotting assay. The FIX:C of normal canine pooled plasma and untreated uPA/SCID mouse plasma (n=4) was approximately 200% and 50% of normal human plasma, respectively. The FIX:C in the recipient uPA/SCID mice with high canine FIX:Ag levels was greater than in untreated mice or recipient uPA/SCID mice with low FIX:Ag levels (R^2=0.8143) (Fig. 1C). These observations confirmed that the secreted FIX protein had functional coagulation activity.

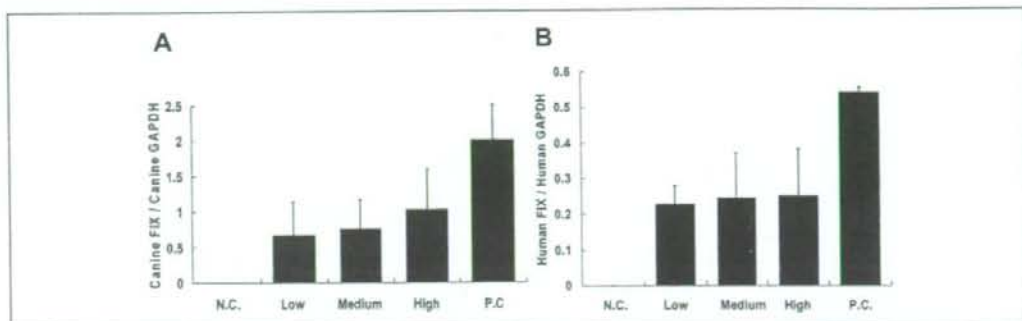

Figure 3: Donor species-specific FIX mRNA expressions in uPA/SCID mouse livers transplanted with either canine or human hepatocytes. A) Canine factor IX (FIX) RNA copy numbers relative to canine GAPDH (reflecting RNA copy numbers per canine hepatocyte), based on plasma cFIX:Ag levels. (Low, <40%; Medium, 41–80%; High, >81% of normal canine plasma. n=4, 4, and 5, respectively). N.C.: negative control: non-transplanted uPA/SCID mouse livers (n=4), P.C.: positive control: normal beagle dog livers (n=3). B) Human FIX RNA copy numbers relative to human GAPDH (reflecting RNA copy numbers per human hepatocyte), based on the repopulation rate (R.R.) estimated from human albumin concentrations as described in *Materials and methods*. (Low, <40%; Medium, 41–65%; High, >66%. n=4, 4, and 4 respectively). N.C., negative control: non-transplanted uPA/SCID mouse livers (n=4); P.C., positive control: normal human liver tissues (n=3).

We also measured canine albumin levels in the plasma of several uPA/SCID mice that received hepatocytes from the seven-year-old beagle, and demonstrated a highly significant correlation between the canine albumin and canine FIX:Ag levels (R^2=0.9963) (Fig. 1D). Assuming that the plasma concentrations of albumin and FIX:Ag in normal dogs are 5 g/dl and 5,000 ng/ml, respectively, the weight ratio of albumin to FIX:Ag in normal canine plasma was calculated to be 10,000:1. These data suggested that the synthesis of canine FIX and albumin in the transplanted animals was similar to that of normal canine liver (i.e. 15% FIX:Ag of normal canine plasma corresponds to 750 ng/ml, and the ratio of 0.6 g/dl to 750 ng/ml approximates to 10,000:1). Immunohistochemical staining for canine albumin in sections obtained at day 55 after transplantation demonstrated a large area of the liver was positive in the recipients with high plasma canine FIX:Ag (33.2%) (Fig. 2B-C), whereas only a small area of liver was positive in mice with low plasma FIX:Ag levels (2.0%) (Fig. 2A). Histological examination of serial liver sections revealed that the canine albumin-positive area was composed of morphologically normal hepatocytes (Fig. 2D-E) indicating that the normal canine hepatocytes had progressively propagated in the uPA/SCID livers.

