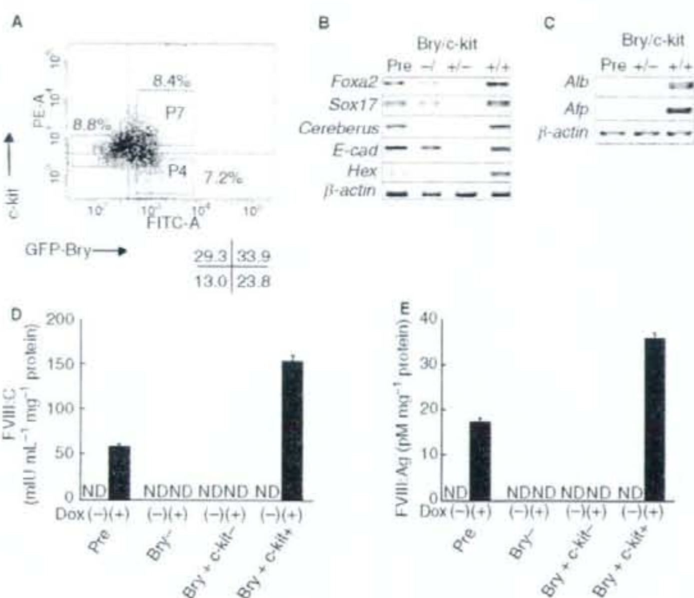

Fig. 4. GFP–Bry$^+$/c-kit$^+$ cells contain the definitive endoderm progenitors and efficiently secrete FVIII. (A) FACS profile for GFP–Bry and c-kit among day 3.5 embryoid bodies cultured in serum-containing medium. (B) Reverse transcription polymerase chain reaction analysis demonstrating the presence of endoderm-related genes in populations derived from presorted cells (pre) or cells sorted on the basis of GFP–Bry and c-kit. (C) Cells from presorted populations or those sorted on the basis of GFP–Bry and c-kit were reaggregated for 1 day, and then cultured in serum replacement medium and replated on day 10. *Alb* and *Afp* mRNA was expressed in GFP–Bry$^+$/c-kit$^+$ cells on day 15. (D, E) Levels of FVIII:C (D) and FVIII:Ag (E) in medium conditioned by presorted cells and those sorted on the basis of GFP–Bry and c-kit, with or without doxycycline (Dox) (1 μg mL^{-1}) induction. The data presented are means of three independent experiments; the error bars represent the SEM. ND, not detected; FITC, fluorescein isothiocyanate; PE, phycoerythrin; GFP, green fluorescent protein; Bry, brachyury.

and GFP–Bry$^+$/c-kit$^-$ cells did not secrete FVIII at all, even after induction with Dox.

Discussion

ES cells capable of secreting human FVIII may represent a unique source for a future cell-based treatment protocol for hemophilia. In the present study, we were able to establish mouse ES cells secreting functional human FVIII with coagulant activity. Tet-WT-*F8* ES cells were established by integrating full-length human *F8* cDNA under the control of the tet operator, which enabled *F8* transcription to be induced by Dox stimulation. We found that levels of FVIII secretion depended on the conditions under which the ES cells were differentiated, regardless of *F8* mRNA expression. Among the conditions that we evaluated, those leading to development of endoderm/liver EBs were the most suitable for efficient FVIII secretion. Furthermore, the efficacy of FVIII secretion was dramatically improved by using 226aa/N6 cDNA, a recently described B-domain variant of *F8* [13]. To our knowledge, this is the first report of an ES/EB system that secretes detectable levels of active human FVIII *in vitro*.

We found it noteworthy that FVIII was present in the supernatant of liver-like EBs, but not in that of undifferentiated ES cells or hematopoietic-like EBs, although the induction of *F8* mRNA was detected in all conditions. It has previously been shown that the transcriptional activity of *F8* is not a critical determinant of plasma FVIII levels, and that mRNA levels are not, themselves, sufficient to predict FVIII secretion [22]. The primary FVIII translation product must be translocated into the lumen of the endoplasmic reticulum (ER), where folding and N-linked glycosylation occurs. Improperly folded FVIII molecules are recognized by chaperones and are not released, but are instead transferred into degradative pathways [23]. Our results indicate that cells with this capacity only appear during differentiation of liver-like EBs, making them more suitable for FVIII secretion than undifferentiated ES cells or hematopoietic-like EBs. Although liver-like EBs expressed hepatocyte-specific marker gene such as *Alb*, *Ttr* and *Tat*, mouse *F8* mRNA was not induced. The reason for this is currently unclear, but liver-like EBs may be still have an immature phenotype for endogeneous *F8* expression. Previous reports have demonstrated that platelets are good targets for the lentivirus-mediated gene therapy of FVIII production [24]. Our hematopoietic EBs were previously showed to contain megakaryocytes, but not platelets [11]. Thus, hematopoietic EBs probably fail to produce FVIII because of the immature differentiation of platelets from megakaryocytes.

