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

Kohei Tatsumi[1], Kazuo Ohashi[2, 5], Miho Kataoka[3], Chise Tateno[3, #], Masaru Shibata[1], Hiroyuki Naka[1], Midori Shima[1], Michiyoshi Hisanaga[2], Hiromichi Kanehiro[2], Teruo Okano[5], Katsutoshi Yoshizato[3, 4, #], Yoshiyuki Nakajima[2], Akira Yoshioka[1]

[1]Department of Pediatrics, Nara Medical University, Nara, Japan; [2]Department of Surgery, Nara Medical University, Nara, Japan; [3]Yoshizato Project, Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER), Hiroshima Prefectural Institute of Industrial Science and Technology, Hiroshima, Japan; [4]Graduate School of Science, Hiroshima University, Hiroshima, Japan; [5]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan

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

Correspondence to:
Kazuo Ohashi, MD, PhD
Institute of Advanced Biomedical Engineering and Science
Tokyo Women's Medical University
8–1 Kawada-cho, Shinjuku-ku, Tokyo, 162–8666, Japan
Tel.: +81 3 3353 8111, ext 66214, Fax: +81 3 3359 6046
E-mail: ohashi@abmes.twmu.ac.jp

Present address: PhoenixBio Co. Ltd, Hiroshima, Japan.

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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

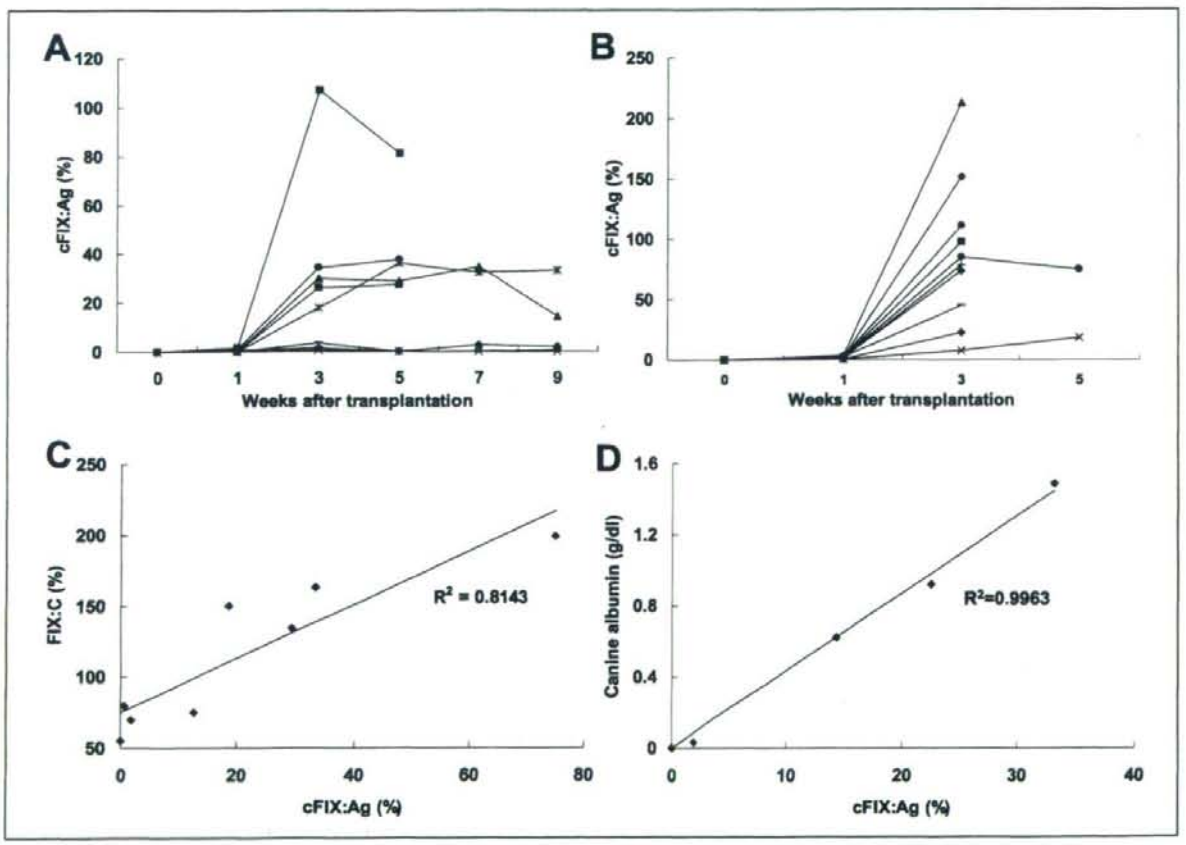

Figure 1: Proliferation of transplanted canine hepatocytes in uPA/SCID mouse livers assessed by recipient plasma analyses. A, B) Plasma canine factor IX (FIX) antigen (cFIX:Ag) levels in uPA/SCID mice after transplantation of hepatocytes isolated from a seven-year-old dog (A) and a one-year-old dog (B)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