The uPA/SCID mice that received canine hepatocytes were divided into three groups according to their plasma canine FIX:Ag levels (low <40%, medium 41–80%, and high >81%). mRNA levels of canine FIX were normalized using canine GAPDH mRNA measurements (FIX / GAPDH). As shown in Figure 3A, canine FIX / canine GAPDH expression was similar in the three groups with no statistically significant difference. This suggests that canine hepatocytes proliferated within the uPA/SCID livers without reducing the steady-state levels of canine FIX gene expression and/or degradation. We confirmed that RNA samples from untreated uPA/SCID livers were not amplified by the primer set used for canine FIX and GAPDH detection (Fig. 3A).

Proliferation of FIX-producing human hepatocytes in uPA/SCID mouse livers

Human hepatocytes were transplanted into the liver of uPA/SCID mice (n=12). The proliferation and propagation status of the transplanted hepatocytes were assessed by periodic measurement of human albumin levels in the recipient blood, and the repopulation rate of human hepatocytes in the uPA/SCID livers was assessed as described previously (35, 38). Human FIX:Ag was detected in the plasma of 75% of the mice (9 out of 12) between 67 and 84 days after transplantation, and the circulating plasma levels ranged between 6–58% found in normal humans. The results from our study demonstrated that the FIX:Ag levels were highly correlated with the human albumin levels (R^2=0.8714) (Fig. 4A). To examine the biological function of the secreted human FIX, we compared the repopulation rate with FIX:C assays (Fig. 4B). Although both murine and human FIX:C could be measured using the clotting assay, we were able to estimate the approximate levels of the *de novo* expressed human FIX:C present in our samples. Plasma levels of FIX:C in untreated uPA/SCID mice were less than 50% of the levels normally found in human plasma, and we expected to increase the FIX:C levels up to 100% following the humanization of the murine livers. Similar to the canine transplants, the results showed that mice with a high repopulation rate had higher FIX:C than those with low repopulation rates (R^2=0.7245). These data suggested that secreted human FIX protein was biologically active. To clarify the proliferation status of transplanted human hepatocytes in uPA/SCID mouse liver, we also transplanted human hepatocytes isolated from another human subject (a 2-year-old Caucasian male) into a new set of uPA/SCID mice (n=9). Using these mice, we measured plasma human FIX:Ag levels, total plasma FIX:C levels, and human plasma albumin concentrations from samples obtained periodically from the recipient mice during an eight-week period after transplantation. As shown in Fig-

