Table 1. Genotypic risk factors for inhibitor development of the patient. The immune response factors related to the risk of inhibitor development were analysed. Low risk was suggested by IL10 and TNFα analysis and high risk was suggested by CTLA-4 analysis.

TNFα
Hap 1 or 3 and -308G/G genotype
(High risk: Hap 2 or 3 and -308G/G genotype)
CTLA-4
–318 C/C
(Low risk: -318 T allele)
IL10
None with allele 134 (138/140)
(High risk: Allele 134 positive)

mRNA obtained from blood is available to observe the state of splicing, and this analysis is widely used to screen for genetic abnormalities. If the mutation exists deep inside the intron, it will give some influence on the transcript. Therefore, examination of the mRNA is very effective to detect unknown genetic mutations or rearrangements. Furthermore, the analysis of ectopic mRNA is also effective to examine the influence that detected gene abnormalities exert on the splice. In fact, the mutation that we found was confirmed to cause the splice abnormality by analysing ectopic mRNA. Although predictive software analysis [17] suggested that this patient's mutation may cause splicing abnormalities, there was no further evidence to prove this.

We analysed ectopic mRNA by using the method that had been reported by El-Maarri *et al.* [10]. This method utilizes the nested PCR technique and is suitable for detection of small amounts of mRNA. At first, the *F8* is divided into four regions, exon 1–8, 8–14, 14–21 and 19–26, and is amplified. Then, each of the first amplification products are further divided into two regions and amplified again. In the present study, because the position of the mutation was identified within intron 10, exon 8–14 was amplified first, then exon 8–11 was amplified. However, consistent amplicons were not obtained from every reaction of the second amplification. We have re-evaluated the preparation of mRNA and the amplification of cDNA to elucidate why the same amplicon was not provided every time. However, we could not resolve the issue. This may reflect the low abundance of such a variant. Therefore, we evaluated the patient's mRNA by the result of 10 independently performed reactions. Although the method mentioned above is an excellent method for analysing ectopic *F8* mRNA, in the case of some splice variants it is suggested that careful evaluation and selection of analyses are necessary.

Originally, the patient was identified as having very mild congenital haemophilia A. The patient had no history of haemorrhage that required treatment until the detection of low FVIII activity level at the age of 60, although he had showed some difficulty of haemostasis, for example in tooth extractions etc. during childhood. The fact that there is agreement between both the FVIII levels at a preoperative examination and the *F8* mRNA levels described in the present study supported the classification of the patient as having mild congenital haemophilia. However, at the present time, the patient has fallen into a very severe state due to development of anti-FVIII antibody. The inhibitor development process of the patient was typical, and took less than 20 exposure days from the first FVIII concentrate injection [18]. Generally, inhibitor development in congenital haemophilia is more frequently observed in the severe patients null mutations [8]. It is comparatively rare that a patient with mild haemophilia A should develop the inhibitor. Inhibitor development in mild haemophilia A is typically observed in patients with molecular abnormalities because endogenous abnormal mutant FVIII, a cross-reacting material (CRM), is recognized as "self" and exogenously infused normal FVIII molecule is recognized as "non-self". The developed antibody is often seen to cross-react with not only "non-self" but also "self".

This patient was diagnosed with congenital mild haemophilia A and has CRM as previously stated. However, analysis of the mRNA might suggest that this patient's CRM would be normal FVIII, produced by the normal mRNA which avoided abnormal splicing. Therefore, this is an interesting case because the inhibitor in this patient raises the possibility that the nature and developing mechanism are different from the inhibitor usually developed in congenital mild haemophilia A. The inhibitor showed a type I inhibition kinetic pattern [19], predominantly IgG4 subclass [20], and multi-clonal epitopes (A2 domain and the light chain of FVIII). These characteristics were most typical of an alloantibody developed in congenital haemophilia. Moreover, we investigated the genetic risk factors in consideration of the possibility that the patient's antibody developed as an autoantibody. The results of the haplotype analysis of the immune response factors suggested that the patient was not at an especially high risk of inhibitor development [21–24]. However, on the other hand, the fact that the patient is aged and had a solid tumour (stomach cancer) may have created a higher risk of an autoantibody development. Therefore, we cannot deny the possibility that the patient's haemophilia is acquired.

The novel factor VIII mutation identified here provides potential insight into the genetic contribution to haemophilia A pathogenesis and inhibitor development. Although the FVIII antibody developed in this patient is interesting, further analysis and knowledge are necessary to judge whether the inhibitor is an alloantibody or an autoantibody.

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基礎・診療の基礎

治療歴のある血友病B患者における血漿由来血液凝固第Ⅸ因子製剤（ノバクト®M静注用）の生体内回収率

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要　　旨

治療歴のある血友病B患者において血漿由来モノクローナル抗体精製血液凝固第Ⅸ因子製剤（ノバクト®M静注用）の薬物動態について検討すると共に，年齢，体重，ヘマトクリット（Hct）値およびbody mass index（BMI）が生体内回収率に影響を与えるか否かについて検討した。その結果，ノバクトM静注用を投与した30分後の生体内回収率は51.8±15.0%であり，第Ⅸ因子活性上昇値（IVR）は1.04±0.30（U/dL per IU/kg）であった。また，年齢による生体内回収率の違いを検討した結果，16歳未満の回収率は44.2±8.9%であったのに対し，16歳以上の回収率は61.3±16.8%であり，両者は大きく異なっていた。更に，体重とBMIは，生体内回収率と有意な正の相関が確認されたが，Hct値は回収率と有意な相関がなかった。

今回の検討におけるノバクトM静注用の生体内回収率は，海外で報告されている血漿由来第Ⅸ因子製剤の回収率とよく一致していた。今回の検討では，生体内回収率に個人差があること，更に同じ患者でも回収率に変動があることが確認されたので，個々の患者で回収率を適宜測定し，その結果を基に適切な投与量を設定することが重要である。

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Recovery of the Plasma-Derived Factor Ⅸ Concentrate (Novact® M) in Previously Treated Patients with Hemophilia B

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はじめに

血友病Bは，血液凝固第Ⅸ因子が欠乏することによる稀な遺伝性の出血性疾患である[1]。この血友病Bの止血管理には，主に血液凝固第Ⅸ因子製剤が使用されており，国内では血漿由来モノクローナル抗体精製第Ⅸ因子製剤と遺伝子組換え第Ⅸ因子製剤が主として使用されている[2]。

血液凝固第Ⅸ因子製剤の薬物動態は，製剤によって異なることが報告されており，特に血漿由来第Ⅸ因子製剤と遺伝子組換え第Ⅸ因子製剤で大きく異なっていることが報告されている[3][4]。血漿由来第Ⅸ因子製剤を患者に投与した時の生体内回収率は，約50%程度であるが，遺伝子組換え第Ⅸ因子製剤の場合には約35%と報告[3]されている。

また，血友病Bの患者毎に生体内回収率が異なることや，成人よりも小児の生体内回収率が低いことが報告[5]されている。同じ患者でも，加齢により生体内回収率が変動することや，加齢以外でも回収率の変動があることが報告[6]されている。

国内では，血漿由来の第Ⅸ因子製剤が3製剤，遺伝子組換え第Ⅸ因子製剤が1製剤の計4製剤が血友病Bの止血管理に使用できる。ノバクト®Mは，1992年の発売より血友病Bの止血管理に使用されているモノクローナル抗体で精製された血漿由来第Ⅸ因子製剤である。このノバクトMは，2010年6月に国際単位で表示された製剤（ノバクト®M静注用400単位・800単位・1600単位，以下ノバクトM静注用と略す）に変更されたが，その国際単位表示の製剤による生体内回収率のデータは乏しいのが現状である。そこで，当施設に通院している血友病B患者でノバクトM静注用の薬物動態について検討すると共に，年齢，体重，ヘマトクリット（Hct）値およびbody mass index（BMI）が生体内回収率に影響を与えるか否かについて検討した。

