2006; Strom *et al.*, 2006). However, the hepatocyte transplantation procedure can be limited in the number of cells that can be infused at one time (Fox *et al.*, 1998; Ohashi *et al.*, 2001; Stephenne *et al.*, 2006, 2007; Strom *et al.*, 2006) and could prevent the necessary level of therapy that may be required to achieve a particular therapeutic modality. For this reason, an alternative approach has been recently developed using isolated hepatocytes in which the individual cells have been engineering into biologically active tissues, which have the ability to simulate whole-organ liver function in an ectopic site (Griffith and Naughton, 2001; Kuge *et al.*, 2006; Ohashi *et al.*, 2000, 2005a, 2005b, 2005c, 2007; Ohashi, 2008; Yokoyama *et al.*, 2006). Unlike the individual hepatocyte infusion, tissue-engineering technology has not been shown to be limited by cell number, but another major obstacle that needs to be overcome is the ability to maintain viable functional hepatocytes in these ectopic sites for extended periods of time (>6 months). By successfully manipulating these engineered liver tissues to provide extended therapy, a greater number of patients with various liver disorders and diseases could be treated.

As tissue-engineering technology has evolved over the past several years, it has spurred significant interest into the field of liver regenerative medicine (Griffith and Naughton, 2001; Ohashi, 2008). To maximize the efficiency of maintaining viable engineered liver tissue in an ectopic site, such as implantation under the kidney capsule (Kuge *et al.*, 2006; Ohashi *et al.*, 2000, 2005a, 2005b, 2005c), in our laboratory a mixture is created of isolated hepatocytes with extracellular matrix components (matrigel), which are rich in laminin and type IV collagen. The functionality of the engineered liver tissues have been confirmed in our previous short- to medium length studies (i.e. 50–200 days), using parameters that include tissue protein expressions of liver enzymes, viral infectivity, uptake of exogenous chemicals resulting in subsequent metabolism and regenerative growth potential (Kuge *et al.*, 2006; Ohashi *et al.*, 2000, 2005a, 2005b, 2005c).

In the present study, we engineered liver tissues under the kidney capsule in mice and assessed the stability of this ectopic liver system for over 450 days, which is nearly the life-span of a normal mouse. In addition, we also assessed liver-specific functions, such as glycogen synthesis, drug metabolism and regenerative potential, within the engineered liver tissues at the end of the experiment (days 450–464) to document the biological efficacy of these liver tissues after such a prolonged period of time.

2. Materials and methods

2.1. Animals

Transgenic mice expressing human α-1 antitrypsin (hA1AT) under the hepatocyte-specific promoter (hA1AT-FVB/N, H-2^q; kindly provided by Dr Bumgardner, Ohio State University, Columbus, Ohio, USA) at 12–13 weeks of age (Bumgardner *et al.*, 1998) were used as donors for hepatocyte isolation. Wild-type female FVB/N mice (aged 11–12 weeks), which were syngenic to the hA1AT-FVB/N, were used as the recipient animals. All mice were maintained in the Animal Center at Nara Medical University, and all of the mouse experiments were conducted in accordance with the institutional guidelines set forth by Nara Medical University Animal Care Committee. Mice were placed in cages within a temperature-controlled room with a 12 h light/12 h dark cycle as well as *ad libitum* access to food and water.

2.2. Hepatocyte isolation and purification

Hepatocytes were isolated from hA1AT transgenic mice using a modified two-step collagenase perfusion method, as previously described (Ohashi *et al.*, 2005a, 2005b, 2007). Isolated cells were filtered through a nylon mesh membrane and hepatocytes were purified by low-speed centrifugation at $50 \times g$ for 5 min, followed by Percoll (Amersham Biosciences, Uppsala, Sweden) isodensity centrifugation. Cells were resuspended with DMEM medium (Sigma, St. Louis, MO, USA) and the cell viability was determined by Trypan blue exclusion. In the present studies, experiments were conducted only when the hepatocyte viabilities exceeded 90%.

2.3. Liver tissue engineering

Hepatocytes were prepared for engineering liver tissue under the kidney capsule space as previously described (Kuge *et al.*, 2006; Ohashi *et al.*, 2000, 2005a, 2005b, 2007; Yokoyama *et al.*, 2006). In brief, isolated hepatocytes were resuspended with serum-free DMEM with an equal volume of EHS-gel (Matrigel; BD Biosciences, Bedford, MA, USA) to a final ratio of 1.5×10^6 hepatocytes/100 µl. A total of 1.5×10^6 hepatocytes were transplanted under the left kidney capsule space. All the surgical procedures were performed under isofluorane (Forane, Abbott Laboratories, Abott Park, IL, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Functional volumes of the engineered liver tissues were assessed by measuring the recipient serum hA1AT concentrations. Serum samples were periodically obtained from the recipient mice and were assayed by ELISA, using a primary antibody against hA1AT (DiaSorin, Stillwater, MN, USA) and a secondary goat antibody conjugated with horseradish peroxidase (HRP; Research Diagnostics, Flander, NJ, USA), as previously described (Ohashi *et al.*, 2000).

2.5. Liver regeneration stimulus

At day 450 of the experiments, a liver regenerative stimulus was induced to some of the recipient mice by performing a 70% partial hepatectomy (two-thirds PH) to the naïve liver. In this procedure, we removed the medial and left lateral lobes of the naïve livers as preciously described (Ohashi *et al.*, 1996, 2005a, 2007). For analysis of the hepatocyte proliferation status in the naïve and engineered livers, 5-bromo-2′-deoxyuridine (BrdU) was administered at a dose of 1 mg/day, using an osmotic mini-pump (Model 2001; Alzet, Palo Alto, CA, USA). This was initiated on the day of the two-thirds PH and was administered for a period of 14 days until day 463. At this point, the naïve livers, the kidneys containing the engineered liver tissues and the duodenum (as a positive control for BrdU incorporation) were removed and processed for histological analysis. During this 14 day period, serum samples were also obtained at various days (450, 456 and 463) to assess the functional activity of the engineered liver tissues.

2.6. Cytochrome P450 induction

At day 450, some recipients received intraperitoneal injection of phenobarbital (PB; Wako Pure Chemical Industries, Osaka, Japan) or 3-methylcholantree (3-MC; Sigma, St. Louis, MO, USA) for 3 consecutive days (Ohashi *et al.*, 2007; Yokoyama *et al.*, 2006). The mice were sacrificed 3 h after the last injection. Naïve livers and engineered liver tissues were processed for histological analyses.

2.7. Histological and immunohistochemical analyses

Naïve liver specimens, engineered liver tissues containing neighbouring kidney, and the duodenum of the recipient mice were harvested and fixed in 10% buffered formalin. Specimens were paraffin-embedded and sliced into 5 μm thick sections, which were subsequently processed for haematoxylin and eosin (H&E) staining or immunohistochemical analyses. For the BrdU and hA1AT immunofluorecent co-staining, deparaffinized sections were treated with 2 N HCl for 90 min. Non-specific binding sites were blocked with 10% normal goat serum. The sections were then incubated overnight at 4 °C with mouse anti-BrdU antibody (1 : 30; Becton-Dickinson, San Jose, CA, USA) and rabbit anti-hA1AT antibody (1 : 100; YLEM, Roma, Italy). Alexa-Fluor-488 and Alexa-Fluor-555 (Molecular Probes, Eugene, OR, USA) were used as a secondary reagent. In each mouse, the hepatocytes' BrdU labelling indices in the naïve liver and engineered liver tissues were determined separately by counting a total of 1000 hepatocytes in 20–30 randomly selected liver fields. The BrdU labelling indices were expressed as a percentage of BrdU-positive hepatocytes. For the CYP2B and CYP1A immunostaining, non-specific binding sites were blocked with normal goat (for CYP2B) and normal rabbit (for CYP1A) serum. Tissues were incubated with either rabbit anti-mouse CYP2B antibody (1 : 500; Chemicon International, Temecula, CA, USA) or sheep anti-mouse CYP1A (1 : 500; Chemicon International) at 4 °C overnight. After several washes, the sections were incubated for 30 min at room temperature with ABC solution (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Visualization of the immune complexes was performed by incubating with 3,3′-diaminobenzidine (DAB). For the cellular glycogen detection, periodic acid–Schiff (PAS) staining was performed as described previously (Ohashi *et al.*, 2007). To confirm the staining specificity of the cellular glycogen, serial sections were pre-treated with salivary amylase for 60 min followed by the same PAS staining procedures.

2.8. Statistical analysis

All the values calculated in the present study were provided as means ± standard deviation (SD). Statistical differences in the values were determined by a Student's *t*-test. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Stable and persistent viability of the liver tissues engineered under the kidney capsule

Functional activity of the engineered liver tissues was assessed by measuring serum hA1AT levels in the recipient mice. As shown in Figure 1A, B, recipient mice showed stable and persistent serum hA1AT levels in the range 9000–30 000 ng/ml, suggesting that the engineered liver tissues could be viable and stably maintained throughout the 450 days experimental period, which was nearly the life-span of the mice themselves. These mice were generated in two separate experiments, in which each group of mice was generated using a different source of donor hepatocytes to demonstrate the reproducibility of this approach. Histological examination at day 463 confirmed that a thin layer of liver tissue had developed and engrafted within the kidney capsule with a thickness of up to 3 cells in height. The hepatocytes that were found to comprise the engineered liver tissues showed normal healthy morphology in terms of presenting a large and eosinophilic cytoplasm (Figure 1C, D). It is important to note that there was no evidence of tumour formation observed in any of the engineered tissues.