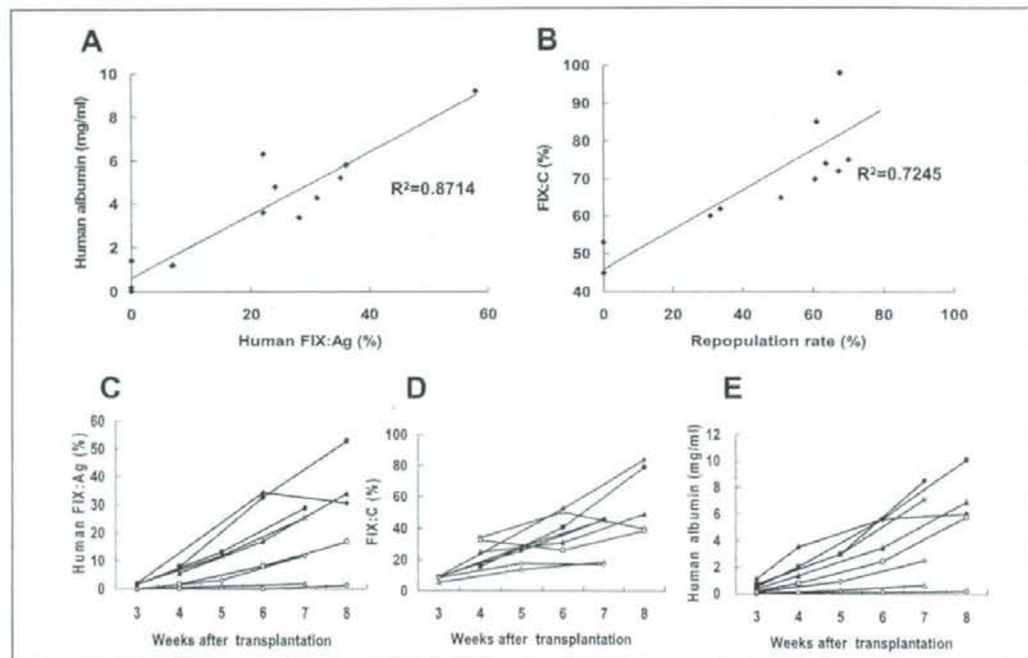

Figure 4: Proliferation of transplanted human hepatocytes in uPA/SCID mouse livers assessed by recipient blood analyses. A) Relationship between blood human albumin and plasma human factor IX (FIX) antigen (hFIX:Ag) concentrations of uPA/SCID mice transplanted with human hepatocytes (n=12). B) Relationship between total plasma FIX coagulation activity (FIX:C; reflecting both murine and human FIX activities) (% of normal human plasma) in uPA/SCID mice transplanted with human hepatocytes based on the repopulation rate estimated from human albumin concentrations as described in *Materials and methods*. The recipient blood samples used for these assays were obtained 67–84 days after transplantation. C-E) Time course of plasma hFIX:Ag levels (C), total plasma FIX:C levels (D), and blood human albumin levels (E) of the recipient mice after human hepatocyte transplantation (n=9) (different set of experimental data from that shown in A and B).

ure 4C-E, each of our measured parameters were found to be increased after transplantation in most of the mice, which was indicative of a robust proliferative status of the transplanted human hepatocytes.

Liver sections obtained from mice with high plasma human FIX:Ag levels were found to have strong positive staining in the hepatocytes for human FIX as determined by immunohistochemistry (Fig. 5B). In marked contrast, only a small portion of the liver stained positive in sections of recipient mice that were detected to have low (i.e. $<1\%$) circulating levels of plasma FIX:Ag level (Fig. 5A). These results were consistent with the findings that the *de novo* production of haemostatically active human FIX in the circulation was dependent on the viability and persistence of the transplanted hepatocytes in the recipient uPA/SCID livers.

The uPA/SCID mice that received human hepatocytes were divided into three groups according to the repopulation rate (low $<40\%$, medium 41–65%, and high $>66\%$). Human FIX mRNA levels were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA measurements (FIX:GAPDH). As shown in Figure 3B, the ratio of human FIX:GAPDH mRNA expression in the recipient livers was not significantly different among the three groups. We further confirmed that extracted total RNA from untreated uPA/SCID livers were not amplified by the primer set used for human FIX and human GAPDH detection (Fig. 3B), demonstrating the specificity of the primers to human and not murine FIX expression. Similar findings were determined in the canine hepatocyte transplantation experiments in which we confirmed that human hepatocytes proliferated in uPA/SCID mouse livers and retained their ability for transcribing the human FIX gene.

It has been reported that differentiated liver function (e.g. mRNA expression of albumin) may be suppressed when hepatocytes are subjected to various proliferative stimuli (40, 41). It is not known, however, if hepatocyte proliferation could directly influence FIX mRNA expression. To address this question, we compared FIX mRNA expression in quiescent and proliferating mouse livers. Liver proliferation was induced by performing a

70% hepatectomy in C57/BL6 wild-type mice, and the remnant liver lobes were subsequently harvested two days after the hepatectomy, which is the time point where hepatocyte proliferation is at its peak. The quiescent (non-proliferative) liver samples used in these experiments were the resected liver lobes obtained from the same mice in which the partial hepatectomy was performed. The relative FIX mRNA expression (FIX:GAPDH) was ~35% lower in the proliferating liver compared to the quiescent liver (p=0.029; Fig. 6). These results would suggest that the proliferative status of the transplanted hepatocytes may affect the production of FIX, and the reason for the lower FIX mRNA expression found in the recipient mice compared to the control livers as shown in Figure 3 may be due to active proliferation by the transplanted hepatocytes. If the FIX mRNA expression levels found in the normal canine and human livers were recalculated to account for a 35% reduction in response to proliferative stimuli, the significant differences between the four groups shown in Figure 3 became non-significant (data not shown). This suggests that proliferating hepatocytes in uPA/SCID mice have the capability of expressing normal levels of canine and human FIX following transplantation.

Discussion

We have established an *in vivo* system to propagate human and canine hepatocytes in uPA/SCID mouse livers, and these transplanted hepatocytes are capable of retaining their cellular machinery to produce coagulation FIX. The capabilities of these propagating transplanted hepatocytes to synthesize FIX were confirmed by the expression of FIX mRNA, FIX-protein production and secretion, and its coagulation activity. The main reason we decided to study canine hepatocyte in addition to human hepatocytes for transplantation, is the availability of a pre-clinical large animal model for haemophilia B studies (42), which will be able to be used for proof-of-concept experiments.