Ⅰ 試験対象

対象は，当施設に通院している血友病B患者9例で，年齢は平均15.7歳（1～34歳）で16歳未満が5例，16歳以上が4例であった。

Ⅱ 試験方法

1．生体内回収率の測定

生体内回収率は，前回の第Ⅸ因子製剤の投与から3日以上の間隔を空け，かつ出血がないことを確認して測定した。ノバクトM静注用は，患者の体重kg当たり50単位（IU）を目安に投与し（平均投与量48.7IU/kg），投与前と投与30分後の採血で第Ⅸ因子活性を測定した。生体内回収率は，投与30分後における第Ⅸ因子活性の実測上昇値を体重kg当たりの投与量の2倍で除して求めた。計算式を以下に示す。

表1　生体内回収率と第Ⅸ因子活性上昇値（IVR）

患者番号	投与量 (IU/kg)	生体内回収率 (%)	第Ⅸ因子活性上昇値 (U/dL per IU/kg)
1	53.3	39.3	0.79
2	84.5	33.0	0.66
3	46.5	45.2	0.90
4	29.8	47.0	0.94
5	48.9	82.9	1.66
6	43.0	65.4	1.31
7	53.1	44.8	0.90
8	54.9	51.9	1.04
9	24.6	56.6	1.13
平均	48.7	51.8	1.04
標準偏差	17.1	15.0	0.30
95%信頼区間	42.0～61.6	42.0～61.6	0.84～1.24

生体内回収率（%）＝

第Ⅸ因子活性の実測上昇値÷投与量(IU/kg)÷2×100

2．第Ⅸ因子活性上昇値（IVR, *in vivo* recovery）の計算

第Ⅸ因子活性上昇値（IVR）は，下記の式のように投与30分後の第Ⅸ因子活性の実測上昇値（%＝U/dL）を体重kg当たりの第Ⅸ因子製剤投与量（IU/kg）で除して求めた。

第Ⅸ因子活性上昇値（IVR）＝

第Ⅸ因子活性の実測上昇値（U/dL）÷第Ⅸ因子製剤投与量（IU/kg）

Ⅲ　結果および考察

1．生体内回収率と第Ⅸ因子活性上昇値（IVR）

ノバクトM静注用を投与した30分後の生体内回収率は51.8±15.0%（95%信頼区間；42.0～61.6）であり，第Ⅸ因子活性上昇値（IVR）は1.04±0.30（U/dL per IU/kg）（95%信頼区間；0.84～1.24）であった（**表1**）。この結果は，海外で報告[3)4)]されている血漿由来第Ⅸ因子製剤の成績とよく一致した成績である。特にPoonら[5)]は，血漿由来第Ⅸ因子製剤の第Ⅸ因子活性上昇値（IVR）が1.06±0.26（U/dL per IU/kg）と報告しており，今回の値と一致している。

2．年齢による生体内回収率の違い

年齢による生体内回収率の違いを検討するために，16歳未満と16歳以上の2群に分け，比較した（**表2**および**図1**）。16歳未満の生体内回収率は，44.2±8.9%であり，16歳以上の回収率は61.3±16.8%であった。16歳以上の生体内回収率を100%とした時に16歳未満の回収率は72%であり，両者は大きく異なっていた。更に，両者の生体内回収率の変動係数（CV）は，20.0%（16歳未満）と26.1%（16歳以上）と大きく，個々の患者毎に回収率が異なることが確認された。これらの結果は，これまでに報告されている小児の生体内回収率が成人に比べて低いこと[5)]，回収率の個人差が大きいこと[7)]とよく一致している。第Ⅸ因子は，生体内に投与された後，速やかに血管内皮細胞に結合するため[8)9)]，生体内回収率の個人差は，血管内皮細胞に対する親和性の差に関連する可能性も示唆されている[10)]。

表2　年齢による生体内回収率の違い

年齢	生体内回収率 (%)	変動係数 (%)	第Ⅸ因子活性上昇値 (U/dL per IU/kg)	変動係数 (%)
16歳未満	44.2±8.9 (72)	20.0	0.88±0.18 (72)	20.1
16歳以上	61.3±16.8 (100)	26.1	1.23±0.34 (100)	27.3

生体内回収率および第Ⅸ因子活性上昇値・平均±標準偏差（16歳以上の値を100%とした時の%）

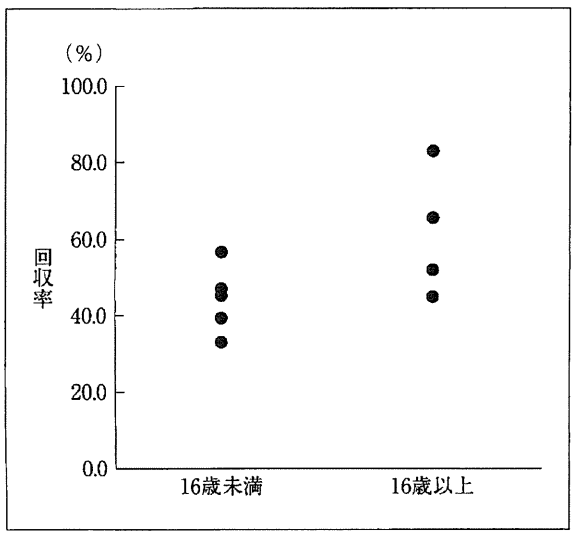

図1　年齢による生体内回収率の違い

また，第Ⅸ因子活性上昇値（IVR）は，16歳未満が0.88±0.18（U/dL per IU/kg）であり，16歳以上が1.23±0.34（U/dL per IU/kg）であった。この結果は，生体内回収率の結果とよく一致している。

3．体重，Hct値およびBMIと生体内回収率の相関

体重と生体内回収率の相関について検討した結果〔**図2**-(a)〕，有意な正の相関が確認された（$P=0.048$，決定係数$R^2=0.448$）。体重は，年齢により増加することから，年齢と生体内回収率の相関についても検討したが，有意な相関はなかった（$P=0.105$）。

次に，Hct値と生体内回収率の相関を確認した結果〔**図2**-(b)〕，有意な相関はなかった（$P=0.564$）。また，BMIと生体内回収率の相関を検討した結果〔**図2**-(c)〕，有意な正の相関が確認された（$P=0.002$，決定係数$R^2=0.805$）。

このように，生体内回収率は，体重およびBMIと正の相関が認められたことから，体重やBMIは第Ⅸ因子製剤の回収率を左右する重要な因子である可能性が示唆された。

4．同一患者の生体内回収率

4例の患者で個人の生体内回収率を比較した結果，0～23カ月の期間で回収率の個人の変動係数は，1.6～21.0%と大きく異なる場合も確認された（**表3**）。同じ月内で測定された生体内回収率は比較的近い値を示したが，12カ月以上の間隔がある場合には回収率が大きく異なっていた。したがって，患者の日常管理として，生体内回収率を測定する場合には1年に1回程度の実施が必要と考えられ，その結果を基に適切な投与量を設定することが患者のQOLの維持・向上に重要である。

なお，大きな手術を実施する場合には，事前に生体内回収率の測定を実施することは十分な止血管理を実施するという手術管理上の必要性ばかりでなく，不必要かつ過剰な投与を避けるという観点からも重要である。

5．ノバクトM静注用と生体内回収率

今回の検討は，第52回日本小児血液学会総会（2010年12月）において発表した内容[11]から血漿由来第Ⅸ因子製剤であるノバクトM静注用のデータのみを抽出し，その後の臨床データを加え，再度解析をした。また，学会発表時には，製剤の表示値（規格値）で生体内回収率等を測定したが，今回の検討では製剤の実測値で求めた。