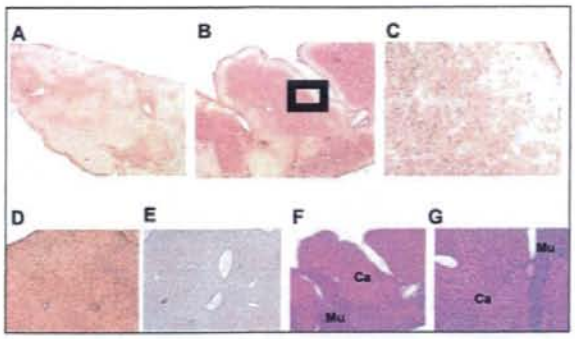

Figure 2: Mouse liver chimerism with proliferated canine hepatocytes. A-E) Immunohistochemical staining of canine albumin in liver sections of uPA/SCID mice transplanted with canine hepatocytes. Representative photomicrographs from a recipient mouse with low plasma cFIX:Ag (2.0% of normal canine plasma)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

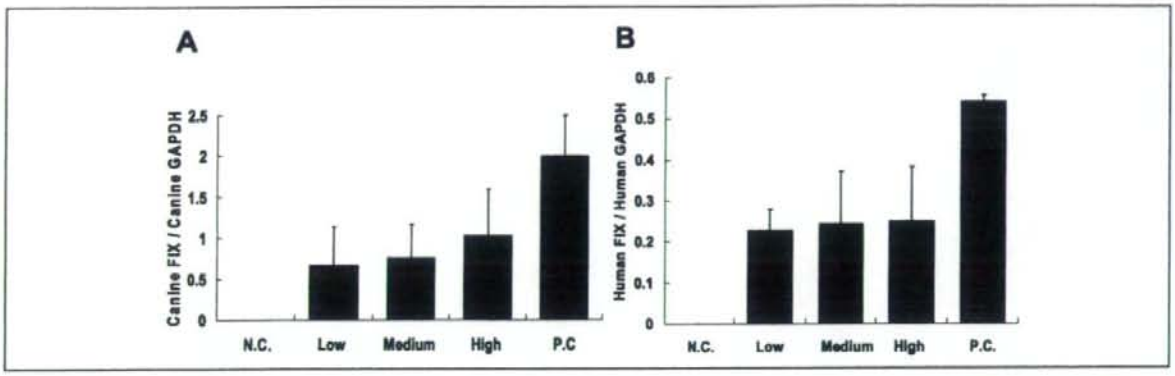

Figure 3: Donor species-specific FIX mRNA expressions in uPA/SCID mouse livers transplanted with either canine or human hepatocytes. A) Canine factor IX (FIX) RNA copy numbers relative to canine GAPDH (reflecting RNA copy numbers per canine hepatocyte), based on plasma cFIX:Ag levels. (Low, <40%; Medium,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

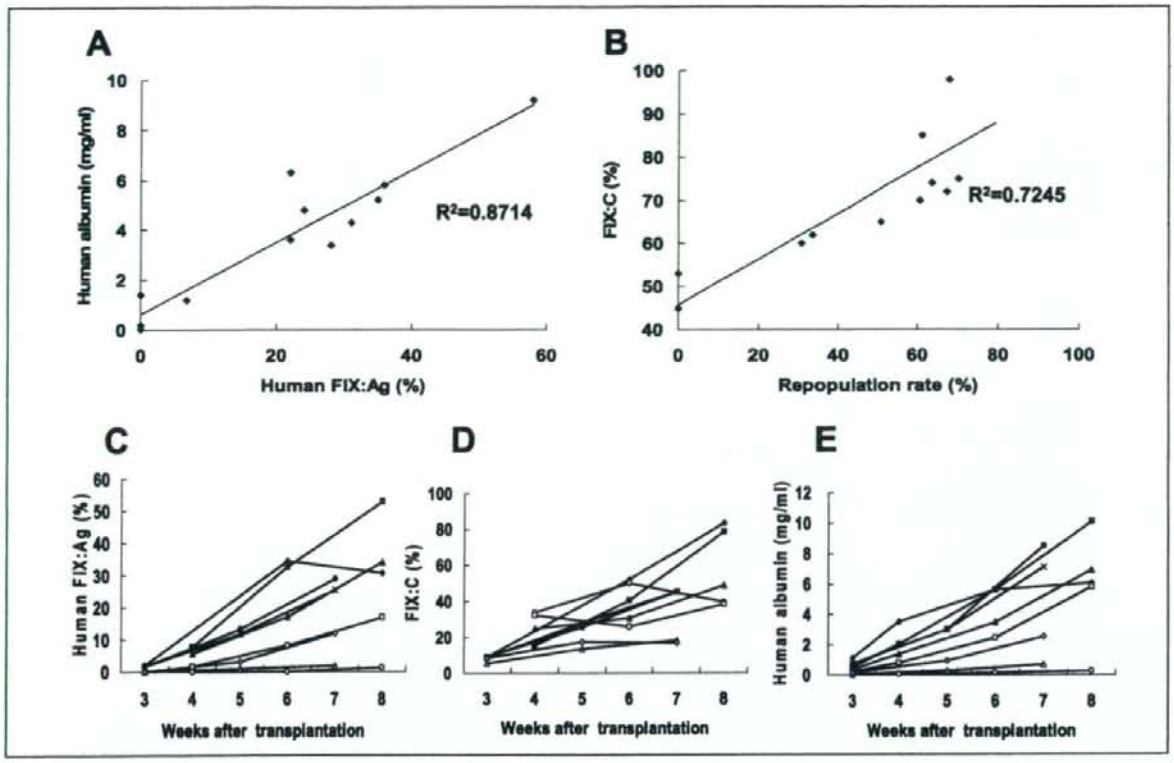

Figure 4: Proliferation of transplanted human hepatocytes in uPA/SCID mouse livers assessed by recipient blood analyses. A) Relationship between blood human albumin and plasma human factor IX (FIX) antigen (hFIX:Ag) concentrations of uPA/SCID mice transplanted with human hepatocytes (n=12). B) Relationship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