Hepatocytes are the only cells that are known to synthesize FIX (18), and successful liver transplantation has resulted in restoration of normal coagulative properties in patients with haemophilia B (17). Although there are obvious benefits in surgically transplanting whole livers in haemophilic patients with critical life-threatening liver diseases such as chronic active hepatitis, this approach is likely not appropriate for most patients due to other obvious negative risks associated with this type of procedure. For this reason, the hepatocyte transplantation approach described in the current study, which is less invasive and requires fewer donor livers, may provide a viable alternative strategy to organ transplantation. Recent trials have highlighted successful application of hepatocyte transplantation in two patients with coagulation factor VII deficiency (31, 43). Following hepatocyte transplantation, both patients were found to have achieved significant and prolonged therapeutic benefit with a marked decrease in the infusion of exogenous recombinant factor VIIa due to episodic bleeding (44, 45).

We demonstrated that canine and human hepatocytes progressively proliferated and propagated in the recipient livers of uPA/SCID mice. Real-time PCR analysis at various stages of hepatocyte propagation showed that FIX mRNA expression per transplanted cell (per donor-specific GAPDH mRNA level) was stably maintained for the duration of the experiment. Plasma FIX:Ag levels were highly correlated with the propagation status of the transplanted hepatocytes as determined by the blood levels of canine and human albumin. Furthermore, the procoagulant function of the secreted canine and human FIX was confirmed by clotting assays. FIX:C increased from baseline levels (less than 50% of normal human plasma) to normal human or canine FIX:C levels (about 100% and 200%, respectively) as repopu-

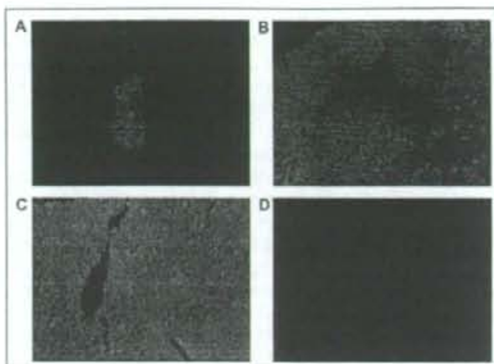

Figure 5: Mouse liver chimerism with proliferated human hepatocytes. Immunofluorecent staining of human FIX in liver sections of uPA/SCID mice after human hepatocyte transplantation. Liver sections of mice with <1% plasma hFIX:Ag level (A) and mice with 34% hFIX:Ag plasma level (B). Red-stained area indicates proliferated human hepatocytes producing hFIX. Positive control (normal human liver tissue) (C) and negative control (non-transplanted uPA/SCID mouse liver) (D) indicate the staining specificity for human FIX. Original magnification, ×100.

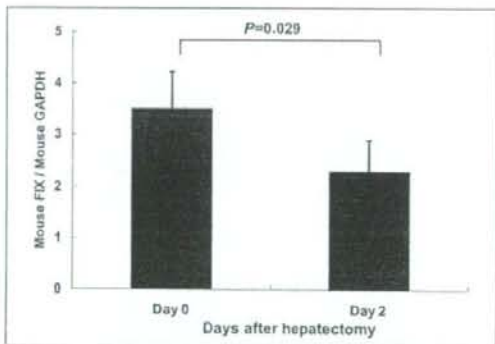

Figure 6: Comparison of factor IX (FIX) mRNA expression levels in quiescent and proliferating livers. Day 0 liver samples (quiescent status) were obtained from C57/BL6 mice at the time of 70% partial hepatectomy (n=6). The remnant liver lobes of the mice were harvested at day 2 were used for the assessment for proliferating status (n=6). Relative FIX mRNA expression was expressed as murine FIX / murine GAPDH.

lation by transplanted hepatocytes progressed. The data from our experiments indicated that post-transcriptional modification of FIX, including cleavage and removal of the pre-pro leader sequence of 46 amino-acids, γ-carboxylation of the first 12 glutamic acid residues, and partial β-hydroxylation of Asp 64 (46), must have occurred within the transplanted hepatocytes to maintain biologically active haemostatic function.