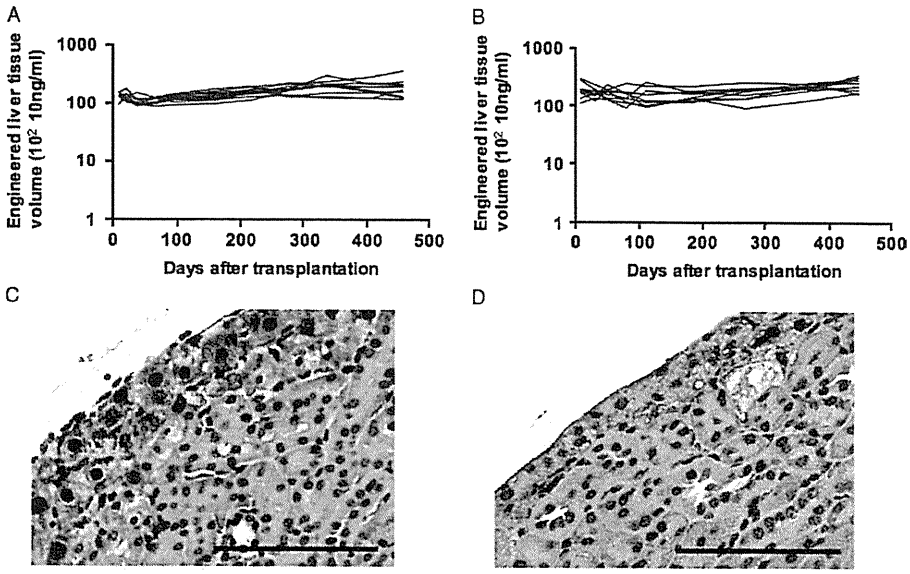

Figure 1. Functional maintenance of the engineered liver tissues under the kidney capsule. (A, B) Functional volume of engineered liver tissues under the kidney capsule was determined by measuring murine serum hAAT levels at various time points up to 450 days. (A) and (B) demonstrate two separate experiments using different donor hepatocytes ($n = 11$ and $n = 9$, respectively). (C) Haematoxylin and eosin staining of the engineered liver tissues are shown at day 453 from the experiment in (A) and (D) from day 464 from experiment in (B). Scale bars = 100 μm

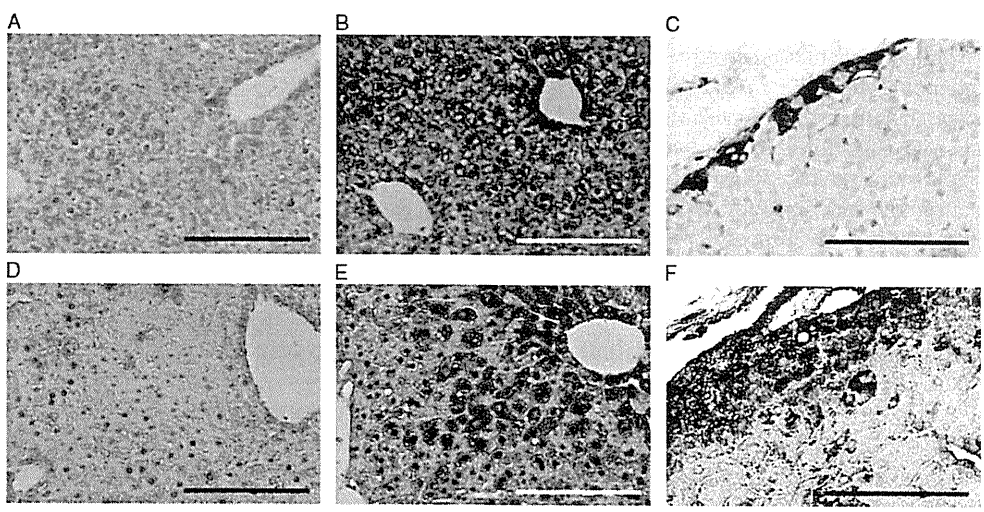

Figure 2. Induction of drug-metabolizing enzymes in naïve mouse livers and engineered liver tissues under the kidney capsule. CYP2B and CYP1A were induced by intraperitoneal injection of PB and 3-MC, respectively. Immunohistochemical staining was performed for CYP2B (A–C) and CYP1A (D–F). (A) Mouse liver without PB treatment. (B) Mouse liver following PB treatment. (C) Engineered liver tissues under kidney capsule following PB treatment. (D) Mouse liver without 3-MC treatment. (E) Mouse liver following 3-MC treatment. (F) Engineered liver tissues under kidney capsule following 3-MC treatment. Scale bars = 100 μm

3.2. Drug-metabolizing functions in engineered liver tissues following long-term implantation under the kidney capsule

To determine whether the engineered liver tissues continued to possess liver-specific drug-metabolizing function after 450 days under the kidney capsule, we inoculated the mice with the engineered liver tissue, using either PB (CYP2B inducer; Waxman and Azaroff, 2006; Yokoyama *et al.*, 2006) or 3-MC (CYP1A inducer; Yokoyama *et al.*, 2006; Zacharova *et al.*, 2003). This enabled us to investigate whether the liver tissue engineered under the kidney capsule long-term after cell transplantation could retain its ability to uptake exogenous compounds as well as induce compound-specific CYP, which would occur in normal livers. As shown in Figure 2, strong immunostaining for CYP2B and CYP1A were found in both the engineered liver tissues and naïve livers. These immunohistochemical findings clearly demonstrate that the engineered liver tissues have retained their ability to take up circulating compounds and have the capability to strongly induce drug-metabolizing

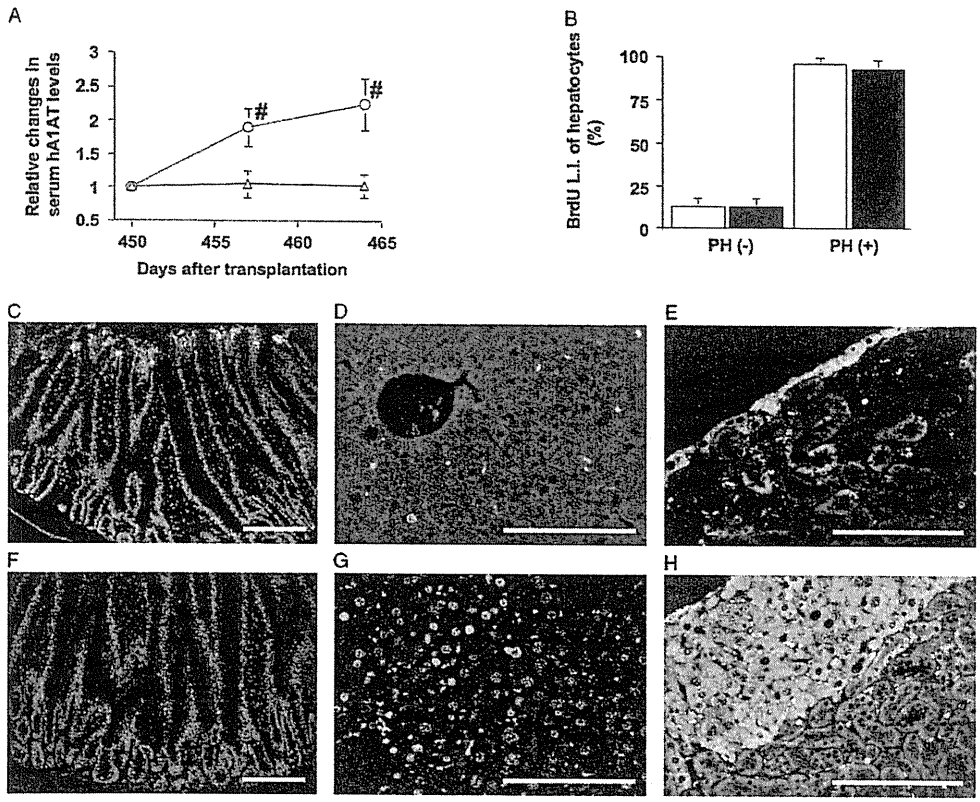

Figure 3. Regenerative ability of the engineered liver tissues within the kidney capsule. We performed sham operation for either control (C–E) or two-thirds PH (F–H) on the recipient mice at day 450. After the surgical procedures, BrdU was continuously delivered through an osmotic minipump for a period of 14 days, starting at day 450, until day 464. (A) Functional activity of the engineered liver tissues was determined by measuring serum hAAT levels by ELISA. At days 450, 457 and 464 the serum was isolated, the hAAT levels were measured and the relative levels of hAAT were calculated by expressing the values relative to day 450. Triangle, mice in sham operation ($n = 5$); circle, mice in two-thirds PH group ($n = 4$). #, $p < 0.05$ between groups. (B) Hepatocyte BrdU labelling index (LI) was examined in naïve livers or engineered liver tissues within the kidney capsule. Representative photomicrographs of BrdU and hAAT co-immunostaining are shown in the duodenum (C, F), naïve liver (D, G) and engineered liver tissues found within the kidney capsule (E, H) from the control, non-PH (C–E) and two-thirds PH (F–H) groups. BrdU-positive nuclei were labelled with Alexa-Fluor-555 and the hAAT-positive cytoplasm from the donor hepatocytes were labelled with Alexa-Fluor-488. Scale bars = 100 μm

enzymes, such as cytochrome P450s, at levels similar to those detected in the naïve livers.