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We also observed that the 226aa/N6 construct is an extremely useful tool for FVIII production from ES cells. It is known that expression of BDD-*F8* results in a seventeen-fold increase in mRNA levels over WT-*F8*, although it yields only a 1.3-fold increase in the amount of secreted protein [22]. As the reason for imbalance between mRNA and protein levels, BDD-*F8* may have a defect in efficient transfer of the primary translation product from the ER to the Golgi via interaction with the 53-kDa ER–Golgi intermediate compartment protein [25]. To overcome this problem, Miao *et al.* [13] created another bioengineered construct, 226aa/N6. They showed that transfecting COS-1 and CHO cells with 226aa/N6 resulted in a 4–11-fold increase in FVIII secretion, as compared to transfection with BDD-*F8*. Consistent with those studies, we found that BDD-*F8* improved FVIII secretion only about 1.5-fold, as compared to WT-*F8*, whereas tet-226aa/N6 ES cells showed a ten-fold increase in FVIII secretion, as compared to tet-WT-*F8* ES cells. Thus 226aa/N6 appears to provide a significant advantage over BDD-*F8* with respect to FVIII production from ES cells, making it the optimal construct for FVIII secretion.

In our data, the levels of FVIII:C seem to be higher than those of FVIII:Ag, especially in tet-226aa/N6 ES cells. To investigate this discrepancy, we also assessed FVIII:C by a COAtest chromogenic assay (Chromogenix, Mölndal, Sweden) with recombinant FVIII as a standard. In this experiment, FVIII:C was detected at lower levels (about 40–50%) than that evaluated by plasma standard (data not shown). These results were in good accordance with previous reports that FVIII:C level against a plasma standard was higher than that against a recombinant FVIII standard by the chromogenic assay [26]. Thus, the discrepancy between FVIII:C and FVIII:Ag may result from the overestimation of FVIII:C by the APTT clotting assay with plasma standard.

Recently, Gouon-Evans *et al.* [20] demonstrated that the GFP–Bry$^+$/c-kit$^+$ cell population contained definitive endoderm progenitors when ES cells were differentiated in serum-free medium with activin stimulation. Using serum differentiation, we also found that the GFP–Bry$^+$/c-kit$^+$ cell population contained endoderm progenitors and that cells with the liver marker genes *Alb* and *Afp* appeared in this fraction. We further showed that cells differentiated from endoderm progenitor (GFP–Bry$^+$/c-kit$^+$) cells secreted FVIII more efficiently on day 14 of differentiation than presorted cells. By contrast, the sorted GFP–Bry$^-$ (ectoderm progenitor) and GFP–Bry$^+$/c-kit$^-$ (mesoderm progenitor) fractions secreted no FVIII, even after induction with Dox. These findings suggest that cells with the capacity for FVIII production are probably present within endoderm-derived tissue such as liver.

When we consider applying these strategies for human therapy, safety issues will be a big concern. An earlier study showed that grafts containing the undifferentiated ES cells rapidly form teratomas, even when only 0.2% of the cells within the transplanted clusters are positive for the undifferentiated marker SSEA-1 [27]. A recent study succeeded in transplanting ES-derived cardiomyocytes without evidence of teratoma formation in *in vivo* mouse models when selectable markers were employed to eliminate undifferentiated ES cells [28]. Thus, it will probably be necessary to develop a system involving selection markers in our tet-226aa/N6 ES cells for further *in vivo* studies.

In conclusion, we established ES cells secreting human FVIII with tetracycline regulation. The combination of endoderm progenitors, liver condition and 226aa/N6 cDNA could improve production to a significant level of human FVIII from ES cells. Our *in vitro* findings will be the first step for ES cell-based therapy as a potentially useful approach to the treatment of hemophilia A. Further *in vivo* studies are anticipated.

Addendum

S. Kasuda performed laboratory studies, data analysis and interpretation and drafted the manuscript. A. Kubo and Y. Sakurai designed the study, interpreted the data and drafted the manuscript. S. Irion and S. W. Pipe contributed vital new reagents and edited the manuscript. K. Ohashi and K. Tatsumi performed laboratory studies and edited the manuscript. Y. Nakajima, Y. Saito and K. Hatake helped to design the study, interpret the analyses and edit the manuscript. A. Yoshioka and M. Shima contributed critical analytical tools and data interpretation, and edited the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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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