Hepatocytes from a one-year-old dog demonstrated high proliferation activity compared with cells from an older (7-year-old) dog as evidenced by the more rapid increase and its higher sustained levels of plasma canine FIX:Ag. These results are consistent with earlier findings by our group in which we reported that human hepatocytes from a younger donor occupied a larger proportion in the recipient uPA/SCID mouse liver compared with transplanted hepatocytes from an older donor (47). One possible reason for the enhanced growth potential of hepatocytes in these circumstances may be due to an elevated expression of cell cycle proteins in hepatocytes from younger compared to older donors (48). With the present study as well as previous work in the literature, we believe that the age of the donor makes a difference on the proliferation and repopulation of the transplanted hepatocytes in uPA/SCID mouse livers.

Human hepatocytes propagated in uPA/SCID mice could be isolated and purified using cell-sorting technology (38). Recently, our group has developed a procedure to isolate human hepatocytes that were propagated in uPA/SCID mouse livers, and these isolated hepatocytes were confirmed to be biologically functional compared to original primary hepatocytes, demonstrating the expression of cytochrome P450 (CYP) (38). We have also been experimentally successful in engineering functional liver tissue using isolated hepatocytes transplanted under the kidney capsule or in the subcutaneous space by demonstrating coagulation factor VIII expression (25–30). More recently, Azuma et al. (49) reported an alternate method to propagate human hepatocytes in living mice that furthers the utility of hepatocyte transplantation. Based on these developments, propagated human hepatocytes with FIX expressions should become a valuable cell source in establishing novel cell-based therapies for direct transplantation or development of tissue engineering strategies in the treatment of haemophilia B.

For the eventual translation of cell-based therapies using the propagated human hepatocytes for haemophilia B to be successful in the clinics, several potential obstacles will need to be considered and overcome. First, contamination of murine cells during the isolation of the transplanted human hepatocytes must be minimized. Second, increasing the engraftment rate of the transplanted hepatocytes into the recipient liver. Lastly, the survival and viability of the transplanted allergenic hepatocytes must be prolonged. With regards to the first issue, the contaminating murine hepatocytes during the isolation of human cells from the mouse liver could be overcome by utilizing recipient transgenic mice that have been incorporated with a inducible suicide gene. In the presence of the inducing agent, the murine cells would be preferentially eliminated and increase the purity of the human hepatocyte mixture leading to enhanced clinical safety. To overcome the low engraftment rate found in the current and previous studies, the recipient livers will require some type of preconditioning regimen to maximize the efficiency and engraftment. Slehria et al. (50) reported an effective and non-invasive pretreatment protocol in which the administration of phentolamine, an adrenergic receptor blocker, resulted in the dilation of the hepatic sinusoidal vasculature leading to enhanced hepatocyte engraftment rate. For the last issue regarding the limited graft survivability of the donor cells due to the activation of the host immune system, it will be important to design an immunosuppressive regimen specific for hepatocyte transplantation and monitoring systems for the early rejection need be established. These issues will need to be studied and overcome to substantiate the utility of this approach for the treatment of haemophilia and other congenital liver disorders.

In all, the present study has demonstrated the utility of hepatocyte transplantation for the therapeutic production of coagulation factor IX. As we continue to overcome the obstacles associated with this approach, this transplantation methodology will evolve into a novel approach to treat not only liver diseases associated with haemophilia but other forms of congenital liver diseases.

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第3は50IU／kgを3〜3・5回／週投与する低用量プロトコールである[34]。

それぞれの成功率は、成功の定義に若干差があるものの73％、80％および83％である。BonnおよびMalmoプロトコールの実施は困難であるが、低用量プロトコールは我が国でも実施されている。

ITIの成功要因については様々な検討がなされているが、現時点で統計学的に明らかにされているのは、ITI開始時のインヒビター力価、今までの最高インヒビター力価および開始後のインヒビター力価である[35]。

その他、ITI成功要因として重要視されているのは、製剤の投与量と製剤の種類（血漿由来製剤vs遺伝子組み換え型製剤、FⅧ vs FⅧ／vWF製剤）である。現在、製剤の投与量をめぐって国際的な多施設無作為化比較対照試験が実施されている。血友病Bインヒビターの有効率は一般に低い。また、アナフィラキシーを含むアレルギー症状を有する症例も稀でなく、今後さらに有効なITIのプロトコールが必要である。