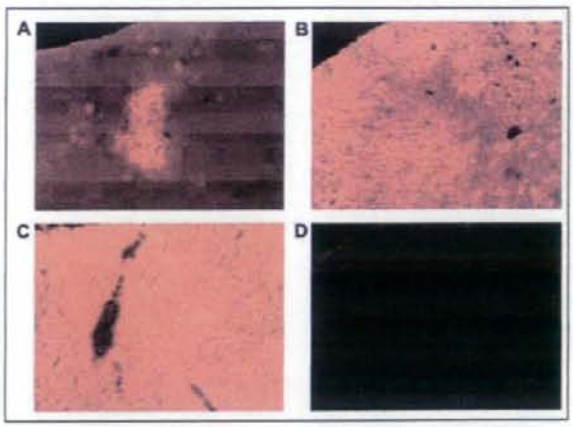

Figure 5: Mouse liver chimerism with proliferated human hepatocytes. Immunofluorecent staining of human FIX in liver sections of uPA/SCID mice after human hepatocyte transplantation. Liver sections of mice with <1% plasma hFIX:Ag level (A) and mice with 34% hFIX:Ag plasma level (B). Red-stained area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

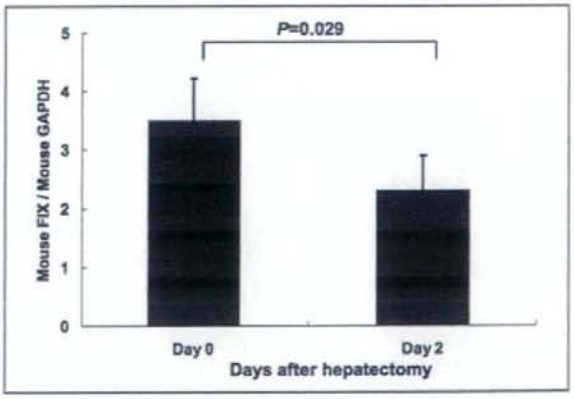

Figure 6: Comparison of factor IX (FIX) mRNA expression levels in quiescent and proliferating livers. Day 0 liver samples (quiescent status) were obtained from C57/BL6 mice at the time of 70% partial hepatectomy (n=6). The remnant liver lobes of the mice were harvested at day 2 were used for the assessment 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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Non-invasive method to detect induction of CYP3A4 in chimeric mice with a humanized liver

C. EMOTO[1], Y. YAMATO[1], Y. SATO[2], H. OHSHITA[3], M. KATOH[4], C. TATENO[5], T. YOKOI[4], K. YOSHIZATO[5], & K. IWASAKI[1]

[1]*Pharmacokinetics Dynamics Metabolism,* [2]*Drug Safety Research & Development, Pfizer Global Research & Development, Nagoya Laboratories, Pfizer Japan, Inc., Aichi, Japan,* [3]*PhoenixBio Co., Ltd., Hiroshima, Japan,* [4]*Faculty of Pharmaceutical Sciences, Kanazawa University, Ishikawa, Japan and* [5]*Developmental Biology Laboratory, Graduate School of Science, Hiroshima University, Hiroshima, Japan*

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Abstract

Chimeric mice with a humanized liver have been previously established by the transplantation of human hepatocytes to urokinase-type plasminogen activator/severe combined immunodeficiency mice. A non-invasive method to detect the induction of cytochrome P450 (CYP) 3A4 was evaluated in chimeric mice with a humanized liver. Dexamethasone (DEX) was used as a probe drug to detect induction; and rifampicin was used as a model drug to induce CYP3A4. Before and after rifampicin treatment (50 mg kg^{-1}, intraperitoneal injection once a day for 4 days) in the chimeric mice, DEX was subcutaneously injected and the urinary excretion of 6β-hydroxydexamethason (6βOHD) and DEX was determined. The metabolic ratio (6βOHD/DEX) significantly increased after rifampicin treatment. Livers from the control and rifampicin-treated chimeric mice were stained immunohistolochemically with antibodies against CYP3A4 and CYP3A5. CYP3A4 and CYP3A5 were detected in the area of humanized liver, but staining was intense for CYP3A4 and very weak for CYP3A5. Only the staining of CYP3A4 was increased after rifampicin treatment. Formation of 6βOHD by human liver microsomes was higher than that formed by mouse liver microsomes. Metabolite formation was catalysed by both CYP3A4 and CYP3A5 and the intrinsic clearance (V_{max}/K_m) by CYP3A4 was found to be 50-fold higher than that of CYP3A5. The results of the present study indicate that estimation of the changes of the urinary metabolic ratio (6βOHD/DEX) in the chimeric mice with a humanized liver is a very useful tool for detecting the induction of CYP3A4 by a non-invasive method.