3.3. Regeneration potential of the engineered liver tissues following long-term implantation under the kidney capsule

One of the unique properties of the liver is its ability to regenerate itself following chemical injury or surgical partial removal. Our previous mouse studies demonstrated that engineered liver tissues implanted under the kidney capsule for at least 70 days possessed their full regenerative growth potential following a two-thirds partial hepatectomy (Ohashi *et al.*, 2005a, 2005b). These results led us to investigate whether engineered liver tissues that were implanted under the kidney capsule for a longer period of time, such as 450 days, as performed in the current study, could still retain their ability to regenerate following a proliferative stimulus. To study this effect, we performed two-thirds partial hepatectomy (PH) in the recipient mice at day 450 following hepatocyte transplantation under the kidney capsule; 14 days later (at day 464 of the experiment), the functional activity of the engineered liver tissues was determined by measuring the serum hA1AT levels, and we detected an increase of 223 ± 37% in the PH mice relative to the levels measured in the non-PH mice (Figure 3).

In addition, we inserted an osmotic minipump in the sham-operated and two-thirds PH recipient mice to administer BrdU, a cell cycle marker, over the 14 day period, starting at day 450, until 464. The BrdU and hA1AT immunofluorescent co-staining of the engineered liver tissues showed significantly greater BrdU-labelling index (LI) in the PH mice (91.1 ± 6.4%) compared to the control sham-operated mice (11.1 ± 5.4%). These BrdU LIs were consistent with the values obtained from the naïve livers in the PH and sham-operated mice (Figure 3B). The strong positive signal intensity of hA1AT detected in the engineered liver tissues implanted under the kidney capsule (Figure 3E, H) and the lack of hA1AT-positive cells in all other organs, including the liver (data not shown), confirmed that the serum hA1AT

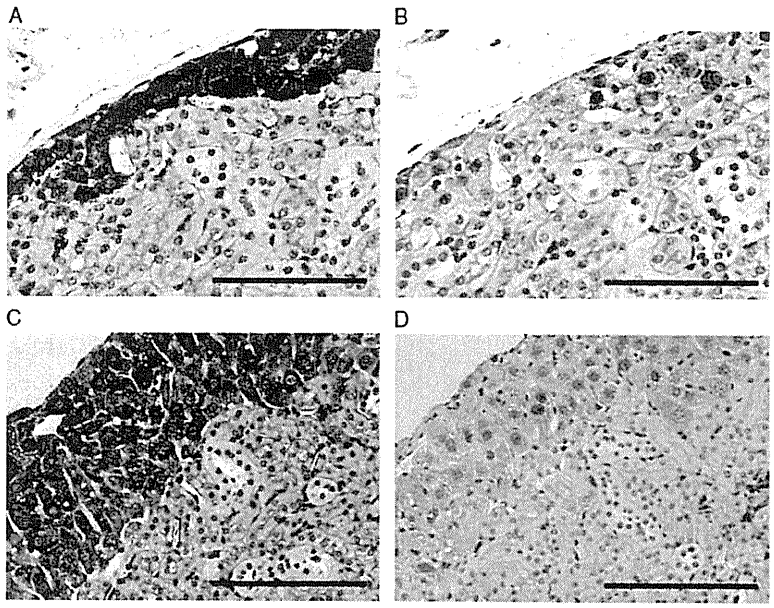

Figure 4. PAS staining of the engineered liver tissues to determine glycogen synthesis. PAS staining was performed to confirm the presence of cellular glycogen in sections that were either non-treated (A, C) or pretreated with salivary amylase (B, D); sections in (A, B) obtained from non-PH mice at day 464, and sections (C, D) obtained from mice that received a two-thirds PH at day 450 and were subsequently sacrificed at day 464. Scale bars = 100 μm

levels were solely originating from the engineered liver tissues.

3.4. Glycogen synthesis in engineered liver tissues following long-term implantation under the kidney capsule

To establish glycogen synthesis, which is another liver function, to the engineered liver tissues implanted under the kidney capsule, we performed PAS staining on sections from sham-operated and two-thirds PH mice at day 464. Both the relatively quiescent hepatocytes in the control mice and the actively proliferating hepatocytes in the two-thirds PH mice showed similarly strong staining for PAS, with the intensity levels being consistent with that observed in the naïve livers (Figure 4). Pretreatment with salivary amylase in the tissue sections significantly diminished the PAS staining, which confirmed that the staining specificity of cellular glycogen in the naïve livers and engineered liver tissues under the kidney capsule.

4. Discussion

The present study achieved life-long stability of *de novo* engineered liver tissues implanted under the kidney capsule. These ectopically transplanted liver tissues were functionally active for at least 450 days, and these bioengineered tissues possessed liver-specific functions in terms of protein expression, drug uptake and metabolism, regenerative growth and glycogen-synthetic capability. At present, we believe that this is the first report to successfully document the ability of bioengineered liver tissues to retain their functionality for the duration observed in this study, i.e. in excess of 450 days.

In our study, we transplanted hepatocytes for the creation of liver tissues under the kidney capsule of mice at 11 weeks of age, and the study was terminated after more than 450 days, since it has been shown that the average life-span of experimental mice is approximately 480 days (Kohn, 1971) and we did not want to risk the loss of the liver tissues and their analyses due to an unscheduled and age-related death. Since we were able to verify stable functional activity throughout the experimental period, we can reasonably speculate that these engineered liver tissues could survive for the duration of the entire life-span of the mouse. At this time, we do not know whether the prolonged ectopic graft survival observed in the present study using small animal models can translate into functional tissues for therapeutic applications in humans. However, the extended 450 day survivability of the engineered liver tissues in the recipient mice is an important advance in the field, and is a necessary proof-of-concept step in the development towards its use as a future clinical application.

Our study examined not only the longevity of the survivability of the engineered liver tissues but also the flexibility of the system to mimic normal liver function. First, the ectopic liver systems were found to have the potential to self-renew hepatocytes, which is a common property of the liver following hepatocellular loss (Fausto, 2001; Michalopoulos, 2007). Previous studies in our laboratory have shown that ~12% of the hepatocytes in the naïve livers entered the cell cycle under normal condition, as measured by BrdU incorporation over a 14 day period (Ohashi *et al.*, 2005a). In the present

study, we administered BrdU over the same number of days, except at a different time point in the age of the mouse (i.e. days 450–464), and we found that 11.6% of hepatocytes were positive for BrdU in the engineered liver tissues under the kidney capsule. These findings demonstrated that the proliferative rate in the engineered liver systems implanted under the kidney capsule is similar to that of normal livers, and that this level of proliferation is sufficient to maintain these ectopically transplanted tissues over a prolonged period of time.

Another important biological property of the liver is its ability to promote active cellular proliferation in the face of a surgical, necrotic and/or chemical injury (Fausto, 2001; Michalopoulos, 2007) and so it was important to assess whether proliferative stimuli could affect ectopically transplanted liver tissues in a similar manner. In general, normally quiescent hepatocytes rapidly progress into the cell cycle following a proliferative stimulus, which leads to robust cellular proliferation until there is a restoration of functional mass within 14 days. It is important to note that this regenerative proliferation of the liver is mediated by mature hepatocytes and is not attributed to a select population of stem cells (Michalopoulos, 2007). Our results demonstrated a high BrdU labelling index in both the naïve liver and the engineered liver tissues (93.8% and 91.1%, respectively), indicating that the regenerative growth detected in the engineered liver tissue was mediated by mature hepatocytes. The synchronized regeneration events between the two sources of liver cells (i.e. naïve liver and engineered liver tissue) are also highlighted by the significantly increased functional volume by over 200% compared to the pre-PH level, as determined by the measurement of the hA1AT. It has previously been shown that liver regeneration can progress based on the signalling crosstalk between the hepatocytes and non-parenchymal cells (Fausto, 2001; Michalopoulos, 2007). Although isolated hepatocytes were the only cell types used in the generation of the engineered liver tissue, the present data strongly suggests that this type of intercellular crosstalk must have been established and maintained even within our heterotopic tissues.

An additional function that we examined in the engineered liver tissue is its ability to uptake exogenous drugs and subsequently activate enzymes involved in its metabolism, which is a critical property found in normal functioning livers. In previous studies using the primary hepatocyte cell culture system, drug metabolic function would decline in the order of hours or days following the plating of the cells, even though multiple modifications were attempted to optimize the media conditions (Fahl *et al.*, 1979; Gomez-Lechon *et al.*, 2004). This is in marked contrast to the results in the current study, in which we were able to manipulate the isolated hepatocytes into a tissue sheet and maintain its drug metabolic effects for at least 450 days. It has been reported that the highly porous endothelial linings along the hepatic cord are essential structures for the filtration of circulating particles or compounds from the systemic circulation into the space of Disse. This anatomical feature allows the foreign matter to reach the hepatocytes (Braet *et al.*, 2001) for removal from the circulation. Once the compounds are taken up by the hepatocytes, a complex cascade of transcription factors are activated to promote the induction of cytochrome P450 (*CYP450*) gene expression, which are essential intracellular enzymes known for their drug metabolism (Gomez-Lechon *et al.*, 2004). Our present findings clearly demonstrated that the engineered liver tissues could simulate normal liver function as a CYP450-inducing and drug-metabolizing system, and it may be possible for these engineered liver tissues not only to metabolize chemical compounds, as shown in this study, but to detoxify endotoxin and ammonia products. These features would be of great importance for the functionality of this liver system, since its therapeutic repertoire would be expanded by enabling its use as a supportive liver-assisting device to eliminate circulating blood waste products in patients suffering from critical liver failure.