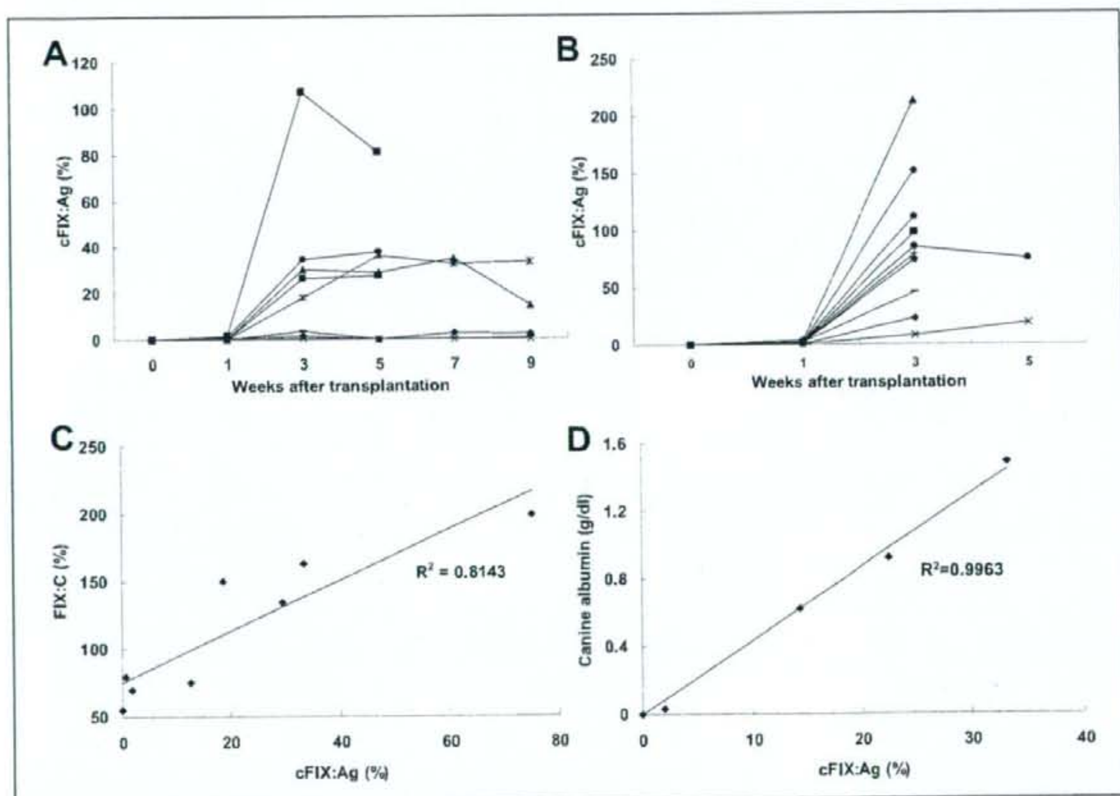

Figure 1: Proliferation of transplanted canine hepatocytes in uPA/SCID mouse livers assessed by recipient plasma analyses. A, B) Plasma canine factor IX (FIX) antigen (cFIX:Ag) levels in uPA/SCID mice after transplantation of hepatocytes isolated from a seven-year-old dog (A) and a one-year-old dog (B) (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.

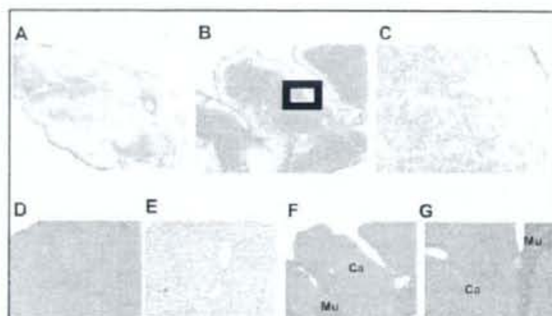

Figure 2: Mouse liver chimerism with proliferated canine hepatocytes. A-E) Immunohistochemical staining of canine albumin in liver sections of uPA/SCID mice transplanted with canine hepatocytes. Representative photomicrographs from a recipient mouse with low plasma cFIX:Ag (2.0% of normal canine plasma) (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.

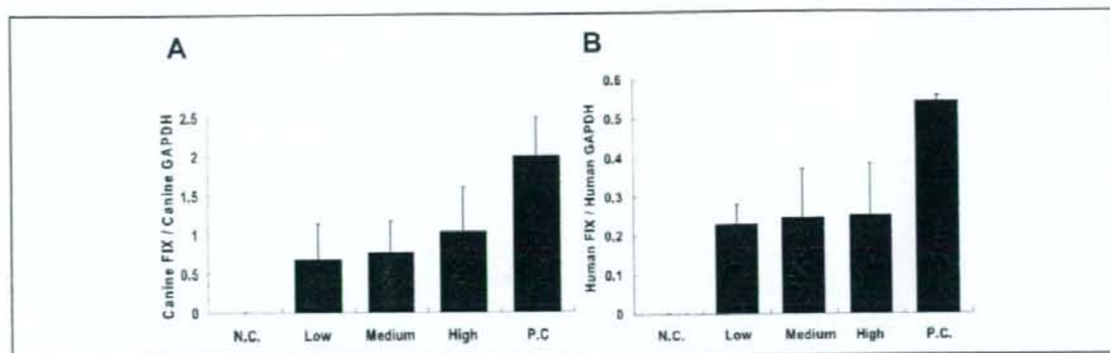

Figure 3: Donor species-specific FIX mRNA expressions in uPA/SCID mouse livers transplanted with either canine or human hepatocytes. A) Canine factor IX (FIX) RNA copy numbers relative to canine GAPDH (reflecting RNA copy numbers per canine hepatocyte), based on plasma cFIX:Ag levels. (Low, <40%; Medium, 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-