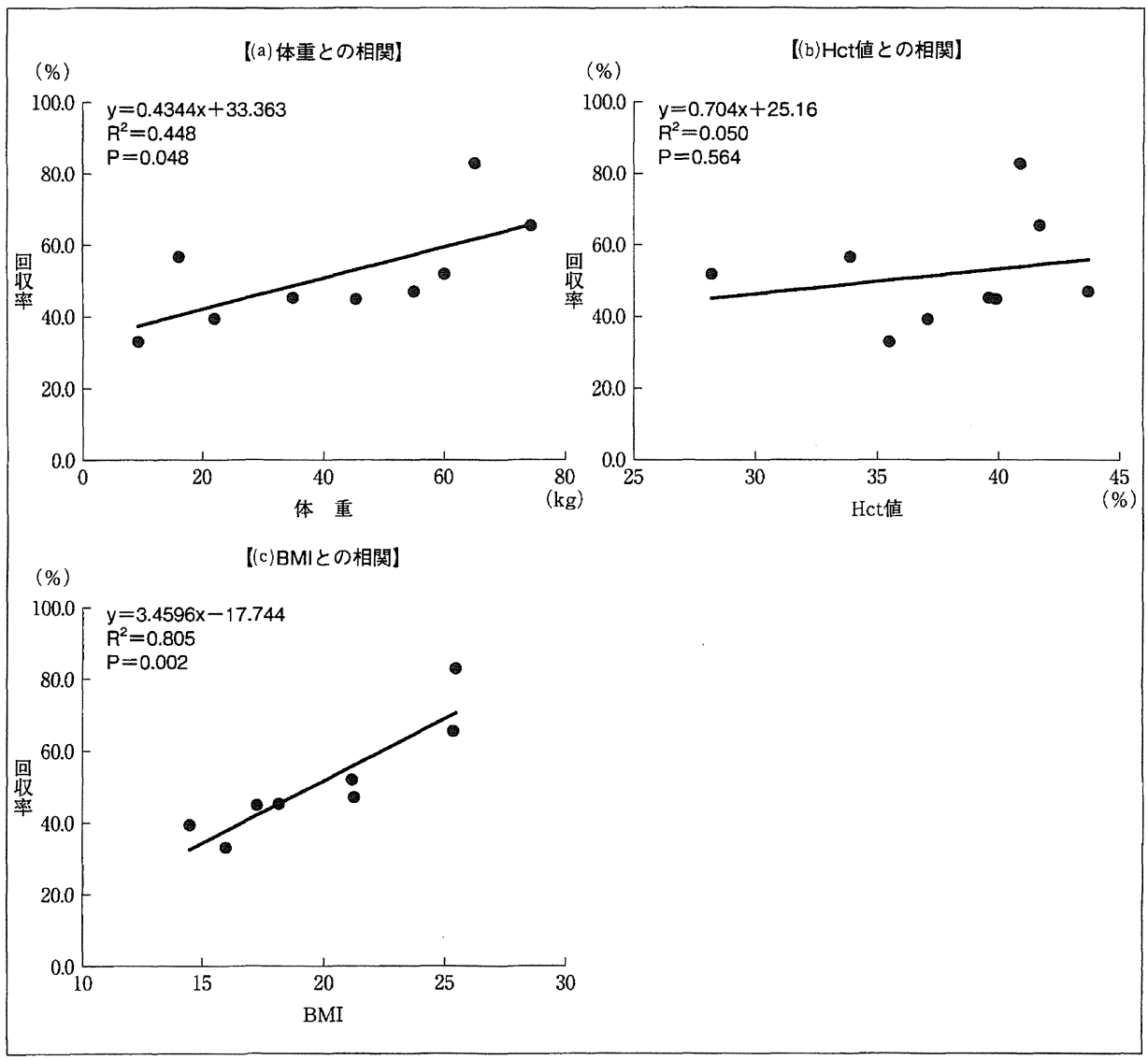

図2　体重，Hct値およびBMIと生体内回収率の相関

旧来のノバクトMの生体内回収率は，臨床試験[12]の成績として平均84.3%と報告されている。しかし，当時のノバクトMは，第Ⅸ因子活性が50単位/mL（倍/mL）の製剤であり，現在の第Ⅸ因子国際標準品と測定方法で評価すると，第Ⅸ因子活性が80国際単位/mL（IU/mL）に相当する。このため，実質的に投与された第Ⅸ因子量は，1.6倍多いことになる。臨床試験の生体内回収率は国際単位で評価すると，見かけ上の投与が増加することに伴い，旧来の生体内回収率の平均84.3%を1.6で除した値である平均52.7%になる[13]。この値は，今回の検討した生体内回収率（平均51.8%）とよく一致している。

ま　と　め

治療歴のある血友病B患者において血漿由来モノクローナル抗体精製血液凝固第Ⅸ因子製剤（ノバクトM静注用）の薬物動態について検討した。

1）生体内回収率は，51.8±15.0%であり，第Ⅸ因子活性上昇値（IVR）は1.04±0.30（U/dL per IU/kg）であった。

2）年齢による生体内回収率の違いを検討

表3　同一患者の生体内回収率

患者番号	測定間隔（カ月）	生体内回収率（%）	平均	標準偏差	変動係数（%）
1	0	51.7	44.2	6.6	14.9
	4	41.7			
	23	39.3			
2	0	44.6	39.4	5.9	14.9
	0	40.5			
	12	33.0			
8	0*	53.1	52.5	0.9	1.6
	0	51.9			
9	0	76.3	66.5	13.9	21.0
	14	56.6			

*：出血時の測定

した結果，16歳未満の回収率は44.2±8.9%であったのに対し，16歳以上の回収率は61.3±16.8%であり，両者は大きく異なっていた。

3）体重とBMIは，生体内回収率と有意な正の相関が確認されたが，Hct値は回収率と有意な相関がなかった。

4）今回の検討では，生体内回収率に個人差があること，更に同じ患者でも回収率に変動があることが確認されたので，個々の患者で回収率を適宜測定し，その結果を基に適切な投与量を設定することが重要である。

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Liver Tissue Engineering Utilizing Hepatocytes Propagated in Mouse Livers In Vivo

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Recent advances in tissue engineering technologies have highlighted the ability to create functional liver systems using isolated hepatocytes in vivo. Considering the serious shortage of donor livers that can be used for hepatocyte isolation, it has remained imperative to establish a hepatocyte propagation protocol to provide highly efficient cell recovery allowing for subsequent tissue engineering procedures. Donor primary hepatocytes were isolated from human α-1 antitrypsin (hA1AT) transgenic mice and were transplanted into the recipient liver of urokinase-type plasminogen activator-severe combined immunodeficiency (uPA/SCID) mice. Transplanted donor hepatocytes actively proliferated within the recipient liver of the uPA/SCID mice. At week 8 or later, full repopulation of the uPA/SCID livers with the transplanted hA1AT hepatocytes were confirmed by blood examination and histological assessment. Proliferated hA1AT hepatocytes were recovered from the recipient uPA/SCID mice, and we generated hepatocyte sheets using these recovered hepatocytes for subsequent transplantation into the subcutaneous space of mice. Stable persistency of the subcutaneously engineered liver tissues was confirmed for up to 90 days, which was the length of our present study. These new data demonstrate the feasibility in propagating murine hepatocytes prior to the development of hepatic cells and bioengineered liver systems. The ability to regenerate and expand hepatocytes has potential clinical value whereby procurement of small amounts of tissue could be expanded to sufficient quantities prior to their use in hepatocyte transplantation or other hepatocyte-based therapies.

Key words: Liver tissue engineering; Cell proliferation; Cell sheet; Tissue engineering; Hepatocyte transplantation; Regenerative medicine

INTRODUCTION

Liver tissue engineering using primary hepatocytes presents the opportunity to create a de novo liver system, which can lead to a new therapeutic modality towards the treatment of various liver diseases (4,10, 15,18,28) and may have the potential to obviate the need for organ liver transplantation. In fact, hepatocyte transplantation into the liver of a recipient patient has proven to be effective in some experimental and clinical settings (3,10,14,20,21,24,26).