In summary, hepatocyte-based therapies have become a viable and alternative therapeutic modality in the treatment of liver diseases, but continued improvement and innovation in this technology would further help in advancing its utilization in clinics (Fox *et al.*, 2006; Ofosu, 2008; Ohashi *et al.*, 2007; Ohashi, 2008). At present, current technologies in this field using bioartificial liver-assisting devices struggle in their efficacy, due in large part to the inability of the hepatocytes to maintain their biological function within the device (Strain and Neuberger, 2002). Although the current study has not addressed the potential for scalability to larger mammals, our results clearly documented that the *de novo* engineered liver tissues were capable of maintaining their morphology and function even after long-term implantation under the kidney capsule for nearly the lifespan of the mouse. These results increase the likelihood that this technology could contribute as a therapeutic modality for treating liver diseases, and the increased longevity and functionality of the liver tissues could greatly expand the type of liver diseases that could be amenable to this therapeutic approach.

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Engineering Liver Tissues Under the Kidney Capsule Site Provides Therapeutic Effects to Hemophilia B Mice

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Recent advances in liver tissue engineering have encouraged further investigation into the evaluation of therapeutic benefits based on animal disease models. In the present study, liver tissues were engineered in coagulation factor IX knockout (FIX-KO) mice, a mouse model of hemophilia B, to determine if the tissue engineering approach would provide therapeutic benefits. Primary hepatocytes were isolated from the liver of wild-type mice and suspended in a mixture of culture medium and extracellular matrix components. The hepatocyte suspension was injected into the space under the bilateral kidney capsules of the FIX-KO mice to engineer liver tissues. The plasma FIX activities (FIX:C) of the untreated FIX-KO mice were undetectable at any time point. In contrast, the liver tissue engineered FIX-KO mice achieved 1.5–2.5% of plasma FIX activities (FIX:C) and this elevated FIX:C level persisted throughout the 90 day experimental period. Significant FIX mRNA expression levels were found in the engineered liver tissues at levels similar to the wild-type livers. The present study demonstrates that liver tissue engineering could provide therapeutic benefits in the treatment of hemophilia B.

Key words: Hemophilia B; Liver tissue engineering; Cell therapy; Blood clotting factors; Drug delivery system; Regenerative medicine

INTRODUCTION

Liver tissue engineering technology has evolved over the past several years, and spurred significant interest as potential new therapies for several types of liver disease, including genetic deficiencies (14,18–21,23). Several studies have succeeded in achieving functional persistency of the liver tissues engineered in subcutaneous sites, under the kidney capsule space, or the omentum (8,9,15–17,19,20,28). Once the liver tissue engineering procedures established clinically applicable success, several types of liver diseases will be treated with the tissue engineering procedures. Because most blood clotting factors are produced by the liver (3,11), liver tissue engineering could be a valuable approach in the treatment of inherited clotting factor deficiencies, including hemophilia A and B. As a proof-of-principle, therapeutic effects were achieved in a mouse model of hemophilia A engineered liver tissue using wild-type hepatocytes (19).

Hemophilia B is a recessive X-chromosome-linked congenital bleeding disorder, and its prevalence is approximately 1 in 30,000 males. The main clinical manifestations of hemophilia B are unpredictable, recurrent, spontaneous bleeding events in various sites including the major joints and internal organs. The bleeding diathesis of hemophilia B is due to a failure in the production of biologically active coagulation factor IX (FIX) (2). It has been well established that achieving plasma FIX activities (FIX:C) as low as 1% markedly changes the phenotype of hemophilia B patients from severe to moderate, and greatly improves the symptoms and quality of life of the individuals (2).

Provided that efficient engraftment of hepatocytes as well as functional maintenance of clotting factor productions can be achieved in individuals with hemophilia B, cell-based therapy could be a feasible and safe therapeutic option for this disease. Because hepatocytes are the primary cell type in the liver involved in FIX clotting factor production (3), hepatocyte-based therapy could be effective in establishing a novel therapy for hemophilia B. Recent experiments on hepatocyte transplantation into the liver of FIX knockout (FIX-KO) mice achieved 1–2% of FIX:C levels that persisted throughout the experimental period (26). These experiments have encour-

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aged further investigation of the potential of a tissue engineering approach for hemophilia B.

The present study was conducted to engineer liver tissues using wild-type hepatocytes under the kidney capsule space of FIX-KO mice. The FIX-related functionality of the engineered liver tissues was assessed, including the plasma FIX activity levels, histological investigations, and FIX mRNA expression of the engineered tissues. The results show that hepatocytes in the engineered liver tissues retained the ability to produce FIX, thus resulting in an elevation of FIX:C at 1.0–2.5% of normal plasma levels. The results demonstrate that a liver tissue engineering approach could be a novel therapeutic option for the treatment of hemophilia B.

MATERIALS AND METHODS

Animals

Factor IX knock-out (FIX-KO) mice and wild-type C57BL/6 mice, which were syngenic to the FIX-KO mice (originally purchased from The Jackson Laboratory, Bar Harbor, ME), were maintained at the Animal Care Center at Nara Medical University. FIX-KO mice, 10–15 weeks old, were used as the recipients of the liver tissue engineering procedure. Wild-type C57BL/6 mice, 10–12 weeks old, were used for hepatocyte donors. All animal studies were conducted under the protocol approved by the Animal Care Committees at Tokyo Women's Medical University and Nara Medical University. Mice were placed in cages within a temperature-controlled room with a 12-h light/dark cycle and ad libitum access to food and water.

Hepatocyte Isolation and Purification

Hepatocytes were isolated from wild-type mice using a modified two-step collagenase perfusion method as previously described (8,16,19,20). Briefly, the liver was primarily perfused with Hank's balanced salt solution (HBSS) (Sigma, St. Louis, MO) containing 0.09% EGTA followed by a second perfusion using HBSS containing 0.03% collagenase (Sigma) and 5 mM $CaCl_2$. Isolated cells were filtered through a nylon mesh membrane and hepatocytes were then purified by centrifuging at $50 \times g$ for 5 min followed by Percoll (GE Healthcare, Buckinghamshire, UK) isodensity centrifugation. Cells were resuspended in DMEM medium (Sigma), and the cell viability was determined by trypan blue exclusion test. In the present studies, experiments were conducted only when the hepatocyte viabilities exceeded 95%.

Liver Tissue Engineering Procedures

Hepatocytes were prepared for engineering liver tissue under the kidney capsule space as previously described (8,15,17,19). In brief, isolated hepatocytes were resuspended with serum-free DMEM with an equal volume of Engelbreth-Holm-Swarm (EHS) matrix (Matrigel, BD Biosciences, Bedford, MA) to a final ratio of 1.5×10^6 hepatocytes/100 μl. A total of 2.4×10^6 hepatocytes were transplanted under the bilateral kidney capsule space of the FIX-KO mice. As an experimental control, several FIX-KO mice received only an abdominal incision and did not receive hepatocyte infusion (sham operation). All the surgical procedures were performed under inhalation anesthesia using isofluorane (Forane, Abbott Laboratories, Abott Park, IL). To avoid excessive surgical procedure-related bleeding, all FIX-KO mice including the sham operation group received an IP injection of 0.5 ml pooled normal mouse plasma twice, 30 min prior and 1 h after the tissue engineering surgical procedure (22).

Coagulation Assay for Plasma FIX Activity

Blood samples were periodically obtained from the retro-orbital plexus of the recipient FIX-KO mice before and after the tissue engineering procedures. The blood samples were immediately anticoagulated with 0.1 vol of 3.8% sodium citrate (Sysmex, Kobe, Japan). After centrifugation, plasma samples were stored at −80°C until being analyzed. The FIX activity (FIX:C) was quantified by one-stage clotting assay based on the activated partial thromboplastin time using human FIX-deficient plasma (Sysmex). Pooled mouse plasma obtained from wild-type C57BL/6 mice was used as FIX:C standard. Each measurement was reported after subtraction of the preoperational baseline FIX:C levels. We have previously confirmed that FIX activity levels as low as 0.25% of normal mouse plasma could be successfully measured using this assay (26). The development of neutralizing antibodies against FIX, called FIX inhibitor, was assessed by the Bethesda method using plasma obtained at sacrifice time (26). Briefly, residual FIX:C was determined after incubating equal volumes of sample plasma with normal mouse plasma at 37°C for 2 h, and was compared with residual FIX:C of a mixture of FIX knockout mouse and normal mouse plasma. The presence of a significant titer of FIX inhibitor was determined as >0.5 Bethesda unit/ml in this assay.

Histological Analyses

Naive liver specimens and engineered liver tissues containing the neighboring kidney of the recipient FIX-KO mice were harvested and fixed in 10% buffered formalin. The specimens were also embedded in Tissue-Tek O.C.T. compound (Sakura, Torrance, CA). Formalin-fixed specimens were paraffin embedded and sliced into 5-μm-thick sections, which were subsequently processed for hematoxylin & eosin (H&E) staining or histochemical staining. For the cellular glycogen detection, Periodic-Acid-Schiff (PAS) histochemical staining was

performed as described previously (19). To confirm the staining specificity of the cellular glycogen, serial sections were pretreated with salivary amylase for 60 min followed by the same PAS staining procedures.

Laser Capture Microdissection (LCM)

The specimens embedded in the Tissue-Tek O.C.T. compound were sliced into 8-μm-thick sections. The sections were then stained with HistoGene LCM Frozen Section Staining Kit (Arcturus, Mountain View, CA) to visualize the engineered liver tissues under the kidney capsule followed by LCM using a PixCell II LCM System (Arcturus). The microdissected tissues were harvested and subjected to total RNA extraction using the PicoPure RNA Isolation Kit (Arcturus). Isolated RNA was amplified using the RiboAmp RNA Amplification Kit (Arcturus) as described previously (28).

