derived FVIII in previously untreated patients with severe hemophilia A: homogeneous results from four published observational studies. *J Thrombos Hemost* 2008; 6: 390–2.

26 Gouw SC, von der Bom JG, Auerswald G *et al.* Recombinant versus plasma-derived factor VIII products and the development of inhibitor in previously untreated patients with severe hemophilia A: the CANAL cohort study. *Blood* 2007; 109: 4693–7.

27 Chalmers EA, Brown SA, Keeling D *et al.* Early factor VIII exposure and subsequent inhibitor development in children with severe haemophilia A. *Haemophilia* 2007; 13: 149–55.

28 Iorio A, Halimeh S, Holzhauer S *et al.* Rate of inhibitor development in previously untreated hemophilia A patients treated with plasma-derived or recombinant factor VIII concentrates: a systematic review. *J Thromb Haemost* 2010; 8: 1256–65.

Haemophilia

Haemophilia (2012), 18, e43–e59

LETTERS TO THE EDITORS

Factor VIII haplotypes of Japanese population show similarity to those of Caucasian populations

H. INABA,* K. SHINOZAWA,† T. HAGIWARA,* T. SUZUKI,* Y. YAMAMOTO,* K. AMANO*† and K. FUKUTAKE*†
**Department of Laboratory Medicine; and †Department of Molecular Genetics of Coagulation Disorders, Tokyo Medical University, Tokyo, Japan*

Haemophilia A is the most common inherited bleeding disorder which is X-linked recessive. This disease is caused by a quantitative or qualitative abnormality of plasma factor VIII (FVIII), which is affected by a genetic mutation located in the coagulation FVIII gene. Diagnosis and replacement therapy strategies in haemophilia A patients are well established; however, some issues remain to be addressed.

The most important issue concerning replacement therapy in haemophilia A patients is the development of inhibitors (alloantibody) against FVIII. This development leads to marked attenuation in the effectiveness of replacement therapy, leading to a substantial deterioration in the quality of life of patients. In general, the incidence of inhibitor development in treatment-naive patients with haemophilia A is estimated to be 20–30% [1]. However, it has been shown that its incidence in the Black population is markedly higher, about twice as high as in other racial groups [2]. The question of why this inhibitor develops with such high incidence in the Black population remains undetermined.

Recently, Viel *et al.* reported that six wild-type FVIII proteins, the H1–H6 haplotypes, had different prevalence rates among racial groups [3]. They speculated that a mismatch of the FVIII haplotype between the FVIII concentrate and its recipients, particularly in immunodominant epitopes, caused an increase in the frequency of inhibitor development. In particular, they focused on four different amino acid polymorphisms (R484H, R776G, D1241E and M2238V), based on a non-synonymous single nucleotide polymorphism. By using a combination of those amino acids, they classified the FVIII protein into six haplotypes, namely, H1: RRDM, H2: RREM, H3: RREV, H4: HREM, H5: RRDV and H6: RGEM. It is presumed that amino acid positions 484 and 2238, located in the A2 and C2 domains, respectively, are components of the immunodominant epitope of FVIII. In Caucasian participants, positions 484 and 2238 have been observed as 'R' and 'M', respectively, and have only shown the haplotypes of H1 and H2. However, Viel *et al.* confirmed the presence of 'H' in place of 'R' at position 484, or 'V' in place of 'M' at position 2238, in approximately 25% of Black participants. Thus, the haplotype frequencies in Black participants (H1: 0.354; H2: 0.374; H3: 0.222; H4: 0.040 and H5: 0.010) are different from those in Caucasian participants. Furthermore, they also analysed haplotypes in ethnic Chinese participants, and observed the H6 haplotype, but not the H3, H4 or H5 haplotype.

We set out to analyse the haplotypes of FVIII proteins among 106 unrelated Japanese subjects at our institution (63 with haemophilia A and 43 with haemophilia B as a control group), as shown in Table 1. The study was approved by the Ethics Committee of Tokyo Medical University and written informed consent was obtained from each patient. The studies were carried out in accordance with the principles of the Declaration of Helsinki. Among the Japanese participants, only the H1 and H2 haplotypes (H1: 0.896 and H2: 0.104) were found at frequencies not significantly different from the Caucasian frequencies (H1: 0.926 and H2: 0.074). Despite the Chinese being geographical neighbours of the Japanese, the H6 haplotype which was observed in approximately 8% of Chinese was not detected in any Japanese.

Table 1. Factor VIII haplotypes in Japanese participants.

Haplotype	Hemophilia A (frequency)	Hemophilia B (frequency)	Total (frequency)
H1	57 (0.905)	38 (0.884)	95 (0.896)
H2	6 (0.095)	5 (0.116)	11 (0.104)
H3–H6	0 (0.000)	0 (0.000)	0 (0.000)

Currently in Japan, approximately 80% of haemophilia A patients receive replacement therapy using the recombinant FVIII concentrates Kogenate (Bayer) or Advate (Baxter). Data regarding the incidence of inhibitor development in Japanese patients with severe haemophilia A were previously presented as poster presentations on post-authorisation safety studies for Kogenate or Advate at the 22nd Congress of the International Society on Thrombosis and Hemostasis (ISTH) (2009) and the Hemophilia World Congress (World Federation of Hemophilia) (2010) by Fukutake *et al.*, who reported that the incidence was similar to that in Caucasians.