We have recently developed a novel approach for engineering a functional ectopic liver system within the subcutaneous space in mice (17). The engineered liver systems have been shown to persist for at least 200 days, and the persistency of the de novo engineered tissue has been largely predicated on two key factors. First, the creation of a vascularized platform using a growth factor-releasing device at the target subcutaneous site prior to the transplantation of the donor hepatocytes (30). The prevascularization process plays a pivotal role in the survival of the transplanted hepatocytes by providing an adequate blood supply to these transplanted cells, due to their high metabolic activities that require an active nutrient supply and waste exchanges (13,16). The second factor is the creation of uniform hepatocyte sheets using the isolated individual donor hepatocytes in culture (17). The in vitro created hepatocyte sheets maintain their intercellular communications (e.g., desmosomes and bile canaliculi) and exhibit other essential cellular microstructures to document their continued hepatocyte phenotype and functionality. Recent studies by our

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group has shown that combining these two factors in which the in vitro-generated hepatocyte sheets were transplanted within a prevascularized subcutaneous site potentiated the formation of a fully functional, long-lasting two-dimensional liver systems (17). Moreover, a more spatially complex, three-dimensional liver systems could be successfully achieved by stratifying additional hepatocyte sheets in the subcutaneous space (17). The confirmed functionality of the engineered liver system includes liver-specific protein productions, chemical uptake and subsequent metabolizing enzyme expressions, and regenerative growth (17).

Although this approach has shown considerable promise in recent years, there are several hurdles that need to be overcome for continual advancement to occur using hepatocyte-based technologies. Most notably, the potential lack of donor hepatocytes could become an issue depending on the abundance of the liver mass obtained for this procedure, since isolated hepatocytes lose their ability to actively proliferate following transfer to culture conditions (9,14). For this reason, an efficient method to propagate donor hepatocytes needs to be established. However, we and others have shown that primary donor hepatocytes can be successfully proliferated in vivo following transplantation in the livers of several mouse models, including the urokinase-type plasminogen activator-severe combined immunodeficiency (uPA/SCID) transgenic mice (1,6,7,19,22,23).

Towards this end, we have designed the present study to determine whether primary donor mouse hepatocytes transplanted into the livers of uPA/SCID recipient mice can be efficiently propagated in vivo for subsequent downstream utilization as a valuable source for the creation of hepatocyte cell sheets. The efficacy in the generation of hepatocyte cell sheets in the prevascularized subcutaneous space of mice and their persistent biological function(s) will be investigated.

MATERIALS AND METHODS

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, OH) (2) at 13 weeks of age were used as donors for hepatocyte isolation. Wild-type female FVB/N mice (11–12 weeks of age), which were syngenic to the hA1AT-FVB/N, were used as the recipient animals. hA1AT transgenic (hA1AT-TG) and FVB/N mice were maintained in the Animal Center at Tokyo Women's Medical University. uPA/SCID mice used in the hepatocyte propagation experiments were generated at Hiroshima Prefectural Institute of Industrial Science and Technology (HPIIST) and PhoenixBio, Co. Ltd. as described previously (22,23,31). Presence of the uPA transgene in each SCID mouse was genotypically confirmed by PCR as described previously (23). Experimental protocols were developed in accordance with the guidelines of the local animal committees at HPIIST, PhoenixBio, Co., Ltd., and Tokyo Women's Medical University. 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.

Hepatocyte Isolation and Purification

Hepatocytes were isolated from hA1AT-TG mice or hA1AT hepatocyte-repopulated uPA/SCID mice using a modified two-step collagenase perfusion method as previously described (5,11,12,16,17,30). Briefly, the livers were 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 slow speed centrifugation at $50 \times g$ for 5 min followed by Percoll (GE Healthcare, Buckinghamshire, UK) isodensity purification. The viabilities of the isolated mouse hepatocytes were determined by trypan blue exclusion test. In studies of hA1AT hepatocyte transplantation to the uPA/SCID mice, experiments were conducted only when the hA1AT hepatocyte viabilities exceeded 95%.

Transplantation of hA1AT Hepatocytes for Propagation in the uPA/SCID Livers

Isolated hA1AT hepatocytes were resuspended with serum-free Dulbecco's modified Eagle medium (DMEM) (Sigma) to a final ratio of 1.5×10^7 hepatocytes/ml. A total of 5×10^5 viable hepatocytes were transplanted into the liver of uPA/SCID mice using an infusion technique into the inferior splenic pole as described previously (22,23,31).

Creation of Two-Dimensional Hepatic Tissue Sheets

The creation of hepatocyte sheets was performed as described previously (17). Briefly, hA1AT hepatocytes were isolated from hA1AT mouse livers and hA1AT hepatocyte-repopulated uPA/SCID livers were plated on the temperature-responsive polymer [poly(*N*-isopropylacrylamide)]-coated (PIPAAm) dishes (UpCell, 35 mm, CellSeed, Tokyo, Japan) at a density of 8×10^5 cells/dish. Cell culture was performed at 37°C. Three days later, when the plated hepatocytes reached confluency, the cultured hepatocytes were detached from the culture dish and harvested as a uniformly connected tissue sheet by lowering the culture temperature to 20°C for 15 min. The harvested hepatocyte tissue sheets were attached to a support membrane (CellShifter, CellSeed) for subsequent transplantation.

Hepatocyte Sheet-Based Liver Tissue Engineering Procedure

Prior to the liver tissue engineering procedure, the basic fibroblast growth factor (bFGF)-releasing device was inserted into the subcutaneous space on the back of FVB/N mice as described previously (17,30). Ten days after the device insertion, a highly vascularized subcutaneous platform was developed. Right before the hepatocyte sheet transplantation, an L-shaped skin incision was made to open the vascularized platform followed by removal of the device. The harvested hepatocyte sheet was transplanted onto the vascularized platform with the support membrane. Approximately 5 min later, the support membrane was removed while leaving the hepatocyte sheet in vivo and procedure was finalized by closing the skin wound.

Enzyme-Linked Immunosorbent Assay (ELISA)

Propagation status of the hA1AT hepatocytes in the uPA/SCID livers and functional volume of the liver tissues engineered in the FVB/N mice were assessed by measuring the recipient serum hA1AT concentrations by ELISA. The ELISA used an antibody against hA1AT (DiaSorin, Stillwater, MN) coupled with a secondary goat IgG antibody conjugated with horseradish peroxidase (Resewarch Diagnostics Inc., Flander, NJ) as previously described (10).

Histological Analyses

In some recipient uPA/SCID mice, livers were harvested at 8 weeks after hA1AT hepatocyte transplantation. Portions of subcutaneous tissues of FVB/N mice containing engineered liver tissues were harvested 90 days after hepatocyte sheet transplantation. Liver specimens and subcutaneous tissues were fixed in 10% buffered formalin and embedded in paraffin. The specimens were sliced into 5-μm-thick sections, which were processed for hematoxylin & eosin (H&E) staining or immunohistochemical staining. Immunohistochemical staining was performed using the avidin-biotin complex method with the rabbit polyclonal hA1AT-specific antibody (1:200, YLEM, Roma, Italy). For the cellular glycogen detection, Periodic Acid Schiff (PAS) histochemical staining was performed as described previously (16). 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.

Statistical Analysis

All of the values calculated in the present study were provided as mean ± 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.