文　献

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の止血療法と同様、大手術時には2時間ごとの投与を1～2日間行い、以後は例えば3、4、6、8、12時間ごとというように、徐々に投与間隔を延ばしていく投与レジメが勧められている[11]。

ⓑrFⅦaの安全性

rFⅦaが国際的に市販開始された1996年以後、70万回以上標準的な投与（90μg／kg）が実施されたが、血栓性の有害事象は16例であった。内訳は10例が心筋梗塞、脳血管障害などの動脈性、6例が静脈性の血栓症であった[26]。症例の多くはaPCC投与後、細菌感染症の併発など血栓症発症リスクの高いものが多く、rFⅦa投与に直接起因したかについては不明である。しかしながら、rFⅦaにおいてもaPCCと同様、感染症や血栓症発症リスクを有する患者においては注意が必要である。

(5)バイパス止血療法の新たな展望

①rFⅦa単回高用量

rFⅦaの1出血エピソードに対する平均投与回数は2・5回で、単回投与で十分な止血効果を得ることは困難である。また、特に小児では半減期も短く、血管の確保が困難な症例もあり、精神的・身体的負担が大きい。

Parameswaranら[27]は、200μg／kg以上の投与量における止血率は97％と、通常量での止血率（84％）を上回ることを報告している。さらにKenetら[28]は通常投与量の3倍量の270μg／kg単回投与が、90μg／kg3回投与と同等の止血効果が得られることを報告し、高用量単回投与の有効性と安全性が国際的にも認知されるようになった。その後、高用量単回投与と標準用量複数回投与群での比較対照試験が実施され、有効性の差もなく、また特記すべき有害事象も見られないことが明らかにされた。

rFⅦa高用量単回投与については、欧州では2007年に正式に承認された。我が国では未承認であるが、最近、高用量単回投与に関する医師主導型のクロスオーバー試験が実施され、標準投与量と同等の有効性が認められた[29]。

②予防的投与

最近、インヒビター保有患者においてもバイパス止血療法による予防的投与法が注目されている。

Dimicheleらの集計[18]では、血友病Aインヒビター保有14症例で15クールのaPCC予防投与が実施されていた。投与期間は平均19・5カ月（0・25～26カ月）で、投与回数は1回／日～／週、投与量は平均69単位／kg（15～100単位／kg）であった。

予防効果は、出血頻度の低下が見られたのは14症例中（15予防投与治療中）11例、QOLの改善が見られた例は3例で、あとは患者から希望された例が1例、外科処置後の予防投与例が1例であった。

Leissingerら[30]も、インヒビター保有5症例に対して6カ月以上にもわたってaPCCを定期的に投与して、いずれの症例においても出血頻度は減少し、かつ、特に副作用も見られなかったことを報告している。

一方、rFⅦaは半減期が短いことから、予防投与への応用は困難と考えられてきたが、最近、rFⅦaの予防投与（200～250μg／kg隔日～220μg／kg連日）を実施した13症例中12例で、実施前に比し出血頻度は減少していたことが報告された[31]。したがって、バイパス療法の定期投与による止血効果に関するエビデンスが蓄積しつつある。

今後、バイパス製剤による予防投与に関する国際的な臨床研究が望まれる。

(6)免疫寛容療法

免疫寛容療法（ITI）はBrackmannら[32]が30年前に最初に報告した方法で、第Ⅷ因子や第Ⅸ因子製剤を大量に反復して投与することによりインヒビターの産生機構をブロックし、インヒビターの消失を図る治療である。

ITIのプロトコールは大きく3種類ある。第1はBrackmannらの原法に基づいた高用量法（Bonnプロトコール）で、ハイレスポンダー症例に200IU／kgの第Ⅷ因子製剤を2回／日連日投与する。

第2はITIの治療期間を短縮させるために、免疫抑制剤、γグロブリンを併用し、さらに、ITI開始時のインヒビター力価を下げるためにプロテインAカラムを用いるMalmoプロトコールである[33]。