Keywords: *CYP3A4, CYP3A5, induction, chimeric mice with a humanized liver, non-invasive method, humanized liver, dexamethasone, rifampicin*

Correspondence: K. Iwasaki, Business Development Department, Contract Research Company, Shin Nippon, Biomedical Laboratories, Ltd., Sumitomo Mitsui Banking Corporation Korai-bashi Building, 2-1-1 Fushimi-machi, Chuo-ku, Osaka 541-0044, Japan. Tel: +81-6-6233-8432. Fax: +81-6-6233-8433. E-mail: iwasaki-kazuhide@snbl.co.jp

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Introduction

Cytochrome P450s (CYPs) consist of a gene super family of haemoproteins and play a critical role in the metabolic clearance of lipophilic compounds including many drugs (Nelson et al. 1996). CYP3A4 is one of the abundant CYPs in human liver (McGinnity et al. 2000) and participates in metabolizing more than half of drugs currently in use (Rendic 2002). Inhibition and induction of CYP-dependent metabolism, especially in the case of CYP3A4, are the causes in the serious problems that can arise in clinical practice and in the attrition of drug candidates through the drug-discovery and development stages (Dresser et al. 2000, Lin 2006). In order to develop a safe drug without induction potency, it is very important to develop a non-clinical system for detecting this characteristic.

Induction of CYP is regulated by a number of nuclear receptors such as pregnane X receptor (PXR) and constitutive androstane receptor (Cheng & Waxman 2006). The induction of CYP3A4 is mainly mediated through activation of PXR, and rifampicin activates human PXR and induces CYP3A4.

Chimeric mice with a humanized liver have been previously established by the transplantation of human hepatocytes to urokinase-type plasminogen activator ($uPA^{+/+}$)/severe combined immunodeficiency (SCID) mice (Tateno et al. 2004). These mice could be a very promising model for examining the absorption, distribution, metabolism and excretion of drugs and drug candidates for predicting problems in clinical practice (Katoh & Yokoi 2007). Induction of CYP3A4 in chimeric mice with a humanized liver has been previously characterized by measuring enzyme activities, protein and mRNA expression levels after treatment with rifampicin (Katoh et al. 2005a, b).

Although the majority of the mouse liver was replaced by the human liver in $uPA^{+/+}$/SCID chimeric mice, 20–30% of the mouse hepatocytes still existed in the mouse liver used in this study. To delete or minimize noise/false information for the sensitive detection of CYP induction in mice, a sensitive probe metabolized more rapidly by human liver microsomes than by mouse liver microsomes should be established. The rate of 6β-hydroxydexamethasone (6βOHD) formation by human liver microsomes is much higher than in mouse liver microsomes (Tomlinson et al. 1997), the reaction being catalysed by CYP3A4 (Gentile et al. 1996). After dexamethasone (DEX) administration to man, 6βOHD is excreted as a major urinary metabolite, and unchanged DEX and DEX glucuronide were detected as a small fraction of the excreted dose (Minagawa et al. 1986).

It is postulated that DEX is a suitable probe to detect induction of CYP3A4 mediated metabolism and in the current study, we have evaluated a non-invasive method in chimeric mice with a humanized liver using DEX as a probe drug and rifampicin as a model drug to induce CYP3A4. Specifically, we have compared the change in the urinary metabolic ratio of 6βOHD to DEX, both before and after rifampicin treatment in the same animal.

Materials and methods

Reagents

DEX was obtained from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan) (chemical purity, 99.9%). 6βOHD was synthesized at KNC Laboratories Co. Ltd (Kobe, Japan) (chemical purity, 97.4%). Rifampicin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Beclomethasone was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Tolubutamide was purchased from Ultrafine (Manchester, UK). β-Nicotinamide adenine

dinucleotide phosphate (reduced form, NADPH) was obtained from Oriental Yeast (Tokyo, Japan). Other reagents were high-performance liquid chromatography (HPLC) grade or better.

Polyclonal rabbit anti-human CYP3A4 and CYP3A5 antibodies were purchased from BD Gentest (Worburn, MA, USA). The CYP3A4 antibodies reacted with CYP3A4 and CYP3A7, and the CYP3A5 antibodies only reacted with CYP3A5 (manufacturer's information).

Recombinant CYP enzymes expressed in insect cells infected with baculovirus containing human CYP enzymes and human NADPH-CYP reductase cDNA inserts with human cytochrome b_5 and pooled liver microsomes from human and mouse (CD-1) liver were obtained from BD Gentest.

Generation of chimeric mice with a humanized liver

The generation of chimeric mice with a humanized liver was conducted as described previously (Tateno et al. 2004; Katoh et al. 2005a, b). All experiments were performed in accordance with Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board, the Ethics Committees of Kanazawa University and the Animal Ethics Committee of Pfizer Global Research and Development Nagoya Laboratories. The extent of the replacement from mouse liver to human liver for the metabolic ratio studies was 70–80% in the chimeric mice as assessed by the method described below. The extent of replacement was 60–90% for the immunohistochemistry study, as assessed by the method described below. The concentration of human albumin in the blood of the mice was determined using an enzyme-linked immunosorbent assay and the extent of the replacement was estimated with a correlation between the extent of the replacement and the human albumin concentration (Tateno et al. 2004).