RT-PCR

One microgram of total RNA was reverse transcribed using Omniscript RT Kit (Qiagen, Hilden, Germany), and the first-strand cDNA samples were subjected to PCR amplification in the StepOnePlus Real-Time PCR Systems (Applied Biosystems, Tokyo, Japan) followed by 2% agarose gel electrophoresis. In addition to the FIX gene expression, albumin (Alb), ornithine transcarbamylase (OTC), and factor VII (FVII) were also analyzed as other gene expression specific to hepatocytes. Housekeeping gene, β-actin (ACTB) was used as reference gene. PCR primers for the mouse sequences (FIX, FVII, Alb, OTC, and ACTB) were purchased from TaqMan Gene Expression Assay (Applied Biosystems, Assay ID: Mm01308427_m1, Mm00487329_m1, Mm 00802090_m1, Mm00493267_m1, and Mm00607939_ s1, respectively).

Statistical Analysis

Significant differences were tested by repeated measures ANOVA with the use of the ystat2006 software program (Igakutoshoshuppan, Tokyo, Japan). If a probability value of $p < 0.05$ was obtained, the Dunnett's test was used for comparison for each individual group. A value of $p < 0.05$ was considered to be significant. Data were presented as the mean ± SD.

RESULTS

Liver Tissue Engineering Procedure Used for the FIX-KO Mice

In order to avoid surgical procedure-related bleeding, all the FIX-KO mice including the sham operation group received IP injections of 0.5 ml pooled normal mouse plasma 30 min before and 30 min after the surgical procedure. Liver tissue engineering procedures based on transplantation of hepatocytes suspended with EHS-matrix or sham operation were successfully carried out using FIX-KO mice without any issues related to bleeding and all the recipient mice survived throughout the experimental period. At day 90, some of the recipient FIX-KO mice were sacrificed for histological analyses and mRNA analyses of the engineered liver tissues.

Effect of Liver Tissue Engineering on Coagulation FIX Activity of Hemophilia B

The FIX activity (FIX:C) in plasma obtained from FIX-KO mice received liver tissue engineering or sham operation was quantified by one-stage clotting assay (Fig. 1). The FIX:C of the FIX-KO mice prior to the tissue engineering procedures and the sham operation groups were consistently below the detection limit (0.25% of normal) and thus the values were expressed as 0. In contrast, the FIX:C of FIX-KO mice that received liver tissue engineering procedures increased to 1.60 ± 0.55 at week 3, and this elevated FIX:C was stably maintained throughout the experimental period. On the other hand, no elevation in the FIX:C levels were detected in the sham-operated mice. Overall, the FIX:C levels were significantly higher in the recipient mice in comparison to the levels found in the sham-operated mice at every time point examined ($p < 0.05$). These results indicated that engineering liver tissues by transferring nonhemophilic hepatocytes into the individuals with hemophilia B could provide a therapeutic effect by de novo production of coagulation factor IX.

Morphology and Functions of the Engineered Liver Tissues

The kidney tissues were harvested at day 90 to determine if the hepatocytes within the engineered liver tis-

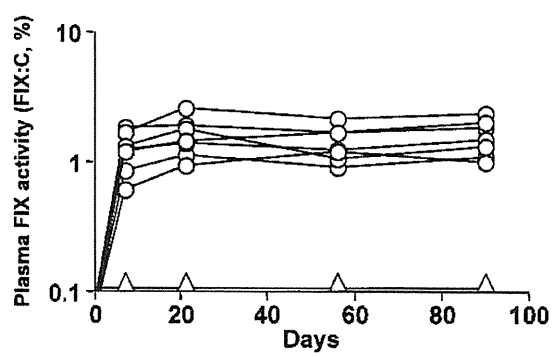

Figure 1. The plasma FIX activity (FIX:C) levels of FIX-KO mice following liver tissue engineering. FIX-KO mice received an injection of hepatocytes resuspended with EHS-matrix into the bilateral under the kidney capsule spaces. Each line with circles ($n = 7$) represents an individual FIX-KO mouse that received hepatocyte injection and the line with triangles ($n = 7$) represents FIX-KO mouse with sham operation.

sues maintained healthy morphology and functions. H&E staining revealed hepatocytes with large cuboidal shapes and eosinophilic cytoplasm next to the renal tubulus (Fig. 2A). Glycogen synthesis, an important liver function, was confirmed by positive PAS staining in the hepatocytes of the engineered liver tissues (Fig. 2B). Staining specificity for cellular glycogen in the PAS staining was confirmed by the finding that pretreatment with salivary amylase in the tissue sections diminished the PAS staining (Fig. 2C).

The liver-specific mRNA expression levels were assessed in the engineered liver tissue specimens. To minimize contamination from the surrounding renal tissues, the engineered liver tissues were selectively dissected from the surrounding kidney tissues by LCM. As shown in Figure 3, the engineered tissue specimens showed significant expression levels of FIX, FVII, albumin, and OTC at levels to those of naive healthy livers. Negative FIX signals for samples from the liver and kidney of FIX-KO mice and positive FIX signal from the liver of wild-type mouse confirmed the detection specificity for mouse FIX mRNA in the present assay. No detectable signals were observed for the hepatocyte-specific mRNAs in the kidney samples.

DISCUSSION

The present study demonstrates a relatively simple procedure to engineer liver tissue in hemophilic animals. After the infusion of wild-type hepatocytes under the kidney capsule space of FIX-KO mice, engrafted hepatocytes functionally survived and small liver tissues were engineered. The functional performance for the FIX production of the engineered liver tissues yielded therapeutic levels of plasma FIX:C. The FIX:C plasma levels persisted for 90 days, the length of the present study. The present report is the first conclusive therapeutic evidence of a liver tissue engineering approach to hemophilia B.

The FIX-KO mouse is a valuable mouse model for hemophilia B. FIX-KO mice fail to produce any hepatic FIX mRNA, have no circulating FIX protein, bleed excessively with hemostatic events, and display changes of bleeding-induced poor wound healing (11). In order to avoid any surgical process related to bleeding events, normal mouse plasma was injected twice prior and after the tissue engineering procedure. With these plasma replenishments, all the recipient mice tolerated the tissue engineering surgical procedures. This uneventful process may highlight the simplicity of the tissue engineering procedures, and they could be reasonably clinically applied to individuals with hemophilia.

One of the approaches toward engineering a functional liver tissue has been the infusion of a hepatocyte mixture with extracellular matrix components under the kidney capsule space (15,17,19). The extracellular matrix rich in laminin and type IV collagen contributes to the stable persistence of the hepatic functions including FIX expression (15). The ability to express clotting factor VIII was documented in previous studies (17,19). The present study demonstrated that elevated FIX:C in the recipient FIX-KO mice persisted throughout the 90-day experimental period. Life-long functional persistence was confirmed in a recent study where the tissue-engineered mice were followed for over 450 days, which is nearly the life span of a normal mouse (unpublished data).

Another important determinant in the liver tissue engineering is an ability to undergo regenerative growth, an important feature of the liver (10). Although the present study did not address to this point, previous investigations established that engineered liver tissues established by the current approach are able to proliferate at similar levels to the naive livers in face of liver regenerative stimuli (16,19,20). Liver regeneration occurs through two distinct pathways: compensatory regeneration as well as the direct hyperplasia mode of regeneration (10). Previous studies have demonstrated engineered liver tissue specimens are also able to undergo

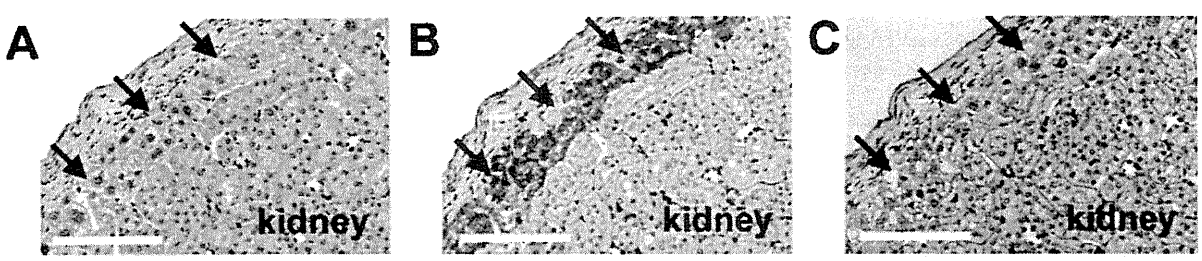

Figure 2. Histological analyses of the engineered liver tissues in the FIX-KO mice. Sections were obtained from FIX-KO mice at day 90 of the experiment. (A) H&E staining and (B, C) PAS staining. PAS staining was performed to confirm the cellular glycogen in the engineered liver tissues that were either nontreated (B) or pretreated with salivary amylase (C). Arrows indicate engineered liver tissues under the kidney capsule. Scale bars: 100 μm.