Although the role of the haplotypes of FVIII proteins as a risk factor for inhibitor development is not yet determined, the similarities of haplotype incidence may partially explain the similarities in inhibitor incidence between Japanese patients and Caucasian patients. Further studies are needed to clarify the role and characteristics of FVIII haplotypes in Japanese population.

Acknowledgements

We are indebted to Mr. Roderick J. Turner, Assistant Professor Edward F. Barroga and Professor J. Patrick Barron, Chairman of the Department of International Medical Communications at Tokyo Medical University, for their review of the English manuscript. This study was partially supported by Health and Labor Sciences Research Grants for Research on HIV/AIDS from the Japanese Ministry of Health, Labor and Welfare.

Disclosures

K. Fukutake has received speaking fees and honoraria from Baxter Healthcare, Bayer HealthCare and Pfizer Inc. K. Shinozawa is an employee of an endowed chair at the Department of Molecular Genetics of Coagulation Disorders of Tokyo Medical University, which received funding from Baxter. The other authors declare that they have no conflicts of interest regarding this study.

Correspondence: Hiroshi Inaba, PhD, Department of Laboratory Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan.
Tel.: +81 3 3342 6111 ext. 5086; fax: +81 3 3340 5448;
e-mail: hrsinb@tokyo-med.ac.jp

Accepted after revision 1 November 2011

DOI: 10.1111/j.1365-2516.2011.02715.x

References

1 Schwartz R, Abildgaard C, Aledort L *et al.* Human recombinant DNA-derived antihemophilic factor (factor VIII) in the treatment of hemophilia A. Recombinant Factor VIII Study Group. *N Engl J Med* 1990; 323: 1800–5.

2 Saint-Remy J, Lacroix-Desmazes S, Oldenburg J. Inhibitors in haemophilia: pathophysiology. *Haemophilia* 2004; 10(Suppl 4): 146–51.

3 Viel K, Ameri A, Abshire T *et al.* Inhibitors of factor VIII in black patients with hemophilia. *N Engl J Med* 2009; 360: 1618–27.

血栓止血誌 22(5) : 259～265, 2011

◆特集：東日本大震災と日本血栓止血学会◆

東日本大震災で被災された血友病患者への支援

Support for patients with hemophilia affected by the Great East Japan Earthquake

田中一郎[*1,2], 天野景裕[*1,3], 瀧　正志[*1,4], 岡　敏明[*1,5], 酒井道生[*1,6], 嶋　緑倫[*1,2], 白幡　聡[*1,7], 竹谷英之[*1,8], 花房秀次[*1,9], 日笠　聡[*1,10], 福武勝幸[*1,3], 藤井輝久[*1,11], 堀越泰雄[*1,12], 松下　正[*1,13], 松本剛史[*1,14], 三室　淳[*1,15], 吉岡　章[*1,16]

Ichiro TANAKA[*1,2], Kagehiro AMANO[*1,3], Masashi TAKI[*1,4], Toshiaki OKA[*1,5], Michio SAKAI[*1,6], Midori SHIMA[*1,2], Akira SHIRAHATA[*1,7], Hideyuki TAKEDANI[*1,8], Hideji HANABUSA[*1,9], Satoshi HIGASA[*1,10], Katsuyuki FUKUTAKE[*1,3], Teruhisa FUJII[*1,11], Yasuo HORIKOSHI[*1,12], Tadashi MATSUSHITA[*1,13], Takeshi MATSUMOTO[*1,14], Jun MIMURO[*1,15], Akira YOSHIOKA[*1,16]