Animal treatment

Chimeric mice with a humanized liver (two females and seven males: body weight, 16–20 g) received an intraperitoneal (i.p.) injection of rifampicin (dissolved in corn oil, 3.3 μl g^{-1} body weight) once a day for 4 days at a dose of 50 mg kg^{-1}. Control chimeric mice with a humanized liver (one female and five males: body weight, 15–20 g) group received an i.p. injection of the same volume of corn oil. On 3 days before and 1 day after the treatment of rifampicin, animals received a subcutaneous (s.c.) injection of DEX (dissolved in polyethylene glycol 400, 5.0 μl g^{-1} body weight) at a dose of 10 mg kg^{-1}. Urine samples were collected every 24 h for 48 or 72 h. Liver samples were obtained from corn oil-treated and rifampicin-treated mice on 1 day after the last refampicin injection. After animals were killed by exsanguination under diethyl ether anaesthesia, livers were dissected and fixed with 4% paraformaldehyde and processed for immunohistochemical analysis.

Analysis of urine samples

Urine samples (0.1 ml) were mixed with an internal standard (beclomethasone, 1 μM) and treated with OASIS HLB μElution Plate (Waters Corporation, Milford, MA, USA). After washing with water, DEX, 6βOHD and beclomethasone were eluted with acetonitrile (0.1 ml). Eluates were diluted with water (0.3 ml) and analysed by HPLC with tandem mass spectrometric detection (LC/MS/MS). The detection of DEX and 6βOHD was performed

using an API-4000 mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Canada), a 1100 series HPLC system (Agilent Technologies, Taufkirchen, Germany), and a CTC-PAL auto injector (CTC Analytics AG, Zwingen, Switzerland). The eluates were injected into a YMC-Pack Pro C8 column (2.0 × 75 mm, YMC, Kyoto, Japan) and eluted by a linear gradient with the mobile phase, which consisted of a mixture of A (10% acetonitrile containing 10 mM ammonium acetate) and B (80% acetonitrile containing 10 mM ammonium acetate). The column temperature and the flow rate were 40°C and 0.5 ml min^{-1}, respectively. The gradient conditions for elution were as follows: 20% B (0.0–0.5 min); 20–100% B (0.5–2.5 min); 100% B (2.5–3.0 min); 100 to 20% B (3.0–3.05 min); and 20% B (3.05–5.5 min).

Immunohistochemistry

Polyclonal rabbit anti-human CYP3A4 and CYP3A5 were used as markers of enzyme induction in immunohistochemical staining. Paraformaldehyde-fixed livers were trimmed, embedded in paraffin, sectioned to a thickness of 4 μm, and mounted on glass slides. The avidin–biotin affinity system (Vectastain Elite ABC, Vector, CA, USA) was used for the immunohistochemistry studies. The dilutions of the CYP3A4 and CYP3A5 antibodies were 1:100 and 1:50, respectively. The liver sections were visualized with 3,3′-diaminobenzidine tetrachloride as the chromogen and counterstained with haematoxylin. Immuno-expression of CYP3A4 and CYP3A5 was analysed quantitatively using two-dimensional evaluation with the Image Processor for Analytical Pathology (IPAP-WIN, Sumika Technoservice, Osaka, Japan), and the positive area in the human hepatocyte area was calculated as a percentage.

Kinetic assay for 6βOHD formation

The reaction mixture consisted of 1.3 mM β-NADPH, 3.3 mM $MgCl_2$, 100 mM phosphate buffer (pH 7.4), microsomes, and a substrate in a final volume of 200 μl. The reaction was started by adding NADPH after pre-incubation for 5 min in a water bath at 37°C. The final human and mouse microsomal concentrations were 0.1 and 0.5 mg ml^{-1}, respectively. The final concentrations of CYP3A4 and CYP3A5 were 2 and 30 pmol ml^{-1}, respectively. Considering microsomal protein binding, the protein concentration in the reaction mixture containing recombinant CYP microsomes was adjusted to 0.23 mg ml^{-1} by the addition of control microsomes. Final substrate concentrations were from 4.4 to 220 μM. The incubation time was 10 min. The reaction was terminated by the addition of 400 μl of methanol containing tolbutamide (25 ng ml^{-1}) as an internal standard. After the centrifugation at 2000 rpm for 15 min at 4°C, the supernatants were subjected to LC/MS/MS analysis.