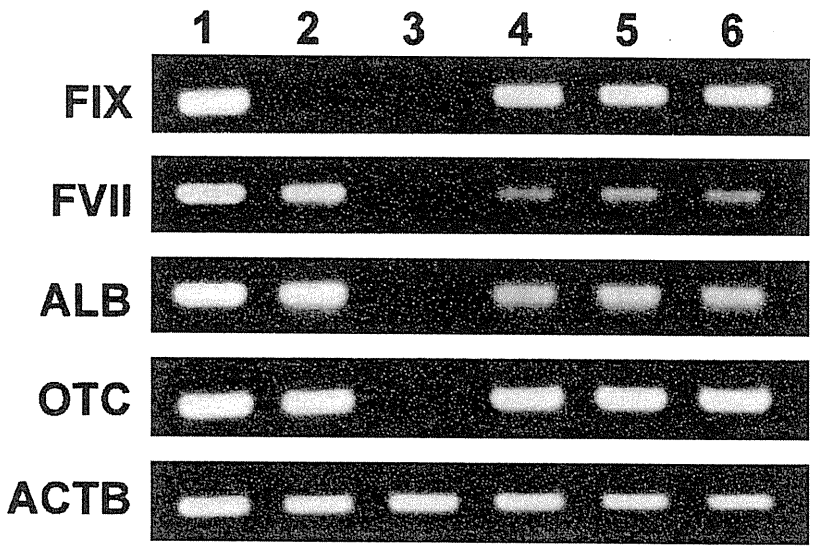

Figure 3. Hepatocyte-specific mRNA expression in the livers, kidney, and engineered liver tissues. The target tissues were selectively dissected under LCM from cryosections obtained at day 90 of the experiment. mRNA expression levels were assessed for coagulation factor IX (FIX), coagulation factor VII (FVII), albumin (ALB), ornithine transcarbamylase (OTC), and β-actin (ACTB). Lane 1: native liver of wild-type C57BL/6 mouse; lane 2: native liver of FIX-KO mouse; lane 3: native kidney tissue of FIX-KO mouse; lanes 4–6: liver tissues engineered under the kidney capsule space composing hepatocytes isolated from wild-type C57BL/6 mouse. Each lane demonstrates samples from three different FIX-KO recipient mice.

both regeneration modes. If it is possible to regulate the in vivo liver growth process, engineered liver tissue could be induced to form a larger tissue. Functional control of the liver volume will allow liver tissue engineering approach to provide the next generation therapy for hemophilia as well as other forms of liver diseases.

The kidney capsule space offers favorable hepatocyte engraftment followed by persistent survival (8,15–17,19). The kidney site offered higher cell engraftment rate in comparison to intrahepatic transplantation when human hepatocytes were transplanted into NOD/SCID mice (17). Successful phenotypic corrections of the disease phenotype of hemophilia A have been described in experiments where wild-type hepatocytes or coagulation factor-producing cells were transplanted into the kidney capsule (13,19). The advantage of the kidney site over the conventional liver site was also documented by Horne et al. (6) describing that humoral and cellular responses to the allogenic hepatocytes engrafted into the kidney site were blunted in comparison to those responses to the intrahepatic engrafted hepatocytes. Tissue factor-dependent coagulation around the transplanted hepatocytes has also been shown to be one of the mechanisms for poor cell engraftment when hepatocytes are transplanted intrahepatically (24). We speculate that transplanting these cells into a closed space (i.e., under the kidney capsule space) may therefore prevent the generation of such coagulation, thus resulting in a higher hepatocyte engraftment rate for the kidney capsule site in comparison to the intrahepatic site.

It is important to note that the hepatocytes within the engineered tissues showed stable functionality for the FIX production throughout the 90 days experimental period. Hepatocytes are known to possess cellular machineries (i.e., posttranslational modification) to produce biologically active forms of clotting factors (1) and thus are considered as a suitable candidate for the cell-based therapy for hemophilia (4,12,21). However, given that clotting factor-producing cells could be successfully and stably engrafted in vivo, the cell source may not be limited to wild-type hepatocytes. The tissue engineering approach could also be highlighted by the recently developed cellular technologies to generate coagulation factor-producing cells (5,7,13,25,27). Considerable progress has recently been made using gene therapy vector systems. The addition of the FIX expression cassette to the naive FIX expression system of the hepatocytes could theoretically enhance the capacity for the FIX production. It would be reasonable to propose that combining tissue engineering and gene therapy technologies will bring higher levels of therapeutic efficacy.

In summary, this study demonstrated that hepatocytes

isolated from wild-type C57BL/6 mice can be effectively engrafted under the kidney capsule space of FIX-KO mice and thus form liver tissue that sufficiently and stably provides therapeutic levels of FIX in the recipients. The therapeutic effect might be enhanced by genetically modifying the hepatocytes to induce the cells to produce biologically active FIX at higher levels. In all, the current work thus can serve as the basis to create a novel cell-based therapy for hemophilia B.

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Regular Article

Lack of association between serum paraoxonase-1 activity and residual platelet aggregation during dual anti-platelet therapy

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ABSTRACT

High residual platelet aggregability during thienopyridine treatment occurs because of low levels of the active drug metabolite, and is associated with an increased rate of major adverse cardiovascular events. Recent findings suggest that paraoxonase-1 (PON1) is a major determinant for clopidogrel efficacy. The aim of this study was to assess the impact of serum PON1 activity on platelet aggregability in thienopyridine-treated patients. In 72 patients receiving treatment with aspirin and ticlopidine after acute coronary syndrome, various laboratory data including the formation of platelet aggregations induced by agonists were compared with serum PON1 activities, measured as paraoxonase and homocysteine thiolactone hydrolase (HTLase). Serum paraoxonase activity was significantly associated with HTLase activity ($R=0.4487$, $P<0.0001$). These PON1 activities were not correlated with any parameters for platelet aggregation, hypertension, sleep apnea, and diabetes mellitus. In contrast, serum PON1 activities seemed to be involved in cardiac function, with brain natriuretic peptide and ejection fraction being significantly correlated with serum HTLase activity ($R=-0.2767$, $P=0.0214$) and paraoxonase activity ($R=0.2558$, $P=0.0339$), respectively. Paraoxonase activity also demonstrated a significant association with increased levels of ankle-brachial index ($R=0.267$, $P=0.0255$). Serum PON1 activities did not influence platelet aggregability during treatment with thienopyridine. However, they might modulate cardiac function after acute coronary syndrome and progression of atherosclerosis.

Introduction

The concept of antiplatelet resistance, particularly poor responsiveness to thienopyridine, has received increasing attention in recent years because of its reported involvement in cardiovascular events after percutaneous coronary artery intervention (PCI) [1-3]. Thienopyridines such as clopidogrel and ticlopidine are rapidly absorbed prodrugs, and must therefore be converted to an active metabolite to exert their inhibitory actions at the target $P2Y_{12}$ ADP nucleotide receptor on platelets. This conversion is *via* a two-step process involving the hepatic cytochrome P450 (CYP) enzyme pathway [4]. Resistance to clopidogrel was thought to result mainly from decreased CYP function leading to reduced active metabolite production [4]. Indeed, individuals carrying the loss-of-function polymorphism of the *CYP2C19* allele had significantly lower levels of the active metabolite of clopidogrel, and a higher rate of major adverse cardiovascular events [5,6]. Drug interaction with the CYP2C19 inhibitor, omeprazole, might also reduce the production of active metabolites [7,8].

Very recently, it was reported that paraoxonase-1 (PON1) is a major and essential factor in the production of active metabolites from clopidogrel [9]. PON1 hydrolyses 2-oxoclopidogrel (an oxidative metabolite of clopidogrel) to form the final active metabolite, a thiol derivative of clopidogrel (Supplemental Fig. 1) [9]. PON1 is a high-density lipoprotein-associated enzyme that prevents oxidative modification of low-density lipoprotein [10]. The *PON1* genotype (Q192 allele) has significant dose-dependent associations with decreased levels of serum PON1 activity and with increased levels of oxidative stress [11]. PON1 has multiple enzyme activities including paraoxonase, arylesterase, and thiolactonase (Supplemental Fig. 1). Although the full range of endogenous substrates hydrolysed by PON1 remains to be elucidated, PON1 has been shown to produce homocysteine from homocysteine thiolactone *via* its homocysteine thiolactone hydrolase (HTLase) activity [12].

Abbreviations: PON1, paraoxonase-1; HTLase, homocysteine thiolactone hydrolase; PCI, percutaneous coronary artery intervention; CYP, cytochrome P450; BNP, brain natriuretic peptide.

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We have previously investigated the mechanisms and clinical backgrounds that determine residual platelet aggregability, and attempted to ascertain whether platelet aggregability is involved in systemic thrombogenicity during dual antiplatelet therapy [13]. Using this previous population, we have retrospectively measured actual serum PON1 activities, measured as paraoxonase and HTLase, in 72 patients treated with ticlopidine and aspirin, and assessed the correlation between PON1 and platelet aggregability.

Methods

Patients

The institutional review board approved all study protocols, and informed consent was obtained from all participants. The design and protocol of this study has been described previously [13]. Briefly, we enrolled consecutive hospitalized patients from July 2006 to April 2007 who were treated by PCI because of symptomatic coronary artery disease. After normalization of cardiac enzymes, patients underwent blood sampling, ankle-brachial index monitoring and cardiorespiratory monitoring.

Blood collection and platelet aggregation

Platelet aggregation was assessed as described previously [14]. A fasting venous sample was carefully collected, and platelet-rich plasma was obtained by centrifugation. The aggregation response was measured based on the light scattering intensities obtained with a PA-200 platelet aggregation analyzer (Kowa Co. Ltd., Tokyo, Japan). This device is particularly sensitive for detecting and classifying the size of platelet aggregates (small, medium, and large) [14]. Platelet aggregation was stimulated with collagen (Hormon-Chemie, Munich, Germany), ADP (MC Medical Co., Tokyo, Japan) and thrombin receptor-activating peptide (TRAP; Invitrogen Co., Carlsbad, CA), a specific agonist for protease activating receptor-1. Blood samples (serum and plasma) were stored at −80 °C until analysis.

Laboratory testing

Plasma levels of plasminogen activator inhibitor-1 antigen, D-dimer, E-selectin and soluble fibrin were assayed using an automated latex agglutination assay (LPIA-S500; Mitsubishi Chemical Medience Co., Tokyo, Japan) based on conjugated monoclonal antibodies. The concentrations of brain natriuretic peptide (BNP) (ShionoRIA BNP kit; Shionogi USA, Inc. Florham Park, NJ) were measured by SRL Inc. (Tokyo, Japan).