Key words: earthquake, hemophilia, network system, consultation service

[*1] 日本血栓止血学会学術標準化委員会血友病部会
Subcommittee of Hemophilia, Scientific Standardization Committee of The Japanese Society on Thrombosis and Hemostasis
[*2] 奈良県立医科大学小児科〔〒634-8522 奈良県橿原市四条町 840〕
Department of Pediatrics, Nara Medical University〔840 Shijo-cho, Kashihara, Nara 634-8522, Japan〕
[*3] 東京医科大学病院臨床検査医学科〔〒160-0023 東京都新宿区西新宿 6-7-1〕
Department of Laboratory Medicine, Tokyo Medical University Hospital〔6-7-1, Nishishinjuku, Shinjuku, Tokyo 160-0023, Japan〕
[*4] 聖マリアンナ医科大学横浜市西部病院小児科〔〒241-0811 神奈川県横浜市旭区矢指町 1197-1〕
Department of Pediatrics, St. Marianna University, School of Medicine, Yokohama City Seibu Hospital〔1197-1 Yasashi-cho, Asahi, Yokohama, Kanagawa 241-0811, Japan〕
[*5] 札幌徳洲会病院小児科〔〒003-0021 北海道札幌市白石区栄通 18-4-10〕
Department of Pediatrics, Sapporo Tokushuukai Hospital〔18-4-10 Sakaedoori, Shiroishi, Sapporo, Hokkaido 003-0021, Japan〕
[*6] 産業医科大学小児科〔〒807-8555 福岡県北九州市八幡西区医生ヶ丘 1-1〕
Department of Pediatrics, University of Occupational and Environmental Health, Japan〔1-1 Iseigaoka, Yahatanishi, Kitakyushu, Fukuoka 807-8555, Japan〕
[*7] 北九州八幡東病院〔〒805-0061 福岡県北九州市八幡東区西本町 2-1-17〕
Kitakyushu Yahata Higashi Hospital〔2-1-17 Nishihonmachi, Yahatahigashi, Kitakyushu, Fukuoka 805-0061, Japan〕
[*8] 東京大学医科学研究所附属病院関節外科〔〒108-8639 東京都港区白金台 4-6-1〕
Department of Joint Surgery, The Institute of Medical Science, The University of Tokyo〔4-6-1 Shiroganedai, Minato, Tokyo 108-8639, Japan〕
[*9] 荻窪病院血液科〔〒167-0035 東京都杉並区今川 3-1-24〕
Department of Hematology, Ogikubo Hospital〔3-1-24 Imagawa, Suginami, Tokyo 167-0035, Japan〕
[*10] 兵庫医科大学血液内科〔〒663-8501 兵庫県西宮市武庫川町 1-1〕
Department of Hematology, Hyogo College of Medicine〔1-1 Mukogawa, Nishinomiya, Hyogo 663-8501, Japan〕
[*11] 広島大学病院輸血部〔〒734-8551 広島県広島市南区霞 1-2-3〕
Division of the Blood Transfusion Services, Hiroshima University Hospital〔1-2-3 Kasumi, Minami-ku, Hiroshima, Hiroshima 734-8551, Japan〕
[*12] 静岡県立こども病院血液腫瘍科〔〒420-8660 静岡県静岡市葵区漆山 860〕
Division of Hematology and Oncology, Shizuoka Children's Hospital〔860 Urushiyama, Aoi, Shizuoka, Shizuoka 420-8660, Japan〕
[*13] 名古屋大学医学部附属病院輸血部〔〒466-8550 愛知県名古屋市昭和区鶴舞町 65〕
Department of Transfusion Medicine, Nagoya University Hospital〔65 Tsurumai, Showa, Nagoya, Aichi 466-8550, Japan〕
[*14] 三重大学医学部附属病院輸血部〔〒514-8507 三重県津市江戸橋 2-174〕
Department of Transfusion Medicine, Mie University Hospital〔2-174 Edobashi, Tsu, Mie 514-8507, Japan〕
[*15] 自治医科大学分子病態治療研究センター分子病態研究部〔〒329-0498 栃木県下野市薬師寺 3311-1〕
Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University〔3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan〕
[*16] 奈良県立医科大学〔〒634-8522 奈良県橿原市四条町 840〕
Nara Medical University〔840 Shijo-cho, Kashihara, Nara 634-8522, Japan〕

はじめに

平成23年3月11日に発生した東日本大震災は東北地方を中心に未曾有の被害をもたらし，被災された方々は死者・行方不明者をあわせて2万人を超えるとされています[1]（8月9日現在，警察庁まとめ）．さらに，今なお避難・転居を余儀なくされている方々は9万人を超えるとされています[2]（7月26日現在，内閣府まとめ）．血液凝固異常症全国調査平成22年度報告書（財団法人エイズ予防財団）[3]によると，青森，岩手，宮城，秋田，山形，福島の東北ブロックで血友病A 345名，血友病B 72名の方々が登録されており，これらの方々の中には深刻な被害に遭われた方もいるものと推測されます．これら被災された血友病患者の方々を血友病部会で支援するようにと震災から8日後の3月19日に池田康夫理事長ならびに坂田洋一学術標準化委員会委員長からご指示をいただきました．それに応える形で血友病部会としていくつかの支援策を行ってきましたので，この誌面をお借りしてご報告させていただきます．現実に，被災地でお困りの血友病患者の方々に対して何ができたのか，また，何ができなかったのかを検証したいと思います．

1．情報の収集と発信

震災直後は，被災地の情報，特に医療関係の情報が全く入らず，混乱の状態が続きましたが，しばらくすると，今度は様々な情報が入り乱れ錯綜するようになりました．テレビや新聞からの情報だけでなく，今日ではインターネットやメーリングリスト，ツイッター，フェイスブックなど情報源は多岐に渡ります．実際，血友病関連のメーリングリストなどでは連日のように現地の情報が流されていましたが，それらの多くは整理されることなく，一方的に情報が流されている状態でした．このような時には情報をいかにコントロールし得るかが極めて重要になりますが，血友病関連でいち早く情報を整理し発信されたのが，日本小児血液学会血友病委員会（嶋緑倫委員長）の血友病診療ネットワークでした．このネットワークは血友病診療を行っている，およそ480の施設が登録されており，主として小児科医で構成されています．震災から4日後の3月15日には相模原協同病院小児科の中舘尚也先生（現，国立成育医療センター総合診療部）が中心となってネットワークの管理を行い，数多くの情報を収集し整理した上で必要な情報をメーリングリストで流すという作業を開始されました．血友病部会でもこれに呼応する形で，3月18日には未登録の部会員に血友病診療ネットワークへの参加を呼びかけ，さらに3月22日には日本血栓止血学会の代議員にも広く参加を呼びかけました．これは，被災された血友病患者の救援・支援という共通の目的のもと，今回の大災害を機に学会や診療科の垣根を越えて実現した画期的な出来事だと思います．実際，このネットワークを利用して様々な有用な情報が発信されました．例えば，保険証や特定疾患の受給者証がなくても診療が受けられるといった厚生労働省からの通達事項[4,5]や血友病治療製剤の供給体制，在庫状況などの情報が発信されました．また，被災患者の受入れの申し出も全国の施設から寄せられたほか，被災地である宮城県や福島県の病院の状況，特に血友病診療の実態などが報告されました．このメーリングリストによって，震災後1か月の間に血友病関連の情報として診療情報が20回，医薬品情報が17回にわたって発信されました．