Analysis of in vitro samples

The detection of 6βOHD was performed using an API-4000 mass spectrometer, a 1100 series HPLC system, and a CTC-PAL auto injector. The supernatants of the reaction mixture were injected into an AtlanticTM dC18 column (4.6 × 150 mm, 5 μm, Waters Corporation) and eluted by a linear gradient with the mobile phase, which consisted of a mixture of A (90% 5 mM ammonium formate/10% acetonitrile containing 0.05% formic acid) and B (15% 5 mM ammonium formate/80% acetonitrile/5% methanol containing

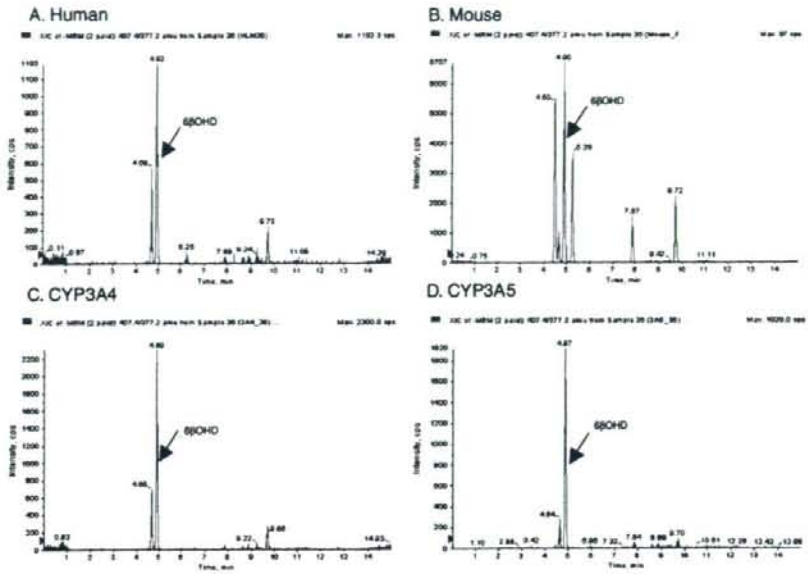

Figure 1. Typical chromatograms of 6βOHD elution formed by liver microsomes and recombinant CYPs. Human liver microsomes (A), mouse liver microsomes (B, male), recombinant 3A4 (C), and recombinant CYP3A5 (D).

0.05% formic acid). The column temperature and the flow rate were 40°C and 0.9 ml min^{-1}, respectively. The gradient conditions for elution were as follows: 100 to 5% B (0.0–9.0 min); 55 to 100% B (9.0–9.1 min); 100% B (9.1–10.5 min); 100 to 5% B (10.5–10.6 min); and 5% B (10.6–15.0 min). The inaccuracy value of each concentrations was <20%.

Kinetic analysis

The kinetic parameters for metabolite formation were calculated using GraphPad Prism 4.02 (GraphPad Software, San Diego, CA, USA).

Statistical analysis

Statistical differences were analysed by a Student's *t*-test.

Results

Kinetics of 6βOHD formation

The kinetic analysis for the DEX 6β-hydroxylation was conducted using liver microsomes from human and mouse, in addition to recombinant CYP3A4 and CYP3A5. As shown in Figure 1, 6βOHD was a major hydroxylated metabolite from DEX in human and mouse

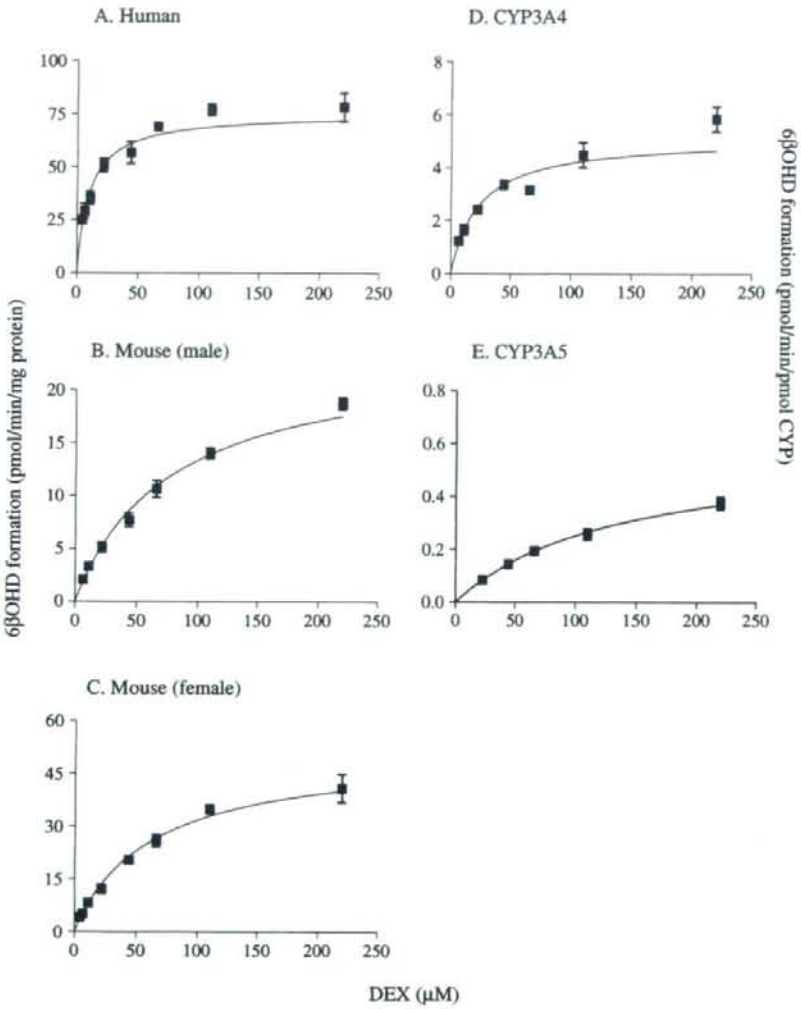

Figure 2. Activity versus substrate concentration profiles for DEX 6β-hydroxylation by liver microsomes and recombinant CYPs. Human liver microsomes (A), mouse liver microsomes (B, male), mouse liver microsomes (C, female), recombinant CYP3A4 (D), and recombinant CYP3A5 (E).