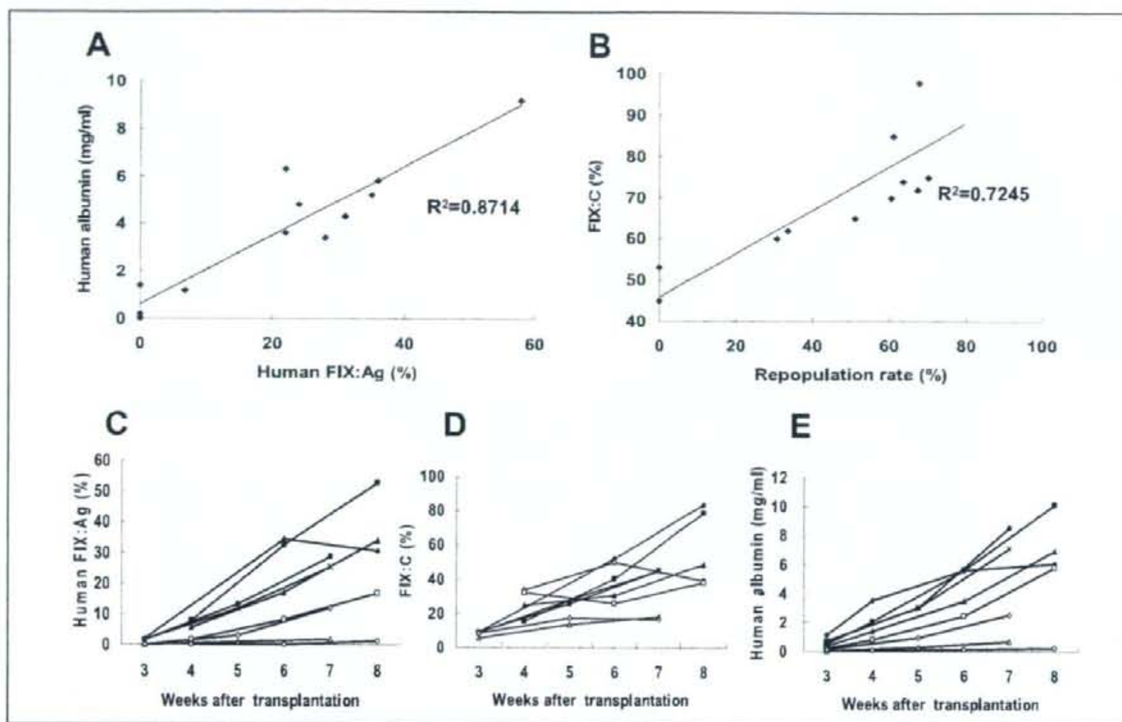

Figure 4: Proliferation of transplanted human hepatocytes in uPA/SCID mouse livers assessed by recipient blood analyses. A) Relationship between blood human albumin and plasma human factor IX (FIX) antigen (hFIX:Ag) concentrations of uPA/SCID mice transplanted with human hepatocytes (n=12). B) Relationship 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.

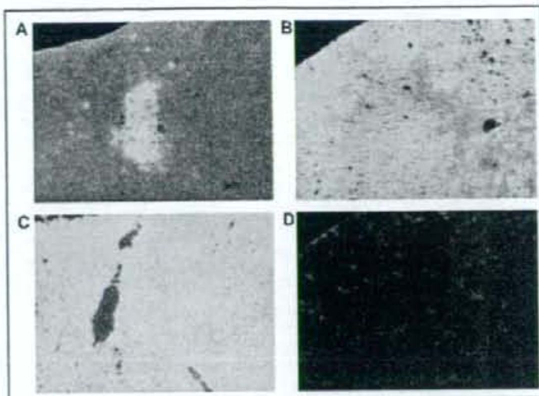

Figure 5: Mouse liver chimerism with proliferated human hepatocytes. Immunofluorecent staining of human FIX in liver sections of uPA/SCID mice after human hepatocyte transplantation. Liver sections of mice with <1% plasma hFIX:Ag level (A) and mice with 34% hFIX:Ag plasma level (B). Red-stained area 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.

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-

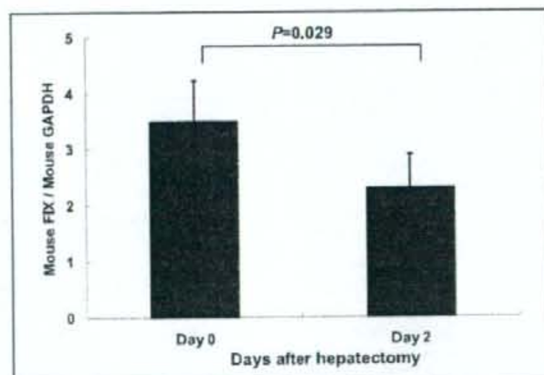

Figure 6: Comparison of factor IX (FIX) mRNA expression levels in quiescent and proliferating livers. Day 0 liver samples (quiescent status) were obtained from C57/BL6 mice at the time of 70% partial hepatectomy (n=6). The remnant liver lobes of the mice were harvested at day 2 were used for the assessment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

軽症血友病Aから検出される第VIII因子R531H変異の第VIII因子活性とその特徴

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日本人軽症血友病A患者2名の第VIII因子遺伝子解析から病因遺伝子異常としてArg531His変異を同定した．患者は共に軽症のフェノタイプであった．両者の第VIII因子は，比活性は同等であるものの，活性および抗原のレベルはかなり異なっていた．患者1の血漿の第VIII因子活性を14種類のAPTT試薬を用いて凝固1段法で測定した結果，25.2%から48.1%の範囲で測定され，用いるAPTT試薬によって大きく異なっていた．この測定値の乖離は，APTT試薬のpHとの間に負の相関関係が認められた．患者2の血漿をサンプルとした検討から，検体の希釈にイミダゾール緩衝液を用いることで乖離を最小化できる可能性が示唆された．これらのことからArg531His変異の第VIII因子レベルは，患者によって異なっており，またさらに測定法によっても大きく影響を受けることが明らかとなった．Arg531Hisのような軽症例を的確に検出・把握できる標準的な検査法を確立する必要がある．

Key words: Mild hemophilia A, Mutation, One-stage clotting assay, Discrepancy

緒　言

先天性血友病Aは第VIII因子（FVIII）をコードする第VIII因子遺伝子（*F8*）の異常によって引き起こされ，X染色体性劣性の遺伝形式をとる．本疾患の呈する出血症状は血中に存在するFVIII活性（FVIII：C）と相関することから，その重症度はFVIII：Cの値により3グループ（重症，中等症，軽症）に分類される．軽症血友病Aは5%を上回るFVIII：Cを有する場合であり，血友病Aの約2割がこれに分類される[1]．

*F8*はXq28に位置する全長186 kbの大きな遺伝子であり，1984年にクローニングされた[2)-5)]．血友病A患者の*F8*解析により，これまでに血友病Aを引き起こす様々な遺伝子異常が同定されてきたが，軽症例についてはその責任遺伝子異常は点変異によるミスセンス変異に限定されるといっても過言ではない．また軽症例から検出されるミスセンス変異は多くが分子異常症であり，FVIII：Cの構造と機能の解析には極めて重要な知見をもたらす．