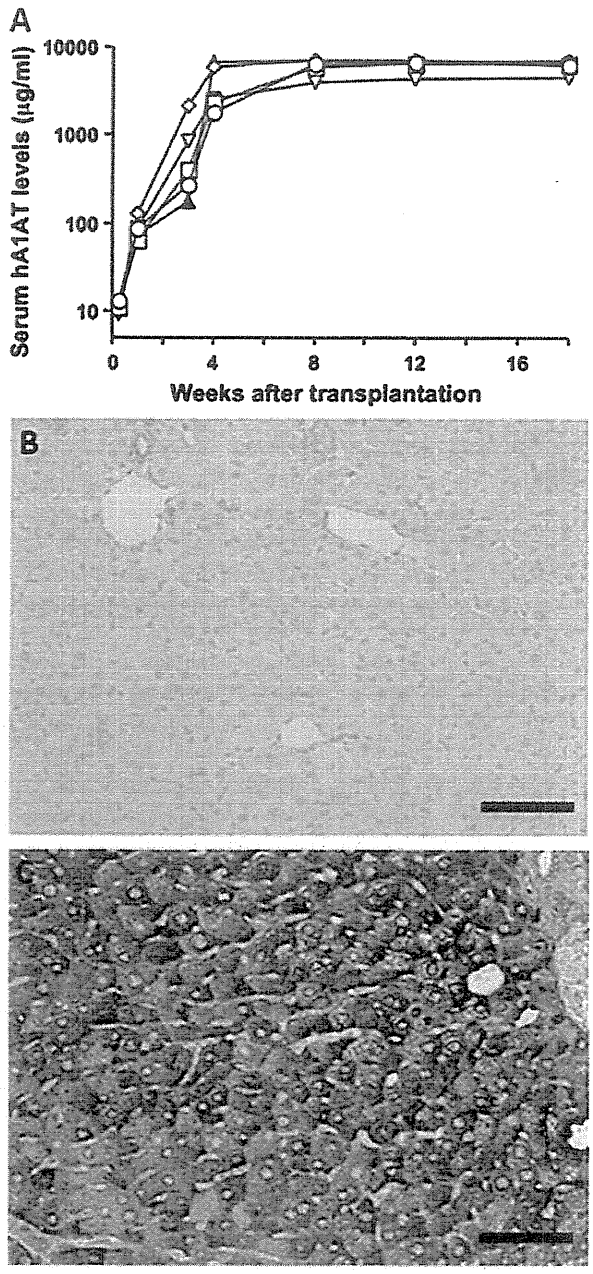

Figure 1. Proliferation and propagation status of the transplanted hA1AT hepatocytes in uPA/SCID mouse livers. (A) Proliferation status of the human α-1 antitrypsin (hA1AT) hepatocytes. At day 0, hA1AT hepatocytes were transplanted into the liver of urokinase-type plasminogen actiovator-severe combined immunodeficient (uPA/SCID) mice ($n = 5$) and recipient serum hA1AT levels were measured by ELISA to determine the level of proliferation and persistence of the transplanted cells. (B, C) hA1AT immunohistochemical staining of the naive uPA/SCID liver (B) and uPA/SCID liver harvested at 8 weeks after the transplantation of hA1AT hepatocytes (C). Scale bars: 100 μm (B, C).

RESULTS

Proliferation of hA1AT Allogenic Hepatocytes in uPA/SCID Livers

Hepatocytes isolated from 13-week-old hA1AT-TG mouse were transplanted into uPA/SCID mice ($n = 5$). Serum hA1AT was detected, which ranged between 8,000 and 14,000 ng/ml, in all of the five recipient uPA/SCID mice at day 2 after transplantation. Following measurements after day 2 showed continuous increases in the serum hA1AT levels through to week 8 (Fig. 1A). In four out of the five recipient mice, the serum hA1AT levels reached the same levels as detected in normal hA1AT-TG mice. The elevated serum hA1AT levels persisted throughout the observation period until the end of the study at week 18.

At random, we selected some of the recipient uPA/SCID mice to assess the state of hA1AT hepatocytes repopulation in the liver samples obtained from the uPA/SCID mice at week 8 after transplantation. hA1AT staining of native liver samples of nontransplanted uPA/SCID mouse (for control) did not show any positively stained cells, confirming the staining specificity for hA1AT (Fig. 1B). In contrast, week 8 liver samples of the recipient uPA/SCID mice showed that hA1AT hepatocytes had invaded the liver throughout the organ (Fig. 1C), indicating that the uPA/SCID livers had been fully repopulated with the transplanted hA1AT hepatocytes during the 8-week period after transplantation.

Isolation of Repopulated hA1AT Hepatocytes From uPA/SCID Livers and Creation of Hepatocyte Sheet

Our confirmation that there was full repopulation of uPA/SCID livers with hA1AT hepatocytes after week 8 allowed us to continue to determine whether these donor repopulated hepatocytes could be a viable option for isolation and recovery to develop them in the hepatocyte sheet protocol. To achieve this goal, we isolated and recovered the repopulated hA1AT hepatocytes form one uPA/SCID recipient at week 12. After the Percoll isodensity centrifugation step, 14.5×10^6 viable hepatocytes were obtained. These recovered hA1AT hepatocytes (recovered hepatocytes) were then plated on the PIPAAm culture dishes at a density of 0.75×10^6 per 35-mm dish as previously described (17) (Fig. 2). We also isolated hA1AT hepatocytes (fresh hepatocytes) from hA1AT mouse and plated the hepatocytes on the PIPAAm culture dishes at the same cell density. Plating efficiency (percent of attached hepatocytes per plated hepatocytes) assessed 24 h after the cell plating were $73.8 \pm 8.8\%$ and $75.5 \pm 5.3\%$ in the recovered hepatocytes and fresh hepatocytes, respectively (four different wells in each group, no statistically significant differences between groups). At day 3 of the culturing protocol, the recovered hepatocytes were found to reach

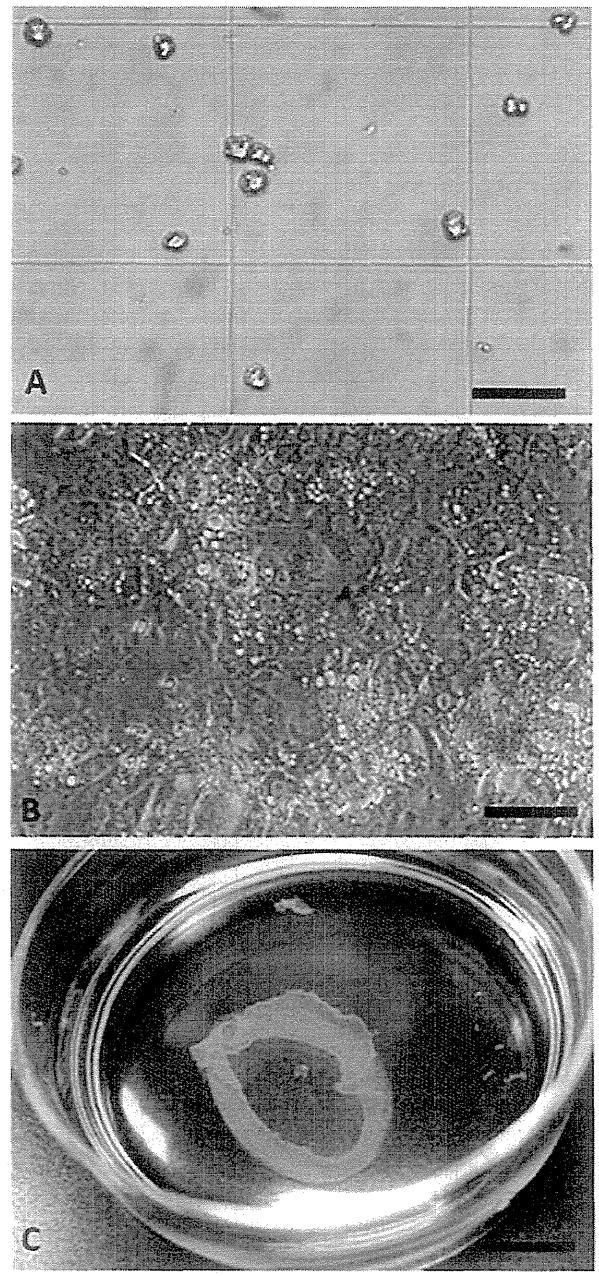

Figure 2. Morphological validation of the recovered hA1AT hepatocytes from the uPA/SCID livers that had been repopulated with the hA1AT hepatocytes. (A) Morphology of the isolated hepatocytes from uPA/SCID recipient. Photo was taken following the trypan blue exclusion test. (B) Recovered hA1AT-hepatocytes cultured on the poly(*N*-isopropylacrylamide) (PIPAAm) dish for 3 days documenting the confluent nature of the cells. (C) Observation of the cultured hA1AT-hepatocytes from the PIPAAm dish as a uniform tissue sheet. Scale bars: 100 μm (A, B), 1 cm (C).

confluence. Lowering the culture temperature from 37°C to 20°C for 15 min resulted in the spontaneous detachment of the cultured hepatocytes from the culture plates as a uniform cell sheet.