liver microsomes, and recombinant CYP3A4 and CYP3A5. 6βOHD formation was linear as a function of reaction time and microsomal protein concentrations used in the current study (data not shown). The activity versus substrate concentration profiles for 6βOHD formation are depicted in Figure 2 and the kinetic parameters are summarized in Table I. The intrinsic clearances (CL_{int}) with human liver microsomes were 23- and 8.9-fold higher than those of male and female mouse liver microsomes, respectively. Both CYP3A4

Table I. Kinetic parameters for 6βOHD formation in pooled liver microsomes and recombinant CYP microsomes.

Enzyme sources	Kinetic parameters		
	K_m (μM)	V_{max}	CL_{int}[a]
Liver microsomes[b]		(pmol min^{-1} mg^{-1} protein)	(μl min^{-1} mg^{-1} protein)
Human	10 ± 1	75 ± 4	7.5
Mouse (male)	76 ± 10	24 ± 2	0.32
Mouse (female)	61 ± 7	51 ± 4	0.84
Recombinant microsomes[c]		(pmol min^{-1} $pmol^{-1}$ CYP)	(μl min^{-1} $pmol^{-1}$ CYP)
CYP3A4	23 ± 3	5.1 ± 0.4	0.22
CYP3A5	140 ± 23	0.60 ± 0.06	0.0043

Kinetic parameters were calculated from the fit curves by non-linear regression using GraphPad Prism 4.02. Kinetic parameters are the mean ± standard error (SE) derived from triplicate determinations.
[a] *In vitro* intrinsic clearance (CL_{int}) = V_{max}/K_m.
[b] Microsomal concentrations of human and mouse liver were 0.1 and 0.5 mg ml^{-1}, respectively.
[c] CYP concentrations of CYP3A4 and CYP3A5 were 2 and 30 pmol ml^{-1}, respectively. Considering the microsomal binding, the protein concentration was adjusted to 0.23 mg ml^{-1} by the addition of control microsomes.

and CYP3A5 catalysed 6βOHD formation and the CL_{int} for CYP3A4 was 51-fold higher than that for CYP3A5.

Excretion of 6βOHD and DEX in chimeric mouse urine after DEX administration

Urinary excretion of 6βOHD was determined after the s.c. injection of DEX to chimeric mice at a dose of 10 mg kg^{-1} (Table II). There were no clear gender-dependent differences and no changes in excretion of 6βOHD and DEX after treatment of urine samples with β-glucuronidase/sulfatase (data not shown). Almost equal amounts (4–5% of dose) of 6βOHD and DEX were excreted in the urine collected for 72 h and the metabolic ratio of 6βOHD to DEX was almost constant (1.1–1.5) throughout collection the periods. Excretion of 6βOHD and DEX was almost complete after the first 48 h following the DEX injection.

Effect of rifampicin treatment on 6βOHD and DEX excretion

Before and after rifampicin or vehicle treatment, urinary samples were collected for 48 h after DEX administration and analysed to determine excretion of 6βOHD and DEX (Table III). When comparing the metabolic ratio of 6βOHD with DEX in the groups that did not receive the i.p. injection of vehicle or rifampicin (in the column titled Pre-rifampicin), the ratio was almost the same between the two groups. The ratio of 6βOHD to DEX was not changed before and after vehicle treatment. This ratio was increased twofold by treatment of rifampicin as shown in the column titled Change (post-/pre-).

Immunohistochemical analysis

Livers from the control and rifampicin-treated chimeric mice were stained immunohistochemically with antibodies against CYP3A4 and CYP3A5 (Figure 3). CYP3A4 and CYP3A5 were detected in the area of humanized liver as described previously (Tateno et al. 2004).

Table II. Excretion of 6βOHD and DEX after treatment of DEX in chimeric mice with a humanized liver.

Time (h)	Excretion (% of dose)		Metabolic ratio (6βOHD/DEX)
	6βOHD	DEX	
0–24	4.23 ± 0.97	3.83 ± 0.43	1.10 ± 0.17
24–48	0.45 ± 0.17	0.34 ± 0.16	1.39 ± 0.37
48–72	0.16 ± 0.12	0.11 ± 0.09	1.54 ± 0.15
Total	4.83 ± 1.22	4.28 ± 0.67	1.12 ± 0.17

Animals received the subcutaneous injection of DEX at a dose of 10 mg kg^{-1}. Urine samples were collected every 24 h for 72 h.
Values are the mean or mean ± standard deviation (SD) from three animals.

Table III. Effect of rifampicin treatment on urinary 6βOHD and DEX excretion in chimeric mice with a humanized liver.