FVIII：Cは凝固1段法，凝固2段法，合成基質法の3つの方法で測定されるが，一部の軽症では測定法によって異なったFVIII：Cを呈することが報告された[6)7)]．立体構造の解析から，これらの変異部位の多くは3つのAサブユニットのインターフェイスに位置することが確認され，FVIII分子からのA2サブユニットの解離の動態が，測定法ごとに異なるFVIII：Cに関与することが明らかにされた[8)-10)]．A2サブ

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ユニット内のArg531His（R531H）変異は，A1サブユニットとのインターフェイスに位置し，測定法によって異なるFVIII：Cを呈する[4]．

今回我々は軽症血友病A症例の*F8*解析から検出した2例のR531Hを対象に，その特徴について検討した．

研究対象，材料および方法

対象

症例1は61歳の男性．手術の準備のため，以前，凝固検査の異常を指摘されていたため精査を目的に近医を受診した．これまでに陸上競技後の膝関節での出血と，扁桃を傷つけた際の止血困難がそれぞれ一度ずつあるのみで，日常生活の中で出血症状を認めないが，母方には出血傾向を有する男性が複数存在する．凝血学的検査ではAPTTに延長を認めなかった．因子定量の結果では唯一FVIIIが約60%に低下していたことから血友病Aが疑われたが，凝血学的検査のみでは血友病Aの診断には至らず東京医科大学病院にて遺伝子解析を施行した．

症例2は30歳の男性．抜歯時の止血困難にて14歳時に血友病Aと診断される．幼少時から鼻出血が多く，激しい運動（サッカー）の後に前下腿部に血腫ができた経験もあったが，日常生活において問題となるような出血症状は認めないため，血液製剤の投与を受けることもなかった．

凝血学的解析

①活性化部分トロンボプラスチン時間（APTT）

APTTは自動凝固測定装置ACL-9000（Instrumentation Laboratory社，IL社）を用いて測定した．通常のAPTT試薬にはHemosIL™ APTT-SP（IL社）を，サンプル血漿の希釈液にはファクターダイリューエント（IL社）を用いた．また，コントロール血漿には凝固因子アッセイコントロール血漿（GK-FACT：ジョージキングバイオメディカル社）を用いた．

②FVIII：C測定（凝固1段法）

凝固1段法によるFVIII：C測定は前述のAPTTを用いた．第VIII因子欠乏血漿にはGK-08（ジョージキングバイオメディカル社）を用いた．

③凝固1段法におけるAPTT試薬の影響の検討

患者1血漿のFVIII：Cを入手可能であった合計14種類（我々が日常用いているHemosIL™ APTT-SPを含む，**表1**）のAPTT試薬を用いて測定し，検討した．対照としてFVIII欠乏血漿と凝固因子アッセイコントロール血漿を6：4で混合して作製したFVIII：C 40%のコントロール血漿を測定し，患者1血漿の成績と比較した．また，用いた各APTT試薬については構成成分（活性化物質，リン脂質）を添付文章から調べ（表1），pHを測定し，FVIII：C測定との関連について検討した．

④凝固1段法における試薬pHの影響の検討

我々が通常用いているHemosIL™ APTT-SP（pH 7.54）のpHを，塩酸または水酸化ナトリウム溶液を用いて6.96，7.17，7.33，7.71に変え，試薬pHのFVIII：C測定に与える影響を検討した．患者2血漿および，FVIII欠乏血漿と凝固因子アッセイコントロール血漿を8：2で混合したFVIII：C約20%のコントロール血漿を測定し比較した．

⑤凝固1段法における乖離の最小化を目的とした検体希釈液の検討

我々が通常用いているHemosIL™ APTT-SPと，R531H症例のFVIII：C測定上HemosIL™ APTT-SPとは明らかに乖離した測定値を精密に再現することのできたトロンボチェックAPTT（S）を用いて乖離の最小化についての検討を行った．患者2血漿と，FVIII欠乏血漿と凝固因子アッセイコントロール血漿を8：2で混合したFVIII：C約20%のコントロール血漿を対照として測定した．希釈液には我々が通常用いているファクター

表1 APTT reagents used in this study

No.	Reagent	Phospholipids	Activator	pH
1	PTT - reagent (Diagnostica Stago)	Cepharin	Kaolin	7.45
2	Actin FS (Dade Behring)	Cephalin (soybean)	Ellagic acid	6.72
3	Automated APTT (bioMerieux)	Cepahlin (rabbit brain)	Silica	7.47
4	Thrombocheck APTT (Sysmex)	Cephalin (rabbit)	Ellagic acid	7.24
5	Platein LS (bioMerieux)	Phospholipids (egg, bovine brain)	Silica	7.54
6	STA Cephascreen (Diagnostica Stago)	Cephalin	Polyphenols	7.61
7	Platelin L (bioMerieux)	Phospholipids (egg, bovine brain)	Silica	7.54
8	HemosIL SynthASil (Instrumentation Laboratory)	Synthetic phospholipids	Colloidal silica	7.23
9	STA APTT (Diagnostica Stago)	Cephalin	Celite	7.41
10	Actin (Dade Behring)	Cepahlin (rabbit brain)	Ellagic acid	6.95
11	Thrombocheck APTT(S) (Sysmex)	Cephalin (soybean)	Ellagic acid	7.29
12	Pathromtin SL (Dade Behring)	Lecithin (soybean)	Silica	7.41
13	Cephotest (Axis-Shield)	Dried bovine brain extract	?	7.49
14	HemosIL APTT-SP (Instrumentation Laboratory)	Synthetic phospholipids	Colloidal silica	7.54