Measurement of serum PON1 activities

We quantified paraoxonase and HTLase activities as a measure of serum PON1 activity (Supplemental Fig. 1). Serum paraoxonase activity was measured by using paraoxon as a substrate (Fully Automated Paraoxonase Activity Measurement Kit, Rel Assay Diagnostics, Gaziantep, Turkey). HTLase activity was measured by a hydrolysis of γ-thiobutyrolactone (Alfresa Auto HTLase, Alfresa Pharma Corp., Osaka, Japan). HTLase hydrolyzes the lactone ring of the substrate γ-thiobutyrolactone, producing free thiols that are detected using Ellman's reagent (DTNB; 5,5'-dithiobis (2-nitrobenzoic acid)). Assay reproducibility was high (coefficient of variation was less than 6%).

Statistical analysis

Statistical analyses were performed using Prism v5 (GraphPad software, Inc, La Jolla, CA). The associations between the individual parameters were calculated using Spearman's correlation method. All reported P values are two-sided; a P value of less than 0.05 was considered to indicate statistical significance.

Results

Patients

Of the 85 patients from our previous study, we selected 72 patients taking 100 mg / day of aspirin and 200 mg / day of ticlopidine after acute coronary syndrome. Base line characteristics of the study population are summarized in Table 1.

Lack of correlation of serum PON1 activities with platelet aggregation

We initially examined serum PON1 activities (measured by paraoxonase and HTLase activity). As show in Fig. 1, serum HTLase activity, but not paraoxonase activity, appeared to be normally distributed across the study population (HTLase: 130.3 ± 36.7 U/L; paraoxonase: 62.65 ± 25.27 U/L). These PON1 activities were significantly correlated ($R = 0.4487$, $P < 0.0001$). To examine whether serum PON1 activities determine platelet aggregability during dual antiplatelet therapy, serum PON1 activities were compared with several parameters of platelet aggregation. However, none of these parameters was significantly associated with PON1 activities (Fig. 2 and Table 2).

Correlation between serum PON-1 activities and cardiac function

We next compared serum PON1 activities with parameters for hypertension, sleep apnea, diabetes mellitus, hyperlipidemia, blood coagulation, arteriosclerosis, and cardiac dysfunction. Using linear regression analysis, we determined that only HDL cholesterol and BNP were correlated with HTLase activity (Table 3). Paraoxonase activity was associated with triglyceride, D-dimer, ankle-brachial index, and ejection fraction (Table 3). The medication including use of diuretics, angiotensin II receptor blocker, angiotensin converting enzyme inhibitor, beta blocker, calcium channel blocker, or statin did not demonstrate a significant association with serum PON1 activities (Supplemental Table 1). These data suggest that decreased levels of PON1 activity might lead to the acceleration of atherosclerosis and cardiac dysfunction after acute coronary syndrome.

Table 1
Characteristics of the study population.

Variables	Total subjects (n = 72)
Age, years	62.15 ± 11.62
Men, n (%)	57 (80)
BMI, kg/m^2	25.11 ± 3.514
Systolic blood pressure (mmHg)	125.3 ± 21.08
Diastolic blood pressure (mmHg)	76.6 ± 11.28
Pulse rate (/min)	72.39 ± 14.59
Blood sugar (mg/dl)	118.3 ± 50.43
HbA1c (%)	6.76 ± 1.891
Triglyceride (mg/dl)	130.2 ± 53.01
Total cholesterol (mg/dl)	167.7 ± 36.77
LDL cholesterol (mg/dl)	100.4 ± 30.35
HDL cholesterol (mg/dl)	41.3 ± 12.72
CPK max (U/L)	2,194 ± 2,211
BNP (pg/ml)	151.4 ± 183.6
Concomitant medications	
Antiplatelet agents, n (%)	
Aspirin + Ticlopidine	72 (100)
Antihypertensive medication, n (%)	66 (91.7)
Statin, n (%)	55 (76.4)
NSAIDs, n (%)	0 (0)

Data for continuous variables are expressed as the mean ± SD. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BNP, brain natriuretic peptide; NSAID, non-steroidal anti-inflammatory drug.

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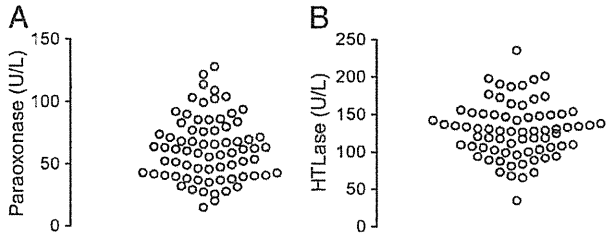

Fig. 1. Serum paraoxonase and HTLase activities in the study population.

Discussion

Inhibition of the $P2Y_{12}$ nucleotide receptor, an ADP receptor on platelets, is currently the gold-standard therapy for the prevention of ischemic events in patients undergoing PCI [15,16]. Although the second-generation thienopyridine, clopidogrel, is recommended by a number of current clinical guidelines, the inter-individual variability of its efficacy is a major drawback in its clinical use [17]. Better understanding of this variability in the efficacy of clopidogrel and other thienopyridines is vital at a time when the number of PCIs is increasingly rapidly. The loss-of-function polymorphism of the CYP2C19 allele has attracted attention as a potential factor in clopidogrel efficacy [4-6], while an elegant recent study suggested that PON1 is a major determinant in the production of the final active metabolite of clopidogrel [9]. In this study, we measured serum two PON1 activities in acute coronary syndrome and compared them with platelet aggregability in patients receiving dual antiplatelet therapy. We could identify no correlation between PON1 activities and any parameter for platelet aggregation in our population.

Several explanations may exist for the discrepancy between our result and the previous report. First, genetic divergence between Caucasian and Japanese patients might affect the result. The Japanese population is reported to express predominantly the 192R allele of *PON1* (192QQ: 18.2%; 192QR: 40.9%; 192RR: 40.9%) [18], whereas the Caucasian population in a large cohort study tended to express the 192Q variant (192QQ: 46.3%; 192QR: 43.9%; 192RR: 9.8%) [11]. The Q allele of *PON1* genotype was significantly and dose-dependently associated with decreased serum PON1 activity, whereby 192QQ, 192QR and 192RR had comparatively low, intermediate and high PON1 activity, respectively [11]. In contrast, the frequency of polymorphism for *CYP2C19*, a key enzyme in clopidogrel oxidation, varies among races, with loss-of-function polymorphisms reportedly being more common in Asian patients [19,20]. However, even in a genetically homogenous population, the *CYP2C19* allele was reported to account for only 12% of the variability in clopidogrel efficacy, whereas the *PON1*

Table 2
Correlation between serum paraoxonase activities and platelet aggregation.

	Paraoxonase		HTLase	
	R	*P* value	*R*	*P* value
ADP 2 μM-LT	−0.1252	0.2945	−0.03844	0.7486
ADP 2 μM-Small	−0.2083	0.0791	−0.07811	0.5143
ADP 2 μM- Med	−0.1335	0.2636	−0.01776	0.8823
ADP 2 μM-Large	−0.03798	0.7514	0.085	0.4777
ADP 5 μM-LT	−0.08351	0.4856	−0.04392	0.7141
ADP 5 μM-Small	−0.2212	0.0619	−0.09755	0.415
ADP 5 μM- Med	−0.2317	0.0501	−0.1055	0.3776
ADP 5 μM-Large	−0.1406	0.2389	−0.06589	0.5824
Coll 1 μg/ml-LT	−0.1072	0.37	0.02695	0.8222
Coll 1 μg/ml-Small	−0.1524	0.2012	0.06594	0.5821
Coll 1 μg/ml- Med	−0.1174	0.3262	0.04772	0.6906
Coll 1 μg/ml-Large	−0.00214	0.9857	0.04327	0.7182
Coll 5 μg/ml-LT	−0.05927	0.6209	−0.01047	0.9304
Coll 5 μg/ml-Small	−0.1489	0.212	−0.1001	0.4029
Coll 5 μg/ml- Med	−0.1269	0.2881	−0.03555	0.7669
Coll 5 μg/ml-Large	−0.1113	0.352	0.007927	0.9473
TRAP 20 μM-LT	−0.1114	0.3515	0.01301	0.9136
TRAP 20 μM -Small	−0.1585	0.1835	−0.1742	0.1434
TRAP 20 μM - Med	−0.09187	0.4427	−0.04854	0.6855
TRAP 20 μM -Large	−0.05235	0.6623	0.03127	0.7943

LT, light transmission; Small, small aggregates; Med, medium aggregates; Large, Large aggregates; Coll, collagen; TRAP, thrombin receptor-activating peptide (SFLLRN). *$P<0.05$.