Liver Tissue Engineering Using Hepatocyte Sheet Made of Recovered Hepatocytes

The recovered hepatocyte sheet was then used for liver tissue engineering with the FVB/N mice using a similar procedure as previously described (17). Hepatocyte sheet was transplanted to the subcutaneous site that had been prevascularized following the insertion of a bFGF-releasing device. Functional activity of the engineered liver tissues was assessed by measuring serum hA1AT levels in the recipient FVB/N mice. As shown in Figure 3A, recipient mice showed stable and persistent serum hA1AT levels (ranged between 2,000 and 5,000 ng/ml), suggesting that the engineered liver tissues could be viable and stably maintained throughout the 90-day experimental period.

Subcutaneous tissue samples taken 90 days after the tissue engineering procedure were assessed for histological examination. H&E staining and hA1AT immunostaining revealed that liver tissues were engineered in the subcutaneous space with the hepatocyte-specific phenotypes (Fig. 3B, C). PAS staining revealed that the engineered liver tissues were positive for the function of glycogen synthesis and storage (Fig. 3D). Negative signals in the PAS staining on salivary amylase-pretreated samples confirmed staining specificity for cellular glycogen (Fig. 3E). It is important to note that there was no evidence of tumor formation observed in any of the engineered tissues.

DISCUSSION

The present study describes an in-mouse hepatocyte propagation system by which uPA/SCID mouse livers were found to fully reconstitute transplanted allogeneic mouse primary hepatocytes. This full reconstitution event was conducted by active and continuous hepatocyte proliferation as evidenced by the progressive increase in the hA1AT serum levels of the recipient uPA/SCID mice and massive occupation of hA1AT staining-positive hepatocytes in the recipient livers. The present study also showed that these propagated donor hepatocytes were able to be recovered, cultured, and subsequently used in the creation of hepatocyte sheets. The de novo generated hepatocytes sheets were able to be transplanted into a prevascularized subcutaneous space resulting in the successful engineering of an ectopic functional liver tissue.

We have established several innovative experimental approaches to create functional liver system in vivo (5,11–13,16,17,30). One of the key technologies in our series of developments is the creation of a uniform hepatocyte sheet using the PIPAAm culture dishes (17,27). The present study demonstrated that the hepatocytes recovered from the propagated livers of uPA/SCID mice were adhesive to the PIPAAm dish surfaces, and appeared to be at a similar efficiency of newly isolated primary hepatocytes that have not been previously isolated and propagated. The attached hepatocytes in the present study showed natural cell extension and became confluent by day 3 of culture. These cells showed the same morphological alterations associated with normal primary hepatocytes. This morphological similarity suggests that the microtubules and microfilaments were likely intact in the recovered hepatocytes.

This study as well as previous studies by our group have shown that hepatocyte sheets can be obtained by natural detachment of the primary cultured hepatocytes from the PIPAAm culture surfaces through a simple change in the ambient temperature for a brief period of time (17) without the need for any proteolytic enzyme treatment (28,29). This simple cell harvesting approach does not damage the cell-to-cell contact allowing for the retention of the intricate cell–cell intercommunication systems (i.e., bile canaliculi, desmosomes, gap junctions) (17). Although the ultrastructural examination was not performed in the present study, it is reasonable to postulate that the hepatocyte sheet made of the recovered hepatocytes possessed intercellular communications previously observed in our system using newly isolated hepatocytes considering that these functional intercellular communications are important in maintaining the structure of monolayered hepatocyte sheet. The integrity of the hepatocyte sheet using the recovered donor hepatocytes is similar to that of de novo isolated cells, and is readily transferable to a support membrane for the transplantation process.

We have previously reported that the uPA/SCID mice have the innate property to actively proliferate donor primary hepatocytes of xenogenic origin, including human hepatocytes, following transplantation into the liver (22, 23,31). The functional preservation of the human hepatocytes during their active proliferation phase in the uPA/SCID livers had been previously established as determined by the production of a hepatocyte-specific coagulation factor IX (FIX) (23). As an extension of the FIX findings, we have recently found that human hepatocytes in the uPA/SCID livers can maintain their cellular machinery to produce other hepatocyte-specific coagulation factors and anticoagulation factors (unpublished data). The availability of the propagated human hepatocytes in the uPA/SCID system for in vitro analyses was established in an earlier study by Yoshitsugu et al. (31). Since the recovered human hepatocytes can be cultured and express various drug-metabolizing enzymes,