ここで，実際にネットワークを利用した事例を紹介したいと思います．患者は被災地にお住まいの血友病Aの小児で，普段から定期補充療法が行われていました．震災後，着の身着のままで首都圏の親戚宅に避難され，どこの病院にかかればいいか困っているということで，3月17日に被災地の主治医からネットワークを通じて緊急支援依頼がありました．実際には聖マリアンナ医科大学横浜市西部病院を通じて東京医科大学病院を紹介され継続して治療を受けることができました．この間，幸いにも大きな出血はなく，本例はネットワークが機能し，専門医間の連携によりうまく対応できたケースでした．

2．相談窓口の開設

血友病部会では上記のネットワークへの参加と

資料1　被災された血友病患者の方々への相談窓口開設の御案内

血友病部会では被災地でお困りの患者・家族の方々を対象にEメールでの相談窓口を設けました．Eメールでの相談が困難な場合はお電話でも受付けます．相談内容に応じて，血友病専門医師もしくは専門看護師が回答させていただきますが，内容によっては回答までにある程度お時間をいただくことがありますのでご了承ください．また，本相談窓口は緊急時には対応しておりませんので，ご理解いただきますようお願い申し上げます．なお，相談内容はプライバシー保護に十分配慮してお取り扱いさせていただきます．ご相談いただく前に，「血友病被災患者・家族の方々へQ&A」もご一読いただければ幸いです．（日本血栓止血学会学術標準化委員会血友病部会）

■Eメールでのご相談：日本血栓止血学会血友病部会事務局（ketuyubyou@gmail.com）
■お電話でのご相談*：東京医科大学病院（代表 03-3342-6111）
＜電話交換手に「血友病相談窓口への電話」とお伝え下さい．＞
平日9時～17時（臨床検査医学科外来まで）
時間外，土日祝祭日（臨床検査医学科当直医まで）
自治医科大学附属病院（代表 0285-44-2111）
平日9時～17時，土日祝祭日除く（分子病態研究部・止血血栓まで）
聖マリアンナ医科大学病院（代表 044-977-8111）
平日9時～17時，土日祝祭日除く（看護部　吉川まで）
奈良県立医科大学附属病院（代表 0744-22-3051）
平日9時～17時，土日祝祭日除く（小児科研究室まで）

*一般診療の業務と重なった場合は対応にお時間をいただくことがあります．

ともにさらなる支援策を検討しました．ネットワークでの情報発信は主として血友病診療を行う医師を対象にしたものであり，それとは別に直接患者さんを支援するような活動ができないものかと考えました．この頃には震災直後の混乱も一段落し，初期の救命・救援活動から，長期に渡る被災者へのケアが求められるようになっていました．血友病患者の方々についても，長期の転居・避難所生活により発生する様々なトラブルや不安にいかに対応するべきかが問われる時期でした．そこで，掛り付けの病院に行きたくても行けない状況に置かれている患者の方々にとって気軽に専門の医師や看護師に直接相談できるような窓口があればいいのではないかということになり，相談窓口開設の準備に入りました．ただ，一口に相談窓口と言っても，普段の診療が多忙な中，どの施設の誰がどのような形態で相談業務を行うのかなど詰めなければいけない課題が数多くありました．しかし，これらの問題も部会員ならびにその施設のスタッフのご協力により，一つずつ解決され，4月6日には学会ホームページ上に相談窓口を開設することができました[6]（**資料1**）．そこではメールでの相談受付を行うとともに東京医科大学，自治医科大学，聖マリアンナ医科大学，奈良県立医科大学の4施設に電話相談の窓口を設けて対応することになりました．また，相談窓口開設と同時に患者の方々からの想定される質問をQ&A形式で掲載しました[7]（**資料2**）．このQ&Aの中には，製剤の室温での保管がどの程度可能かといった情報や製剤の販売元の問い合わせ先情報などが含まれています．さらに，4月22日には製剤が入手可能な被災地の施設リストも追加掲載しました[8]（**資料3**）．

3．広報活動

いかに有用な情報でも被災された患者の方々に届かなければ意味がありません．いかに多くの方々にこれらの情報をお知らせするのかが次の大きな課題でした．実際，患者の方々の手元に相談窓口開設の情報をお届けするために，様々なルートを通じて広報活動を行いました．具体的には岩手，宮城，福島県の各災害対策室や厚生労働省にご案内したほか，患者会を通じて可能な限り患者の方々にも情報を伝えていただきました．また，製剤関連メーカーを通じて被災地の病院や施設にも広報していただきました．さらに，インターネット上では，厚生労働省や宮城県災害保健医療支援室のウェブサイトのほか，患者会やその支援組織，日本赤十字社，そして製薬企業の患者向けサイト

資料2　血友病被災患者・家族の方々へ Q&A

Q1. 自分の使っている製剤についての情報を知りたい.