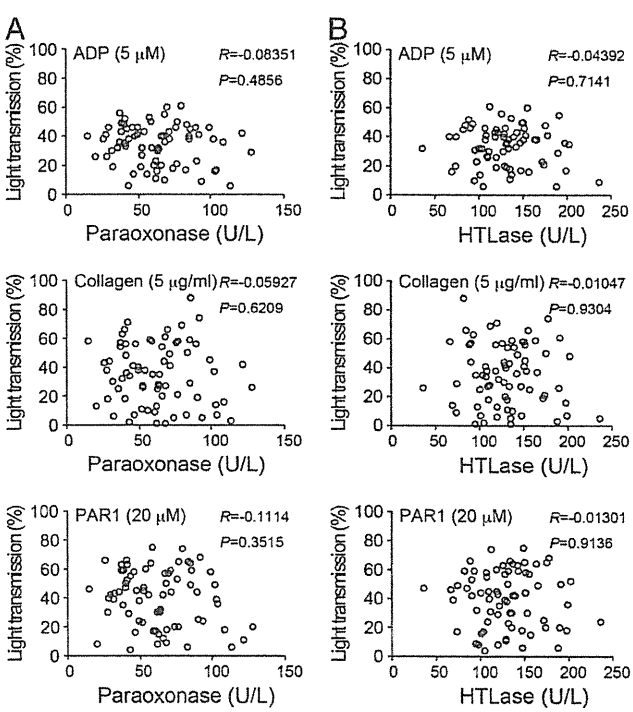

Fig. 2. Association between PON1 activities and platelet aggregation. Platelet aggregation induced by 5 μM ADP, or 5 μg / ml of collagen, or 20 μM TRAP was assessed by aggregometry, and was expressed as light transmission (%). Serum paraoxonase activities (U / L) (A) and HTLase activities (U / L) (B) were compared with platelet aggregation using Spearman's rank correlation coefficient.

Table 3
Correlation between paraoxonase activities and other laboratory data.

	Paraoxonase		HTLase	
	R	*P* value	*R*	*P* value
SBP	−0.0953	0.443	−0.118	0.3417
DBP	−0.121	0.3294	−0.00797	0.949
HR	−0.1096	0.3774	0.02895	0.8161
AHI	0.06843	0.5735	0.05563	0.6474
Blood suger	−0.06266	0.609	0.06714	0.5836
HbAlc	−0.08087	0.5121	−0.09806	0.4263
Triglyceride	0.2958	0.0129*	0.00273	0.9821
Total cholesterol	0.01199	0.9215	0.1166	0.3365
LDL cholesterol	−0.0158	0.8975	0.05602	0.6475
HDL cholesterol	−0.01299	0.915	0.2646	0.0269*
PAI-1	−0.08214	0.4928	0.01523	0.899
E-selectin	−0.0007075	0.9953	−0.1618	0.1746
Soluble Fibrin	−0.03224	0.788	−0.08483	0.4787
D-dimer	−0.2348	0.0471*	−0.2229	0.0598
max CPK	−0.1691	0.1616	−0.02521	0.8359
BNP	−0.1306	0.2849	−0.2767	0.0214*
Pulse wave velocity	−0.1665	0.1682	−0.04456	0.7141
ABI	0.267	0.0255*	−0.01957	0.8722
Ejection fraction	0.2558	0.0339*	0.1632	0.1803

SBP, systolic blood pressure; DBP, diastolic blood pressure; AHI, apnea-hypopnea index; LDL, low-density lipoprotein; HDL, high-density lipopurotein; PAI-1, plasminogen activator inhibitor-1; BNP, brain natriuretic peptide; ABI, ankle-brachial index. *$P<0.05$.

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Q192R polymorphism was estimated to be responsible for 72.5% of the variability in ADP-stimulated platelet aggregation after clopidogrel administration [9]. It is therefore important that we clarify which polymorphism combinations (of *PON1* and *CYP2C19*) are the most relevant in the metabolism of thienopyridines in our population.

The first-generation thienopyridine, ticlopidine, was used instead of clopidogrel in our study because ticlopidine was the only approved drug for acute coronary syndrome in Japan during our study period. We acknowledge the possibility that the rate-limiting enzyme for ticlopidine metabolism to its active metabolite may differ from that of clopidogrel. All thienopyridines including ticlopidine, clopidogrel, and prasugrel are prodrugs that need to be converted into active metabolite through the formation of thiolactone metabolites (2-oxo-ticlopidine, 2-oxo-cloipdogrel, and prasugrel thiolactone, respectively (see Supplemental Fig. 1)) [4]. The free active thiol of these active metabolites forms disulfide bonds with, and therefore binds irreversibly to, cysteine residues Cys17 and Cys270 of $P2Y_{12}$ [21]. It is of great importance, therefore, to understand whether thiolactone metabolites of all thienopyridines are hydrolyzed mainly by PON1, or are instead oxidized by CYP.

We found correlations between PON1 activities and cardiac function in our study population. PON1 has a protective effect against oxidation of lipoproteins, and a *PON1* polymorphism (the 192Q allele) that produces decreased levels of PON1 activity was associated with systemic oxidative stress and higher rates of major cardiovascular events [11]. It is possible that decreased levels of PON1 activities enhance the progression of atherosclerosis in the coronary artery, resulting in decreased cardiac function after acute coronary syndromes. Indeed, reduced paraoxonase activity was significantly associated with a decreased ankle-brachial index in our study. Further studies are needed to assess the possible mechanisms and biological effect of PON1, particularly the severity of its effects on cardiac function after coronary artery disease.

Some limitations in this study merit discussion. First, we could assess platelet function testing in the patients treated with ticlopidine, but not clopidogrel. We cannot exclude the possibility that results may differ with other thienopyridines, as described above. In addition, we assessed only the correlation between serum PON1 activities and platelet response to ticlopidine, and we did not assess gene polymorphisms. Although it is accepted that serum PON1 activities are determined by *PON1* polymorphism, more data regarding genetic variation in *CYP* and *PON1* may have extended our findings relating to the mechanism(s) of the platelet response during dual antiplatelet therapy. Finally, the analysis reported here is *post hoc* analysis of a previously reported population and the number of participants is limited. We previously estimated that at least 62–85 participants would be required for the study ($\alpha = 0.05$, $\beta = 0.20$, and expected correlation coefficient, $R = 0.30$–0.35) [13]. Weak association due to β-error may affect the strength of any conclusions based on these data.

Conclusions

The current study has demonstrated that serum PON1 activities did not influence platelet aggregation in patients receiving thienopyridine treatment, but was involved in cardiac function. Our data suggest the need for a re-evaluation of the importance of PON1 (and/or CYP) in the production of active metabolites from thienopyridines. We may also need to consider how expression of the rate-limiting enzymes for thienopyridine metabolism differs between individual drugs and racial populations. During the preparation of this article, it was reported that no association exists between *PON1* genotype and platelet response to clopidogrel and stent thrombosis in a *post hoc* analysis of prospective studies [22]. Further large-scale prospective studies are required to determine which enzyme (PON1 or CYP) is critical for the production of active metabolites from thienopyridines, and therefore for cardiovascular events during thienopyridine administration.

Supplementary materials related to this article can be found online at doi:10.1016/j.thromres.2011.10.033.

Conflict of interests statement

T.O. has received financial support from Daiichi Sankyo. The other authors declare that they have no competing interest.

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Haemophilia

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ORIGINAL ARTICLE

Immune response against serial infusion of factor VIII antigen through an implantable venous-access device system in haemophilia A mice

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Summary. Haemophilia A is a life long bleeding disorder caused by an inherited deficiency of factor VIII (FVIII). About 30% of haemophilia A patients develop neutralizing antibodies as a consequence of treatment with FVIII concentrates. Immune tolerance protocols for the eradication of inhibitors require daily delivery of intravenous FVIII. We evaluated the immune responses to serial intravenous administration of FVIII in preimmunized haemophilia A mice. We introduced an implantable venous-access device (iVAD) system into haemophilia A mice to facilitate sequential infusion of FVIII. After preimmunization with FVIII, the haemophilia A mice were subjected to serial intravenous administration of FVIII through the iVAD system. In all mice with serial infusion of FVIII, high titers of anti-FVIII inhibitory antibodies developed at 10 exposure days (EDs). However, the anti-FVIII IgG titers were decreased after 150 EDs of sequential low-dose infusion of FVIII [0.05 U g^{-1} body weight (BW) five times per week]. Proliferative response to *ex vivo* FVIII stimulation was significantly suppressed in splenic $CD4^+$ T cells from mice with serial low-dose FVIII infusion compared with those from mice with high-dose FVIII infusion (0.5 U g^{-1} BW five times per week) or preimmunized mice. Moreover, splenic $CD4^+$ T cells from mice with serial low-dose infusion of FVIII failed to produce interleukin-2 and interferon-γ. These data suggest that serial infusion of FVIII could induce T-cell anergy in haemophilia A mice with inhibitor antibodies.

Keywords: anergy, factor VIII, haemophilia A mice, inhibitor, venous-access device

Introduction

Haemophilia A is a life-long bleeding disorder caused by an inherited deficiency of factor VIII (FVIII) because of mutations in the FVIII gene [1]. About 30% of severe haemophilia A patients who received replacement therapy with intravenous FVIII products develop neutralizing antibodies that inhibit the function of substituted FVIII [2,3]. Once an inhibitor develops, treatment of bleeding episodes is quite difficult due to partial or complete lack of efficacy of replacement therapy. Immune tolerance induction (ITI) therapy using regular applications of FVIII is the only strategy that has been proven successfully to combine eradication of FVIII inhibitors and induction of FVIII-specific immune tolerance [2,4].

Central venous-access devices (VADs) are often used in haemophiliacs undergoing ITI to overcome difficulties of regular venous puncture [5,6]. The fully implantable devices offer many advantages compared with external catheters, because they generally have longer useful duration with lower rate of infectious complication and cannot be accidentally displaced [7]. Although ITI approach was introduced several decades ago, little is known about the immunological mechanisms that cause down-modulation of FVIII-specific immune responses and the induction of long-lasting immune tolerance against FVIII.

In this study we introduced an implantable VAD (iVAD) system into haemophilia A mice to facilitate serial intravenous infusion of FVIII and evaluated immune responses against FVIII in preimmunized haemophilia A mice. We demonstrated that sequential administration of FVIII through the iVAD system could induce T-cell anergy in adult haemophilia A mice with inhibitors.

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