Treatment	Metabolic ratio (6βOHD/DEX)		
	Pre-rifampicin	Post-rifampicin	Change (post-/pre-)
Control	1.00 ± 0.49 (0.57–1.89)	1.64 ± 0.72 (0.64–2.42)	1.75 ± 0.73 (0.94–2.63)
Rifampicin	0.84 ± 0.35 (0.25–1.35)	2.56 ± 1.03 (1.15–3.73)	3.50 ± 1.96* (1.70–6.96)

Animals received the i.p. injection of vehicle or rifampicin once a day for 4 days at a dose of 50 mg kg^{-1}. On 3 days before and 1 day after vehicle or rifampicin treatment, animals received the s.c. injection of DEX at a dose of 10 mg kg^{-1}. Urine samples were collected for 48 h.
Values represent mean ± standard deviation (SD) from six to nine animals. Values in the parentheses represent the range of minimal and maximal values.
*Significantly different from control values ($p < 0.05$).

The immunohistochemical staining was intense for CYP3A4 (CYP% = 50.0%) and faint for CYP3A5 (CYP% = 15.2%). The positive reaction for CYP3A4 was increased (CYP% = 73.7%) after rifampicin treatment. Only CYP3A4 staining significantly increased after rifampicin treatment ($p < 0.01$) and CYP3A5 staining did not changed.

Discussion

6βOHD formation has been previously reported to be catalysed by CYP3A4 (Gentile et al. 1996), but participation of CYP3A5, another member of the CYP3A subfamily, has not been previously determined. As shown in the current study, 6βOHD formation is catalysed by both CYP3A5 and CYP3A4. The intrinsic clearance by CYP3A4 is more than 50-fold higher than that of CYP3A5. These results indicate that DEX 6β-hydroxylation is a suitable substrate that partially differentiates CYP3A4 and CYP3A5 activities.

Although mouse liver was highly replaced by human liver in uPA$^{+/+}$/SCID chimeric mice, 20–30% of mouse liver was still present. For sensitive detection of CYP induction in the mouse, a probe should be metabolized more rapidly in human liver microsomes than in mouse liver microsomes. Activities of DEX 6β-hydroxylation by human liver microsomes are higher than those in mouse liver microsomes (Tomlinson et al. 1997) and the reaction is catalysed by CYP3A4 (Gentile et al. 1996). As shown in the current study,

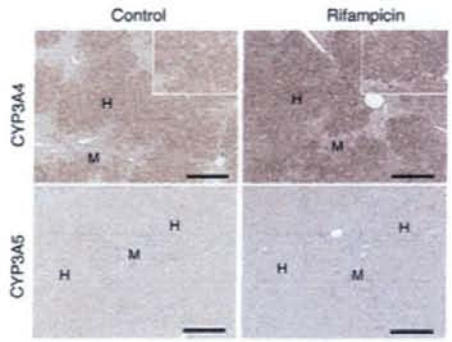

Figure 3. Immunohistochemical analyses of CYP3A4 and CYP3A5 in chimeric mice with a humanized liver. Immunohistochemical staining of CYP3A4 and CYP3A5 revealed cytoplasmic positive reactions in large clear human hepatocytes. The immuno-expression (CYP%, mean± SD from five animals) of CYP3A4 and CYP3A5 were 50.0% ± 5.2% and 15.2% ± 21.9%, respectively, in control mice. The expression of CYP3A4 and CYP3A5 were 73.7% ± 9.8% and 11.2% ± 11.8%, respectively, in rifampicin-treated animals. H, human hepatocyte area; M, mouse hepatocyte area; bar, 100 μm; and insert, higher magnification (× 2.1) for each figure.

6βOHD formation by CYP3A5 was much lower that that by CYP3A4. After DEX administration to man, 6βOHD is excreted as a major urinary metabolite, and only a small fraction of unchanged DEX and DEX glucuronide were detected (Minagawa et al. 1986). Accordingly, it is concluded that DEX is a suitable probe to detect induction of CYP3A4-mediated metabolism in chimeric mice with a humanized liver by a non-invasive method.

In the current study, the ratio of 6βOHD to DEX after DEX treatment was 1.1 in the urine of the chimeric mice with a humanized liver. Although the first study reported that after DEX administration to man, 6βOHD was detected as a major urinary metabolite (about 30% of the dose) and DEX as only a small portion (about 2% of the dose) (Minagawa et al. 1986), a recent study reported a ratio of 1.4 by an LC/MS method (Zurbonsen et al. 2004). The cause of the differences in these latter two studies is not clear but may arise to different assay methods, individual differences, different population of subjects administered, etc.

Although DEX is also reported as an inducer of CYP3A4, a metabolic ratio of 6βOHD to DEX is not changed before and after vehicle treatment. The result indicates that CYP3A4 is not induced by a single s.c. treatment of DEX at a dose of $10\,mg\,kg^{-1}$.

The present paper has reported a non-invasive method to detect induction of CYP3A4 in chimeric mice with a humanized liver. A change of the metabolic ratio (6βOHD/DEX) before and after inducer treatment in the same animal minimizes inter-individual differences of any factors which could affect the ratio. It is concluded that the chimeric mice with a humanized liver coupled with determining the changes of the urinary metabolic ratio (6βOHD/DEX) before and after inducer treatment are a useful animal model for detecting the induction of CYP3A4 by a non-invasive method.

Further studies such as induction study using another CYP3A4 inducers and co-administration study of CYP3A4 substrates with inhibitors will provide further information on usefulness of the chimeric mice with a humanized liver for detecting CYP3A4 induction by a non-invasive method.