A1. 以下の各メーカーのお問い合わせ窓口をご利用ください.

各メーカーのお問い合わせ窓口				
血友病A治療製剤	クロスエイトM®	日本赤十字社	03-3437-7579 090-4380-3895	日中時間帯 その他時間
	コージネイト®FS バイオセット	バイエル薬品	0120-106-398 090-9090-8945	平日9：30～17：30 時間外
	アドベイト®	バクスター	03-6204-3800	平日10：00～17：00 土日祝祭日除く
	コンファクト®F	化血研／アステラス	0120-345-724	平日8：30～17：30 土日祝祭日除く
血友病B治療製剤	ノバクト®M	化血研／アステラス	0120-345-724	平日8：30～17：30 土日祝祭日除く
	ベネフィクス®	ファイザー	0120-664-467 0120-168-365	平日9：00～17：30 土日祝祭日除く 24時間対応（溶解操作関連）
インヒビター治療製剤	ファイバ®	バクスター	03-6204-3800	平日10：00～17：00 土日祝祭日除く
	ノボセブン®HI	ノボ ノルディスク ファーマ	0120-180-363 0120-359-516	平日9：00～18：00 夜間，土日祝日，会社休日
フォンヴィレブランド病治療製剤	コンファクト®F	化血研／アステラス	0120-345-724	平日8：30～17：30 土日祝祭日除く

Q2. 製剤は室温でも保管できるのですか.

A2. 室温安定性は製剤によって以下の表のように異なります．詳しくはA1．のメーカー窓口にお問い合わせください．なお，いずれの製剤も凍結は避けて保管してください.

各製剤の室温安定性			
血友病A治療製剤	クロスエイトM®	日本赤十字社	室温保存可（1～30℃）
	コージネイト®FS バイオセット	バイエル薬品	3か月まで室温保存可（25℃以下）
	アドベイト®	バクスター	通常，2～8℃で保存 ただし，6か月まで室温保存可（1～30℃） 室温保管後は再度冷蔵庫に戻さないこと
	コンファクト®F	化血研／アステラス	10℃以下保存
血友病B治療製剤	ノバクト®M	化血研／アステラス	室温保存可（30℃以下）
	ベネフィクス®	ファイザー	6か月まで室温保存可（30℃以下）
インヒビター治療製剤	ファイバ®	バクスター	2～8℃で保存
	ノボセブン®HI	ノボ ノルディスク ファーマ	室温保存可（1～30℃）
フォンヴィレブランド病治療製剤	コンファクト®F	化血研／アステラス	10℃以下保存

Q3. 手元にいつも使っている製剤がないのですが，他の製剤に変更できますか.

A3. 緊急の場合は変更可能です．どの製剤も止血効果および安全性に大きな差はありません．また，製剤を変更することによってインヒビターが発生する可能性は極めて低いと考えられます．具体的には，血友病Aではクロスエイト M®（日本赤十字社），コージネイト®FSバイオセット（バイエル薬品），アドベイト®（バクスター），コンファクト®F（化血研／アステラス）が使用可能です．一方，血友病Bではノバクト®M（化血研／アステラス），ベネフィクス®（ファイザー），クリスマシン®-M（ベネシス／田辺三菱），PPSB®-HT（日本製薬）が使用可能です.

Q4. 今回の震災で全国的に製剤が不足しているのですか.

A4. 各製剤とも国内の在庫は全く問題ありません．被災地の病院への配送も徐々に回復しているようですが，一部地域では依然として配送が困難な所もあるようです（4月1日現在の情報）．詳しくはA1．にある各メーカーの窓口にお問い合わせください.

Q5. 震災で泥まみれになった製剤は使用できますか.

A5. 容器が破損している恐れもありますので，そういった製剤は使用しないで下さい.

Q6. 定期補充療法をしていたのですが，急にやめても大丈夫ですか．

A6. 被災状況によっては，出血時補充療法に切り替えざるを得ない方々もおられると思います．定期補充療法に比べて出血の機会が増えることが予想されますが，可能な限り出血後早期の治療を心がけるようにしていただけたらと思います．

Q7. 大きな出血をおこしたら，どうしたらいいですか．

A7. 可能であれば製剤を自己注射していただいた上で，最寄りの医師の診察を受けるようにして下さい．緊急の場合は救急車の出動を要請して下さい．

Q8. インヒビターが陽性で現在，免疫寛容導入療法を始めたところです．途中でやめてもいいのですか．

A8. 状況が落ち着いて普段通りになるまでは仕方がないと思います．免疫寛容導入療法を中断した場合，インヒビターがどうなるかは個人によって違いますので一概には予測できません．