ダイリューエント（生理食塩水）以外にベロナール緩衝液（28 mM バルビタールナトリウム，125 mM 塩化ナトリウム，pH 7.35），イミダゾール緩衝液（50 mM イミダゾール，100 mM 塩化ナトリウム，pH 7.3），トリス緩衝液（20 mM トリス塩酸緩衝液，150 mM 塩化ナトリウム，pH 7.4）を検討した．

⑥ FVIII : C 測定（発色性合成基質法）

発色性合成基質法による FVIII : C 測定にはコアテスト SP FVIII キット（Chromogenix 社）を用い，製造者の指示に従いマニュアル通りに測定した．

⑦第 VIII 因子抗原（FVIII : Ag）

FVIII : Ag はアセラクロム VIII : Ag キット（Diagnostica Stago 社）を用い，製造者の指示に従いマニュアル通りに測定した．

⑧遺伝子解析

F8 を構成する 26 のエクソンは，それぞれのイントロンと境界領域を含めて PCR にて増幅し，PCR 産物のダイレクトシークエンスによって塩基配列を決定した．エクソン 14 は 3.1 kb と大きいことから，互いにオーバーラップする 8 つの領域に分けて解析した．本研究は東京医科大学医学倫理委員会の審査を経て，東京医科大学学長により承認されている『先天性血液凝固線溶因子欠損（欠乏）および異常症に関する遺伝子研究』に従い，患者には文章による説明と同意を取得したうえで遺伝子解析を行った．

成　績

1. 遺伝子解析結果

患者 1，2 の *F8* 解析から R531H 変異が検出された．今回の研究において塩基配列を決定した *F8* の他の領域には両者間に差異を認めなかった．

2. 各種測定法における FVIII : C 測定結果

両患者の FVIII : C（凝固 1 段法，合成基質法）および FVIII : Ag を**表 2** に示す．FVIII : Ag に比べ FVIII : C は著しく低値を示しており，こ

表2　APTT and FVIII level of the two R531H patients

	APTT (cont. 32. 2)	FVIII : C		FVIII:Ag
		One-stage clotting assay	Chromogenic assay	
Patient 1	34.6sec	34.5%	32.9%	70.7%
Patient 2	48.3sec	19.9%	13.4%	48.1%

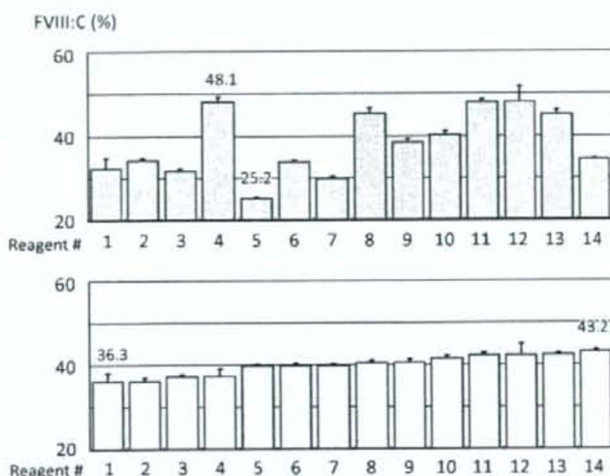

図1　FVIII : C levels of R531H (patient 1) and 40% control plasma measured by 14 different APTT reagents.
The FVIII : C levels of patient 1 and 40% control plasma were measured by one-stage clotting assay with 14 different APTT reagents. The 40% control plasma showed similar values when assayed with each of the 14 reagents (range, 36.3%–43.2%) (lower graph). However, measurements of R531H differed with reagent (range, 25.2%–48.1%) (upper graph)

のことからR531H変異は分子異常症であることが確認された．14種類のAPTT試薬を用いてのFVIII : C測定では，40% FVIII : Cコントロール血漿の測定値はAPTT試薬ごとに多少のばらつきが確認されるものの40%を中心とした約±10%の範囲で測定されたのに対し，患者1血漿の測定値は最低25.2%から最高48.1%の広範囲で測定され，試薬ごとに著しい乖離が確認された（**図1**）．

3. 乖離に影響する要因の解析

FVIII : C乖離に影響する要因の解析のため，APTT試薬の構成成分（活性化物質，リン脂質）とpHの影響について検討した（**表1，図2**）．FVIII : Cは，活性化物質とリン脂質との間に何ら相関関係は認められなかった．しかしながら，pHはR531Hの活性値に大きく関与し，pH7.2～7.4で最も高値に測定される傾向があり，またpHが7未満であった2試薬を除いた12種類のAPTT試薬では負の相関関係（$r = -0.748$ $(p = 0.005)$）が認められた．

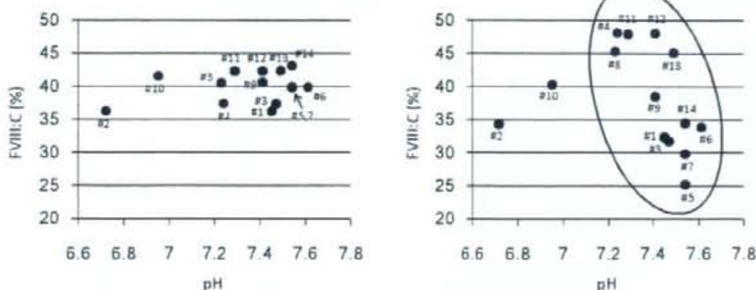

図2 Influence of APTT reagent pH on 40% control plasma and R531H (patient 1) FVIII:C measurement.
Excluding the reagent of the #2 and the #10, a negative correlation (r = -0.748) between pH and R531H measurement was observed (right graph).