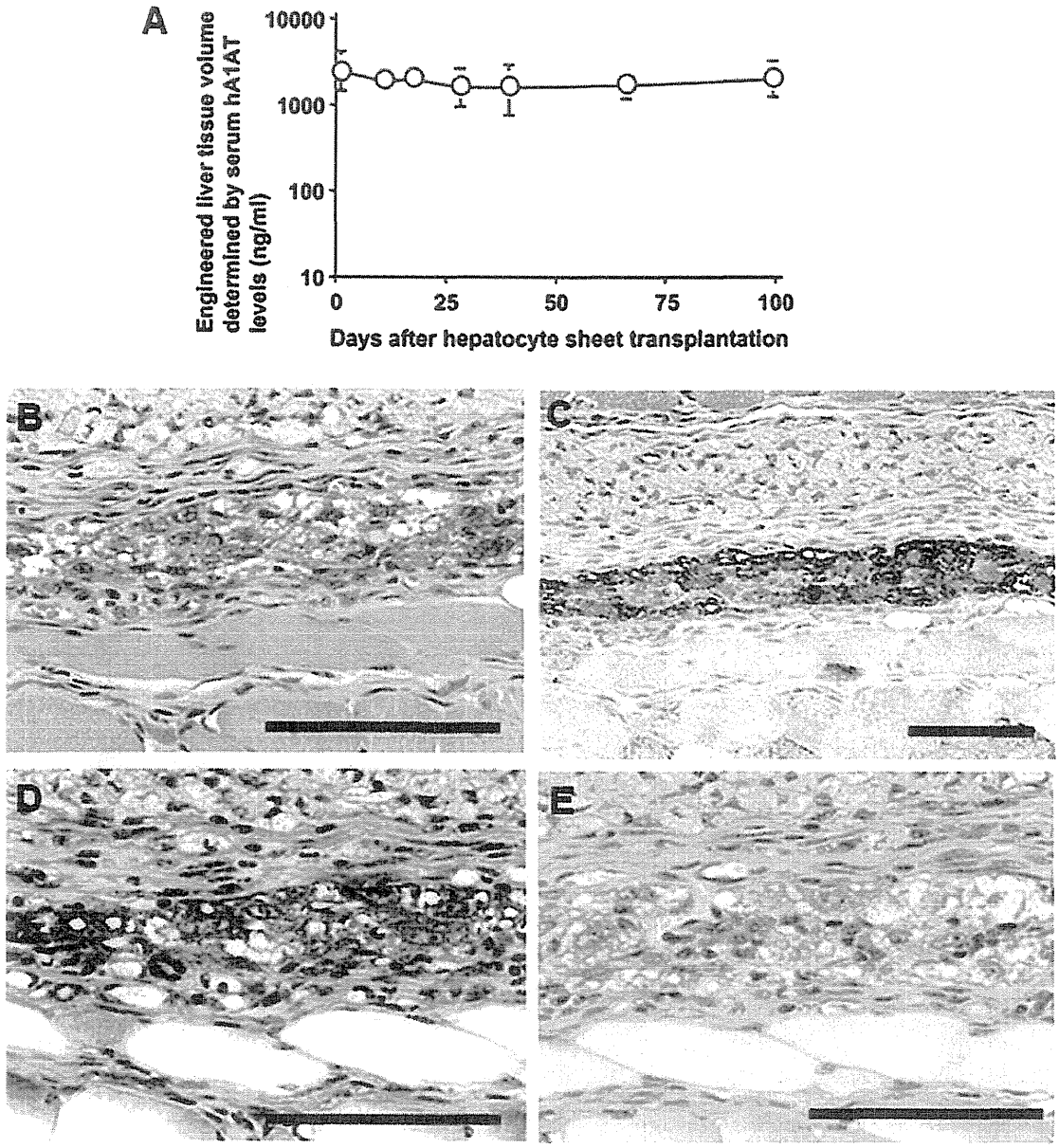

Figure 3. Functional maintenance of the engineered liver tissue within the subcutaneous space. (A) Functional volume of the liver tissues engineered in the subcutaneous space was determined by measuring recipient serum hA1AT levels by ELISA. Hepatocyte sheet composed of the hA1AT hepatocytes as shown in Figure 2 was generated and this harvested hepatocyte sheet was then transplanted into the subcutaneous space where an active vascular network had been induced using a basic fibroblast growth factor (bFGF)-releasing device. Histological findings by (B) H&E staining, (C) immunohistochemical staining for hA1AT, and (D, E) Periodic Acid Schiff (PAS) staining of the engineered liver tissue from samples obtained at day 90 after the hepatocytes sheet transplantation. Functions for cellular glycogen synthesis and storage were confirmed by PAS staining in sections that were either nontreated (D) or pretreated with salivary amylase (E). Scale bars: 100 μm (B–E).

it was demonstrated that these recovered hepatocytes could be a valuable cell source for the study of a variety of liver functions (31). From these previous studies as well as the findings in our current study, we suggest that the propagated hepatocytes in the uPA/SCID livers could be as effective as the original primary hepatocytes with respect to their cellular functionalities. Although the present study clearly demonstrated that the hepatocytes obtained by in-mouse propagation system are useful for the creation of hepatocyte sheets, their availability for other hepatocyte-based approaches have not been investigated. Future studies will need to be conducted if the recovered hepatocytes can be transplanted and/or engraftable to other sites, such as the liver parenchyma itself or other ectopic sites within the body other than the subcutaneous space.

For potential clinical application for this type of hepatocyte-based therapeutic approach, the potential risk of oncogenesis deriving from the transplanted hepatocytes needs to be addressed, particularly due to the active level of proliferation that is being undergone following the transplantation procedure (8). In the present study, the gross morphology of all six of the recipient uPA/SCID livers at the time of sacrifice and/or hepatocyte isolation was normal. Histological investigation of the liver specimens did not show any cancerous or precancerous lesions. In parallel to these findings, no cancer development was observed in our previous study in which human hepatocytes were transplanted into the same strain of mice following the active proliferative phase of the donor hepatocytes (23). The best to our knowledge and as recently reviewed by Marongiu et al. (8), cancer cell development from the transplanted normal primary hepatocytes has not been reported in the uPA/SCID system. Other types of small animal models that are able to coordinate the process of proliferation of transplanted hepatocytes toward the replacement of recipient livers have been established, including the fumaryl-acetoacetatehydroxylase-null Rag2$^-$ mouse (1), the retrorsine-treated (6–8), or monocrotaline-treated rat models (25). In none of the models has there been any evidence of cancer cell development being documented. As far as we are aware from the previously documented and the present findings, it could be summarized that the risk of the cancer cell development from the normal hepatocytes during in-animal hepatocyte proliferation process appears minimal.

In all, we have shown that the use of the uPA/SCID mice as a viable recipient model to actively proliferate allogenic primary hepatocytes in their livers for subsequent use in the development of newly propagated hepatocytes or as a cell sheet system. These results taken together provide strong evidence that isolated hepatocytes can be efficiently propagated using an in-mouse liver procedure, and can be a potential valuable method to generate a new source of hepatocytes for hepatocyte-based therapies.

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Human Hepatocyte Propagation System in the Mouse Livers: Functional Maintenance of the Production of Coagulation and Anticoagulation Factors

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We previously reported that cell-based therapies using isolated hepatocytes including hepatocyte transplantation and liver tissue engineering approaches provide therapeutic benefits to hemophilia. For clinical application of these approaches, it is important to establish an active hepatocyte proliferation system that enables providing a sufficient number of hepatocytes. We also reported that human hepatocytes, which were transplanted into the liver of urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mice, were able to proliferate while retaining their ability to produce coagulation factor IX. The objective of this study was to explore the functionalities of other coagulation and anticoagulation factors of the propagated human hepatocytes in uPA/SCID mice. Human hepatocytes were transplanted into the liver of uPA/SCID mice, and the propagation status of human hepatocytes in the mice was monitored by the increase in serum human albumin levels and immunohistochemical evaluation on the liver sections. Using uPA/SCID livers with various stages of human hepatocyte propagation, we analyzed the gene expression levels of coagulation factors (prothrombin, factor VII, factor X, and factor VIII) and anticoagulation factors (protein C and protein S) by real-time polymerase chain reaction (PCR) using human-specific primers. As a result, the total amount of raw messenger RNA expression levels increased in all genes analyzed according to the progress of hepatocyte propagation and proliferation. Except for factor VIII, the gene expression levels of the highly repopulated uPA/SCID mouse livers with human hepatocyte showed higher levels than those of normal human livers, indicating that propagated human hepatocytes in the uPA/SCID system possess full functions to produce most of the coagulation-related factors. The current work demonstrated that human hepatocytes can be propagated in experimental animals while maintaining normal gene expression levels of coagulation-related factors. It could be speculated that the propagated cells serve as a cell source for the treatment of various types of coagulation factor deficiencies.

Key words: Hepatocyte; Cell therapy; Hepatocyte transplantation; Coagulation factor; Urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mouse; Anticoagulation factor

INTRODUCTION

Production of coagulation and anticoagulation factors is one of the important functions of the liver, and most of these coagulation-related factors are produced by hepatocytes (4,6,30,31,33). There are various types of congenital bleeding disorders that lack a production of coagulation factor in the liver, showing a symptomatic bleeding tendency. Hemophilia A or B is well known as a representative bleeding disorder, which is caused by a failure in the production of functional coagulation factor VIII (FVIII) or factor IX (FIX) from the liver. Although the ultimate cure for hemophilia patients could be obtained by liver transplantation (10,13,14), world-wide donor organ shortage is the most critical obstacle. For patients with hemophilia and other congenital coagulation factor deficiencies, the elevation of the responsible factor level to 1–2% of normal levels can provide a phenotypic change from severe to moderate form, resulting in a marked improvement in the symptomology and the

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quality of life (5). Cell-based approaches using isolated hepatocytes could be a feasible therapeutic option toward these coagulation factor deficiencies (18,20,21,24).

Proof-of-concept studies for hepatocyte-based approaches have been accomplished in both laboratory animals and humans. We recently reported that hepatocyte transplantation provided an increase of 1–2% of coagulation activities in a mouse model of hemophilia B, FIX-knockout mice (31). We also reported that engineering functional liver tissues beneath the kidney capsule were able to provide therapeutic effects in the mouse model of hemophilia A as well as hemophilia B (20,21). As reported by Dhawan et al. (7), hepatocyte transplantation was also successful in the clinic for the treatment of congenital factor VII deficiency. In utero liver cell transplantation was also investigated by Rosen et al. (25). They described phenotypic improvements in the mouse model of factor X deficiency. It is important to note that these hepatocyte-based approaches could be employed with a simple procedure and in a less invasive manner compared with organ transplantation (18,22).