Q9. 県外の避難先で診てもらえる病院はありますか．

A9. 日本小児血液学会の血友病診療ネットワークでは被災された患者さんの受入れ支援を全国の施設から名乗り出ていただいています．Eメール（ketuyubyou@gmail.com）でお問い合わせいただければ，ご紹介することは可能です．

Q10. 避難先の県でも医療費はかからないのですか．

A10. 厚生労働省から，「公費負担医療を受けている被災者が，医療機関において手帳，患者票等の提出ができない場合においても，受診が可能である」という通知が各都道府県に出されています．また，「緊急の場合は，小児慢性特定疾患治療研究事業や特定疾患治療研究事業の受託契約を結んだ医療機関以外の医療機関でも受診できるものとする」とされています．詳しくはおかかりの病院のソーシャルワーカーにお問い合わせください．

などに相談窓口へのリンクを貼っていただきました（**資料4**）．ただ，現時点では被災者の方々からのご相談はほとんどなく，実際にお困りになられている方々が幸いにも少ないのか，現地の方々まで情報が届いていないのか，窓口開設のタイミングが遅かったのか，患者の方々にとって窓口の敷居が高いのかなど今後の広報活動のあり方について課題を残した形になっています．

4. 今後の対応

今回の大震災の経験を踏まえ，痛切に感じたのは災害に対する事前の備えの重要性でした．地震や津波以外にも災害はいつ，いかなる場所にも前触れなしに起こるものです．そのため，今回用意したような災害時の医療情報や医薬品情報などを前もって整理しておくとともに，製剤の入手可能な施設のリストを全国各地で作成しておく必要があると感じました．これは広く他の関連学会にも呼びかけて進めていきたいと考えています．また，現在，血友病部会で進めている血友病センター構想もより重要になってきます．センター構想ではセンターを中心として各地域の診療ネットワークを構築し，さらには全国レベルのネットワークに発展させることを目指していますが，それが実現すると血友病関連の様々な情報共有，診療協力が可能になります．また，各センターで疾患登録を行い，全国的なデータベースを構築することによって，患者情報の共有，安否確認などがよりスムーズになり，災害時に役立つのではないかと考えています．

おわりに

今回の大震災は原発問題も相まっていまだ収束の目処が立たない状況であり，長期の避難所生活を余儀なくされておられる被災者の方々のご苦労は察するに余りあります．ただ，テレビや新聞報道で被災地での復興を目指して頑張っておられる地元の方々，また，被災地を応援するために日本中，世界中から寄せられた数々の支援を見るにつけ，勇気と希望が与えられる思いがします．われわれ医療従事者は今回の震災を教訓として学ぶとともに，次に起こる可能性のある災害に備えることが重要ではないかと考えます．決して他人事でなく，また，はるか先のことでなく，真摯にそして着実に対応していくことが求められています．

今回の大震災で被災された皆様にはあらためて心よりお見舞い申し上げますとともに一日も早い復興を祈念して止みません．

謝　辞

今回の原稿執筆にあたり，快く貴重な資料を提供

資料3　血友病等治療製剤の入手可能施設

※　常に院内在庫があるとは限りませんので，受診前に必ず各施設にお問い合わせください．
※　施設によって取り扱っている製剤の規格（単位数）が異なる可能性がありますので，こちらもご確認ください．
※　平成23年4月18日現在，ホームページ掲載のご許可をいただいた施設のリストであり，他にも取り扱っておられる施設はあると思われます．