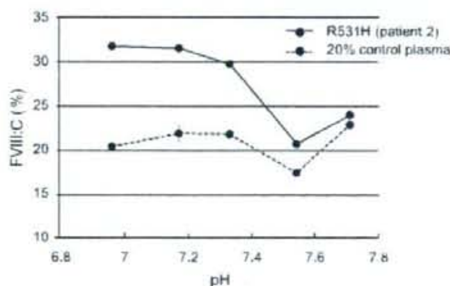

図3 Influence of APTT reagent pH on 20% control plasma and R531H (patient 2) FVIII : C measurement.
The FVIII : C levels of patient 2 and 20% control plasma were measured by one-stage clotting assay using different pH of HemosIL APTT-SP. Although pH of the APTT reagent similarly influenced FVIII : C measurement of both R531H and control plasma, it was more remarkable in the R531H.

4. 試薬 pH の影響の検討

HemosIL™ APTT-SP の pH (7.54) を，塩酸または水酸化ナトリウム溶液を用いて 6.96，7.17，7.33，7.71 に変えて試薬 pH の FVIII：C 測定に与える影響を検討した．R531H，20% FVIII：C コントロール血漿ともに，pH の変化により同様の測定値の変動がみられたが，R531H でのそれはより顕著であった（図3）．

5. 乖離の最小化に関する検討

患者2の FVIII：C の乖離は希釈液に緩衝液を用いることで軽減し，イミダゾール緩衝液を用いた場合に最小であった（図4）．

考　案

本論文では軽症血友病 A 症例の遺伝子解析から2例の血縁関係のない R531H 症例を検出し，その FVIII：C 測定における特徴について検討した．

R531H 変異は点変異のホットスポットである CpG ダイヌクレオチドでのグアニン（CGC）からアデニン（CAC）への置換によって引き起こされる．2007年8月現在，血友病 A 変異データベース（The Haemophilia A Mutation, Structure, Test and Resource Site;HAMSTeRS, http://europium.csc.mrc.ac.uk/usr/WWW/WebPages/main）において同変異は17例の登録があり，点変異の 0.87%（17/1994），軽症例の 2.21%（17/769）を占める．17例の FVIII：C は 20～40% 程度での報告が多いが，最低 5% から最高 96% までの広範囲の値が記載されている．これまでに R531H 症例では，FVIII：C が凝固1段法と合成基質法間で乖離することが報告されており[8,9]，また最近，凝固1段法でも，

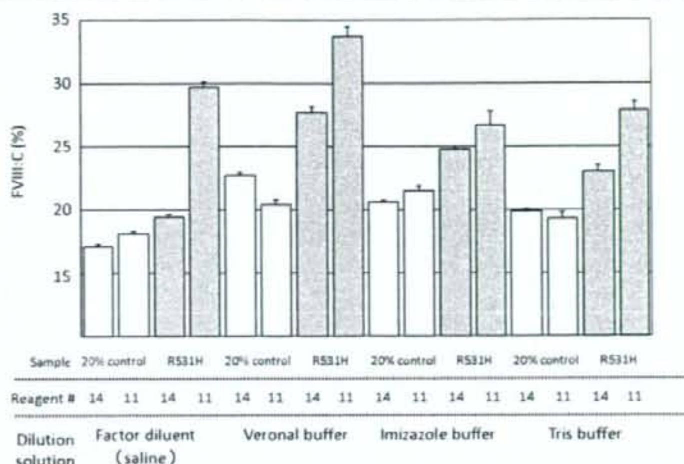

図4　Influence of sample dilution solution on R531H (patient 2) FVIII : C measurement.
The FVIII : C levels of patient 2 and 20% control plasma were measured by one-stage clotting assay with four different sample dilution solutions. Two APTT reagents (Reagents #11 and HemosIL™ APTT-SP) that showed high reproducibility under ordinary assay conditions were used. Differences in R531H FVIII:C measurements were reduced considerably with the use of imidazole buffer as the sample dilution solution.

一部のAPTT試薬間でFVIII：Cに乖離が認められるとの報告がなされている[10]．今回の患者1の解析では，我々が日常用いているAPTT試薬（HemosIL™ APTT-SP）による測定値が，合成基質法による測定値と同程度となっていたため凝固1段法と合成基質法間で大きな乖離は認められなかったが，凝固1段法ではAPTT試薬の違いによって大きな差異をもって測定された．これらのことから，FVIII：C測定に関する情報がないことから断定はできないが，おそらくデータベースにみられる著しいFVIII：Cの差異は，測定法に起因するものが大きいと推測される．今回の解析においても，患者1が最初に近医にてAPTT試薬としてアクチンFS（**表1 #2**）を用いて測定したFVIII：Cが約60%であったのに対し，我々の研究室でHemosIL™ APTT-SP（**表1 #14**）を用いて測定した結果が34.5%であったことも，APTT試薬を含めた測定法の違いが関与していると考えられる．しかしながらR531H変異に限らず血友病Aでは，von Willebrand因子量，血液型，ポリモルフィズム，日差などの要因が関与しているためか，同一の変異を有する患者であっても同一のFVIII：Cやクリニカルフェノタイプとはならないことはしばしば経験されることであり，実際，今回検討した2例では同一の方法で測定されたFVIII：Cでありながら患者1のFVIII：Cは約1.7倍高値であった．したがって，患者個人の要因のみならず，さらに検査法の要因が通常より大きく関与するR531HのFVIII：C測定では，得られた値が本当に患者の病態を把握するために適正な値であるのかについては慎重に検討する必要がある．また，このような変異の場合，軽症血友病Aと診断されていないケースが多く存在するのではないかということも懸念される．通常，血友病Aの診断は，まずAPTTによるスクリーニングを行い，延長が認められた症例に対してFVIIIの定量が行われるが，患者1のようなラボラトリーフェノタイプでは，スクリーニングとして行われるAPTTにおいて明ら