One of the major hurdles in establishing hepatocyte-based approaches is the limited availability of biologically functional hepatocytes. At present, the number of donor livers for hepatocyte isolation remains severely limited. In most of cases, donor livers are of marginal quality that makes it difficult to obtain functional hepatocytes (23). An additional issue is that current technology for hepatocyte primary culture appears to be unable to support extensive cell proliferation (19). Under these circumstances, we previously proposed urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPA/SCID) mice as a feasible in-mouse hepatocyte propagation tool. uPA/SCID mice have a feature to develop an active damage of their own hepatic parenchymal cell and subsequent occurrence of continuous release of regenerative stimulus. Because of this nature, uPA/SCID mice provide a hepatic environment that is more conducive to the engraftment of human hepatocytes and a selective advantage for transplanted cells to proliferate (29). An important property of uPA/SCID mice was recently reported to allow human hepatocytes transplanted into the liver of uPA/SCID mice to actively propagate while retaining their ability to produce and secrete human FIX (30). From these data, it was reasonably speculated that the propagated human hepatocytes can serve as a cell source for future hepatocyte-based therapies toward hemophilia B. The functional preservation of FIX production of the propagated human hepatocytes encourages us to further assess functionalities for the production of other coagulation or anticoagulation factors.

We hypothesized that propagated human hepatocytes in the uPA/SCID mouse livers retained a normal gene expression of other coagulation and anticoagulation factors including prothrombin, factor VII, factor VIII, factor X, protein C, and protein. This report documents the first comprehensive analyses of coagulation factor-related gene expressions during in-mouse propagation status of human hepatocytes.

MATERIALS AND METHODS

Animals

Recipient uPA/SCID mice were generated at Phoenix Bio (Higashihiroshima, Hiroshima, Japan) as described previously (29). Genotyping for the presence of uPA transgene in SCID mice was confirmed by polymerase chain reaction assay of isolated genomic DNA as described previously (11,29). Experimental protocols were developed in accordance with the guidelines of the local animal committees located at both PhoenixBio and Nara Medical University.

Transplantation of Human Hepatocytes for Propagation in the uPA/SCID Livers

Human hepatocytes, isolated from a 1-year-old white male and a 6-year-old Afro-American female were purchased from In Vitro Technologies (Baltimore, MD). The cryopreserved hepatocytes were thawed and suspended in transplant medium (9,29). The cell viability of the human hepatocytes was determined to be 64.4% and 49.2% by trypan blue exclusion test, respectively. One day prior to the transplantation and 1 week after the transplantation, uPA/SCID mice, 20–30 days old, received intraperitoneal injections of 0.1 mg of anti-asialo GM1 rabbit serum (Wako Pure Chemical Industries, Osaka, Japan) to inhibit recipient natural killer cell activity against the transplanted hepatocytes. Viable human hepatocytes (0.75×10^6) 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 ($n = 18$). After the transplantation, uPA/SCID mice were treated with nafamostat mesilate to inhibit complement factors activated by human hepatocytes as described elsewhere (29).

Determination of Replacement Ratio

Blood samples were collected periodically from the tail vein, and the levels of human albumin were determined with a Human Albumin ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX) to estimate the status of proliferation and propagation of the transplanted human hepatocytes as previously described (29). For accurate determination of the ratio that transplanted human hepatocytes occupied in the recipient mouse livers (the replacement ratio), the harvested liver section were stained with antibodies against human-specific

cytokeratins 8 and 18 (hCK8/18), as described elsewhere (29). The replacement ratios of the mouse liver that received human hepatocytes were calculated as the ratio of area occupied by hCK8/18-positive hepatocytes to the entire area examined immunohistochemical sections of six lobes.

RNA Isolation and Quality Controls

Total RNA was extracted from the liver of the recipient mice with various stages of the replacement and normal human liver tissue samples by a RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Normal human liver tissue portions were obtained from surgical specimens during liver surgery for metastatic liver tumors after acquiring a written informed consent for the experimental use of harvested liver samples. DNase I was used to eliminate genomic DNA contamination, and the concentration of the RNA was determined by UV spectrometry. All of the RNA samples used in this study had an absorbance ratio (260/280 nm) between 1.9 and 2.1, and the integrity of RNA samples was confirmed by electrophoresis on a 1% agarose gel.

Reverse Transcription Coupled to Quantitative Real-Time PCR (Real-Time RT-PCR)

Total RNA (1 μg) was reverse-transcribed using oligo d(T)16 primers as described by the manufacturer (Omniscript RT Kit; QIAGEN). First-strand cDNA samples were subjected to quantitative PCR amplification using a StepOne Real-time PCR system (Applied Biosystems, Tokyo). For this experiment, we examined the following gene groups: 1) seven housekeeping reference genes, including glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), β-actin (*Actb*), peptidylprolyl isomerase A (*Ppia*), ribosomal protein L4 (*Rpl4*), transferrin receptor (*Tfrc*), β-glucuronidase (*Gusb*), and hypoxanthine phosphoribosyltransferase (*Hprt1*); 2) the genes of five vitamin K-dependent coagulation factors, including prothrombin (*F2*), factor VII (*F7*), factor X (*F10*), protein C (*Prosc*), and protein S (*Pros1*); and 3) factor VIII (*F8*) gene. TaqMan probes and primers for these genes were chosen from a TaqMan Gene Expression Assay (Applied Biosystems), and the information regarding these primer sets are listed in Table 1. All PCR analyses were performed using the following cycling conditions: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The specificity of the primers was verified by 2% agarose gel electrophoresis of the amplicons derived from naive human liver cDNAs. The PCR primers used in this study were confirmed to be human specific and to have no cross-reactions with mouse-derived genes. For quantification of gene expression, the cDNAs derived from total RNA extracted from normal human liver tissues were serially diluted and used to generate calibrations.

Statistical Analysis

Correlation coefficients between the repopulation rate and each gene expression were determined using Excel (Microsoft).

RESULTS

Propagation of Human Hepatocytes in uPA/SCID Mouse Liver

Human hepatocytes were transplanted to uPA/SCID mice, which were sacrificed to excise the liver tissues at various time periods after the transplantation with monitoring the levels of blood human albumin. Using the collected liver samples, hCK8/18 immunostaing on the liver sections was performed to accurately assess the replacement ratios with human hepatocytes as described in Materials and Methods. As a result, the repopulation ratios ranged from 0% to 98%, and the number of mice in each repopulation category was 2, 4, 4, 4, and 4 in 0–20%, 21–40%, 41–60%, 61–80%, and 81–100%, respectively.

Selection of an Appropriate Reference Gene

The expressions of seven commonly used housekeeping genes (HKG) specific to human cells were evaluated in the recipient uPA/SCID mouse livers. Figure 1 shows that the raw expression levels of all seven HKGs increased in parallel to the replacement ratios. The correlation coefficiency with the replacement ratios of each gene was 0.68 for h*Gapdh*; 0.86 for h*Actb*; 0.72 for h*Ppia*; 0.82 for h*Rpl4*; 0.68 for h*Tfrc*; 0.75 for h*Gusb*; and 0.78 for h*Hprt1*. When the replacement ratios exceeded 80%, the expression levels of all HKGs but *Hprt1* became higher than those of normal human liver samples. h*Gapdh* expression levels in the repopulated uPA/SCID livers were also observed to be beyond the levels of normal human livers at the repopulation ratio as low as 40%. Eventually, h*Gapdh* expression levels reached approximately sixfold of normal human liver levels when the repopulation ratios exceeded 80%. In contrast, gene expression levels of h*Actb* failed to reach to comparable levels with normal human liver until the repopulation ratios were close to 100%. Under the condition of varied gene expression levels of HKGs, it is important to select the most stably expressed HKG to assess the expression of target human genes in the uPA/SCID livers. For achieving this, *Actb* gene, which demonstrated the best correlation coefficient with the replacement ratios, was selected as a reference normalizing gene in the following gene expression analyses.