遺伝子組み換え活性型第Ⅶ因子製剤（rFⅦa，ノボセブン®）の2製剤である（表1）。

①aPCC

ⓐaPCCによる急性出血症状に対する止血治療

aPCCはビタミンK依存性因子であるプロトロンビン、第Ⅶ因子、第Ⅸ因子および第Ⅹ因子を主成分とするが、活性型第Ⅻ因子（Ⅻa）、活性型第Ⅸ因子(Ⅸa)、活性型第Ⅹ因子（Ⅹa）、活性型第Ⅶ因子（Ⅶa）およびトロンビンなどの活性型凝固因子も含まれている。aPCCの止血作用の主なメカニズムは第Ⅴ因子依存性にプロトロンビンと活性型第Ⅹ因子がプロトロンビナーゼ活性を増幅してトロンビン産生を促すことと考えられている[16]。

欧州の多施設後ろ向き調査によると、aPCCの急性出血症状に対する有効率は81・3％で、関節内出血に限定すると有効例のうち50・7％が1回、31・2％が2回、7・4％が3回投与されていた[17]。さらに、最近、欧米で実施された後ろ向き調査では、169エピソード中110エピソード（65％）が治療中に止血したが、45エピソードで止血が遅延し、無効例は7例であった[18]。aPCCの単回投与で止血できた例が21％、2回投与が16％、3回投与が48％であった。したがって、aPCCの単回投与では止血が困難な例も少なからず存在することが分かる。

ⓑaPCCによる外科処置・手術時の止血管理

従来、高力価のHRインヒビター保有例の外科的処置や手術をaPCC単独で実施することは、止血効果の不確実性、高額な医療費、血栓症や播種性血管内凝固症候群（DIC）発症の危惧、さらにエビデンスが少ないなどの理由によりきわめて困難であった。

しかし、近年、aPCC単独使用による実施例の報告が増加している。Negrierらの多施設後ろ向き調査[17]によると、19例の小外科処置に対してaPCCが使用されていた。最も多いのが関節穿刺（10例）であった。大手術では膝関節滑膜切除術、膝関節形成術、皮膚筋肉形成術、前立腺腫摘出術に使用されていた。Dimicheleらの調査報告[18]では、足関節、膝関節形成術、下肢のコンパートメント症候群などで使用されていた。

我が国では、aPCCに関して、インヒビター力価10BU／mℓ以上、投与期間3日の使用制限があったために、大手術に使用された経験は少なかった。しかしながら、平成20年度よりこれらの制限が撤廃されたために、我が国でも手術や外科処置時の止血管理にもaPCCを使用できるようになった。

ⓒaPCCの安全性

Ehrlichら[19]の報告によると、計39万5000回のaPCC投与で発生した有害事象（adverse event：AE）は55例で、そのうちの16例がDICと心筋梗塞などの血栓症関連のAEである。しかしながら、AE発症16例中13例（81％）に何らかの血栓症発症リスクが認められた。半数の8例が過剰投与であった。AE発症リスクを十分に考慮して適正な投与量を選択することが重要である。

②rFⅦa

rFⅦaは、インヒビター保有血友病症例に対する止血治療製剤として1988年に初めて臨床応用され、今やaPCCと並んで重要なバイパス止血療法製剤である[20]。本製剤の止血メカニズムは大量投与により組織因子非依存性に活性化血小板上でトロンビンバーストを惹起することが考えられている。

rFⅦaの有効性は、出血後製剤投与までの時間経過に強く依存している。実際、出血症状の出現後、6時間以内に投与を開始することにより、明らかな止血効果を得られることが報告されている[21]。さらに、家庭内投与を基盤に早期投与を開始することにより、より少ない投与回数で止血効果が得られることも明らかにされている[22]。我が国で実施された市販後調査によると、3時間以内投与群では有意に有効率が高かった[23]。

ⓐrFⅦaによる外科処置・手術時の止血管理

大手術21例、小手術57例、抜歯関係25例の計103例の外科手術におけるrFⅦaの有効性を検討した報告[24]によると、rFⅦaの著効＋有効例はそれぞれ81％、86％、92％であった。我が国でも、偽腫瘍摘出術などの大きな整形外科的な手術の止血管理に使用されている[25]。

ガイドラインでは重篤な出血時