かな延長が認められないため血友病Aの診断に至らない場合も少なくないと想像される．したがって，血友病AのスクリーニングおよびFVIIIの定量は，FVIIIに対する感受性の高いAPTT試薬を用いて行うことが重要であろうと考える．

活性化FVIII分子は，A1，A2およびA3-C1-C2の3つのサブユニットで構成されるヘテロトリマー構造をとっており，R531HはA2サブユニットに位置する．トロンビンによって活性化されたFVIIIのA2サブユニットは，他の2つのAサブユニットとの非共有結合にて複合体を形成しており，このA2サブユニットの解離はFVIIIの失活につながる[10]．Pipeら[8][9]はリコンビナントFVIIIの実験から，R531Hでは野生型に比べA2サブユニットが解離しやすいことを確認しており，基質反応までの加温時間が長い2段法では，A2サブユニットの解離の亢進によりFVIII：Cが低値となってしまうことを報告している．またRodgersら[11]は古典的な凝固2段法と合成基質法の両法について加温時間の検討を行い，短い加温時間の方が高値を呈することを確認している．我々も凝固1段法での加温時間について検討し，加温時間の延長による活性低下の度合いがR531Hでは正常FVIIIのそれに比べて顕著であり，R531Hが加温に対してより不安定であることを確認している（データ非提示）．これらのことから加温時間は乖離に関与する重要な要因であることは間違いない．しかし加温時間以外にも，試薬に起因する何らかの別の要因が乖離に関与することが今回の解析において確認され，各APTT試薬の構成成分とpHの検討を行った．APTT試薬の組成は製造メーカーが独自に培ってきた製造技術情報をもとに，自社の試薬に何らかの特徴を持たせるような工夫もなされており，その詳細が把握できないため，添付文章に記載されている限られた情報を参考に乖離に関連する因子の検討を行った．その結果，FVIII：Cと試薬構成成分であるリン脂質や活性化物質との間には特定の関連を認めなかったが，pHとの間には相関関係が示唆された．Lollarら[12][13]はブタのFVIIIの実験から，pHが高い条件ではA2サブユニットの解離が速やかになり，活性化FVIIIの凝固活性が速やかに減少することを確認している．またFayら[14]もヒトのFVIIIを用いた実験で同様の結果を報告している．今回得られたpHの影響は，これらの報告のpHの影響と一致しており，APTT試薬のpHがA2サブユニットの解離に影響しFVIII：Cを変動させる要因であると考えられた．一方，Perssonら[15]はA2サブユニット解離に関与するpH以外の要因として，イオン強度，カルシウムイオン濃度を報告しており，これらの影響も併存している可能性も考えられる．また今回は由来のみの調査であったが，リン脂質に関しては詳細な組成の検討により乖離との関与が認められる可能性がある．

APTT試薬のpHがFVIII：C測定，特にR531Hのような変異体のFVIII：C測定に重要であることが確認されたことから，反応液のpHを整えることは精密な検査を行ううえで有効であると考え，試薬と検体の希釈に3種類の緩衝液を用いその効果について検討ところ，緩衝液の効果は一様とはならず，イミダゾール緩衝液が最も効果的に作用した．イミダゾール緩衝液は凝固検査の際に用いられることが多く，FVIIIではインヒビターの測定法として一般的に知られているベセスダ法における検体の希釈液に用いられる．なぜイミダゾール緩衝液が最も有効に働いたかについては不明である．今後，#11と#14以外のAPTT試薬の場合や，他の緩衝液を用いた場合などの解析を行い，検討する必要がある．

今回検討したR531Hの2症例は共に臨床的には軽症型であるが，いずれの試薬を用いたFVIII：Cの結果が臨床症状を最も反映しているのかは不明である．同一変異を有する他の症例の詳細な臨床症状とFVIII：Cの関係を含めて検討をする必要がある．

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Features of factor VIII activity measurement in Arg531His mutant detected in patients with mild hemophilia A

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Arg531His (R531H) mutation was identified from unrelated two Japanese patients with cross reacting material positive (CRM+) mild hemophilia A. Although these patients have the same genetic defect and mild clinical phenotype, FVIII activity differed considerably, between the two. In Patient 1, FVIII activity, measured with 14 different APTT reagents, ranged widely, from 25.2% to 48.1%. There was a negative correlation between reagent pH and R531H measurement. Although the FVIII activity in Patient 2 also showed a discrepancy, the difference was considerably lessened by use of an imidazole buffer for sample dilution solution. This finding suggests that the use of imidazole buffer as a sample diluent is an effective means for reducing the wide range of assay results. As for the results of the present study, FVIII levels associated with the R531H mutation differed with the patients and were greatly influenced by measurement method. It is necessary to establish a standard method that can detect and characterize the mild hemophilia phenotype caused by a particular mutation, such as Arg531His.