製剤		県名	病院名	診療科	住所	代表電話番号
血友病A治療製剤	クロスエイトM®（日本赤十字社）	岩手	盛岡赤十字病院	小児科	岩手県盛岡市三本柳6地割1番地1	019-637-3111
			奥州市総合水沢病院	小児科	岩手県奥州市水沢区大手町3丁目1番	0197-25-3833
		宮城	国立病院機構仙台医療センター	内科	宮城県仙台市宮城野区宮城野2-8-8	022-293-1111
			宮城県立こども病院	血液腫瘍科	宮城県仙台市青葉区落合4丁目3-17	022-391-5111
	コージネイト®FSバイオセット（バイエル薬品）	岩手	岩手医科大学附属病院	小児科	岩手県盛岡市内丸19-1	019-651-5111
			盛岡赤十字病院	小児科	岩手県盛岡市三本柳6地割1番地1	019-637-3111
		宮城	国立病院機構仙台医療センター	内科	宮城県仙台市宮城野区宮城野2-8-8	022-293-1111
			宮城県立こども病院	小児科	宮城県仙台市青葉区落合4丁目3-17	022-391-5111
			大崎市民病院	小児科	宮城県大崎市古川千手寺町2-3-10	0229-23-3311
			真壁病院	内科	宮城県東松島市矢本字鹿石前109-4	0225-82-7111
		福島	福島県立医科大学附属病院	小児科	福島県福島市光が丘1番地	024-547-1111
			いわき市立磐城共立病院	小児科	福島県いわき市内郷御厩町久世原16	0246-26-3151
			大原総合病院	小児科	福島県福島市大町6-11	024-526-0300
			すやま小児科医院	小児科	福島県福島市御山町6-29	024-534-3018
	アドベイト®（バクスター）	岩手	岩手医科大学附属病院	小児科	岩手県盛岡市内丸19-1	019-651-5111
			盛岡赤十字病院	小児科	岩手県盛岡市三本柳6地割1番地1	019-637-3111
		宮城	国立病院機構仙台医療センター	内科	宮城県仙台市宮城野区宮城野2-8-8	022-293-1111
			宮城県立こども病院	血液腫瘍科	宮城県仙台市青葉区落合4丁目3-17	022-391-5111
		福島	福島県立医科大学附属病院	小児科，循環器・血液内科	福島県福島市光が丘1番地	024-547-1111
			おおがチャイルドクリニック	小児科	福島県郡山市大槻町字土瓜204-75	024-962-0600
			いいづかファミリークリニック	小児科・内科	福島県会津若松市一箕町大字鶴賀字下居合59	0242-32-3330
	コンファクト®F（化血研／アステラス）	岩手	岩手医科大学附属病院	小児科	岩手県盛岡市内丸19-1	019-651-5111
		福島	いわき市立磐城共立病院	血液内科	福島県いわき市内郷御厩町久世原16	0246-26-3151
血友病B治療製剤	ノバクト®M（化血研／アステラス）	岩手	岩手医科大学附属病院	小児科	岩手県盛岡市内丸19-1	019-651-5111
		福島	いわき市立磐城共立病院	血液内科	福島県いわき市内郷御厩町久世原16	0246-26-3151
	ベネフィクス®（ファイザー）	岩手	岩手医科大学附属病院	小児科	岩手県盛岡市内丸19-1	019-651-5111
			盛岡赤十字病院	小児科	岩手県盛岡市三本柳6地割1番地1	019-637-3111
		宮城	国立病院機構仙台医療センター	小児科	宮城県仙台市宮城野区宮城野2-8-8	022-293-1111
		福島	いわき市立磐城共立病院	血液内科	福島県いわき市内郷御厩町久世原16	0246-26-3151
インヒビター治療製剤	ファイバ®（バクスター）	岩手	岩手医科大学附属病院	小児科	岩手県盛岡市内丸19-1	019-651-5111
			盛岡赤十字病院	小児科	岩手県盛岡市三本柳6地割1番地1	019-637-3111
		宮城	国立病院機構仙台医療センター	内科	宮城県仙台市宮城野区宮城野2-8-8	022-293-1111
			宮城県立こども病院	血液腫瘍科	宮城県仙台市青葉区落合4丁目3-17	022-391-5111
		福島	福島県立医科大学附属病院	小児科，循環器・血液内科	福島県福島市光が丘1番地	024-547-1111
			おおがチャイルドクリニック	小児科	福島県郡山市大槻町字土瓜204-75	024-962-0600
			いいづかファミリークリニック	小児科・内科	福島県会津若松市一箕町大字鶴賀字下居合59	0242-32-3330
	ノボセブン®HI（ノボノルディスクファーマ）	宮城	宮城県立こども病院	血液腫瘍科	宮城県仙台市青葉区落合4丁目3-17	022-391-5111
フォンヴィレブランド病治療製剤	コンファクト®F（化血研／アステラス）	岩手	岩手医科大学附属病院	小児科	岩手県盛岡市内丸19-1	019-651-5111
		福島	いわき市立磐城共立病院	血液内科	福島県いわき市内郷御厩町久世原16	0246-26-3151

資料4　相談窓口開設情報の広報・リンク先

1. 行政	
(1) 厚生労働省 HP「東日本大震災関連情報」	http://www.mhlw.go.jp/stf/houdou/2r985200000155g1.html
(2) 宮城県災害保健医療支援室 HP「保健医療についての情報集」	http://www.dcrc.tohoku.ac.jp/wiki/index.php
2. 学会・大学・医療施設	
(1) 日本小児血液学会血友病委員会「血友病診療ネットワーク」	
(2) 日本医学会 HP「東日本大震災関連情報」	http://jams.med.or.jp/japanquake2011/information.html - na
(3) 日本内科学会 HP「東日本大震災に関する関連学会等のリンク集」	http://www.naika.or.jp/info/info110318.html
(4) 東京医科大学臨床検査医学講座 HP	http://labo-med.tokyo-med.ac.jp/
(5) 国立国際医療研究センター病院　エイズ治療・研究開発センター HP「東北地方太平洋沖地震に関連した HIV 感染症診療情報」	http://www.acc.go.jp/earthquake/index.html
3. 患者会・患者支援サイト	
(1) ヘモフィリア友の会全国ネットワーク HP	http://hemophilia.web.fc2.com/earthquake.html
(2) 血友病の子供とその親たちのためのネットワーク CHPnet の HP「被災者支援血友病関連情報」	http://disaster.chpnet.info/
(3) 血友病関連情報の発信サイト Hemophilia Group の HP	http://csws.tokyo-med.ac.jp/csws/hemophilia/index.html
(4) 血友病患者のコミュニティーサイト，ライフパレット for Hemophilia の HP	http://hemophilia.lifepalette.jp/contributions/trend_2011earthquake_info
4. 日本赤十字社・製薬企業	
(1) 日本赤十字社 HP「血液事業　ニュース一覧」	http://www.jrc.or.jp/blood/news/14/Vcms4_00002155.html
(2) 千葉県赤十字血液センター HP「お知らせ」	http://www.chiba.bc.jrc.or.jp/news/page.php?eid=00104
(3) バイエル薬品株式会社の患者向け HP「ヘモフィリアヴィレッジ」	http://www.hemophilia.jp/html/index.html
(4) バクスター株式会社の患者向け HP「ヘモフィリアギャラクシー」	http://www.hemophiliagalaxy.org/
(5) ファイザー株式会社の患者向け HP「ヘモフィリアライフ」	http://hemophilia-life.jp/
(6) ノボノルディスクファーマ株式会社の HP	http://www.novonordisk.co.jp/documents/home_page/document/index.asp
5. その他	
(1) 医療安全全国共同行動 HP「災害時の医療安全」	http://kyodokodo.jp/saigai.html
(2) 地域医療振興協会 HP「公衆衛生ねっと」	http://www.koshu-eisei.net/cgi/topics/disp.cgi?mode=detail&id=1444
(3) 保健・医療系図書館員のサイト，リテリス HP「災害時の健康・医療」	http://plaza.umin.ac.jp/~literis/cgi-bin/fswiki/wiki.cgi?page=Earthquake-Health%26Diseases

HP：ホームページ

していただいた中舘尚也先生（国立成育医療センター総合診療部）に深謝致します．

Disclosure of Conflict of Interests

The authors indicated no potential conflict of interest.

文　献

1) 警察庁緊急災害警備本部：平成 23 年（2011 年）東北地方太平洋沖地震の被害状況と警察措置（平成 23 年 8 月 9 日発表資料）http://www.npa.go.jp/archive/keibi/biki/higaijokyo.pdf
2) 内閣府緊急災害対策本部：平成 23 年(2011 年)東北地方太平洋沖地震(東日本大震災)について(平成 23 年 7 月 26 日発表資料) http://www.kantei.go.jp/saigai/pdf/201107261700jisin.pdf
3) 財団法人エイズ予防財団：血液凝固異常症全国調査 平成 22 年度報告書，2011.
4) 厚生労働省保険局医療課：東北地方太平洋沖地震による被災者に係る被保険者証等の提示について（平成 23 年 3 月 11 日事務連絡）http://www.mhlw.go.jp/stf/houdou/2r98520000014tr1-img/2r9852000001573g.pdf
5) 厚生労働省健康局：東北地方太平洋沖地震による被災者の公費負担医療の取扱いについて（平成 23 年 3 月 11 日事務連絡）http://www.mhlw.go.jp/stf/houdou/2r98520000014tr1-img/2r985200000156kq.pdf
6) 日本血栓止血学会：被災された血友病患者の方々への相談窓口開設の御案内．http://www.jsth.org/news/0095.html
7) 日本血栓止血学会：血友病被災患者・家族の方々へ Q&A. http://www.jsth.org/news/docs/Q&Ahemo.pdf
8) 日本血栓止血学会：岩手，宮城，福島三県における血友病等治療製剤の入手可能施設リスト．http://www.jsth.org/news/0101.html

Reversal of Diabetes by the Creation of Neo-Islet Tissues Into a Subcutaneous Site Using Islet Cell Sheets

Takahiro Saito,[1,2] Kazuo Ohashi,[2,3] Rie Utoh,[2] Hirofumi Shimizu,[1] Kazuya Ise,[1] Hiroyuki Suzuki,[1] Masayuki Yamato,[2] Teruo Okano,[2] and Mitsukazu Gotoh[1]

Background. There remains a paucity of therapeutic approaches to completely treat diabetes mellitus. This study was designed to develop a dispersed islet cell-based tissue engineering approach to engineer functional neo-islet tissues in the absence of traditional bioabsorbable scaffold matrices.

Methods. Specialized coated plastic dishes were prepared by covalently immobilizing a temperature-responsive polymer, poly(*N*-isopropylacrylamide), onto the plastic followed by coating with laminin-5. Dispersed rat islet cells were plated on the laminin-5-poly(*N*-isopropylacrylamide) dishes. After 2 days of culturing, islet cells were harvested as a uniformly connected tissue sheet by lowering the culture temperature from 37°C to 20°C for 30 min. Two harvested islet cell sheets were transplanted into the subcutaneous space of streptozotocin-induced diabetic severe combined immunodeficiency (SCID) mice to engineer neo-islet tissues in vivo. Therapeutic effects were investigated after the tissue engineering procedures.

Results. In all of the diabetic SCID mice transplanted with the islet sheets, serum hyperglycemia was successfully reverted to a steady normoglycemic level. The recipient SCID mice demonstrated positive for serum rat C-peptide and elevated serum insulin levels. Moreover, the islet cell sheet-transplanted SCID mice demonstrated rapid glucose clearance and return of serum glucose levels after intraperitoneal glucose tolerance test. Histological examination revealed that the transplanted islet cell sheets were structured as flat clusters of islet tissues in which an active vascular network manifested within and surrounding the newly formed tissues.

Conclusions. This study describes a new proof-of-concept therapeutic approach to engineer functional neo-islet tissues for the treatment of type 1 diabetes mellitus.

Keywords: Islet, Diabetes mellitus, Cell sheet engineering, Dispersed islet cells.

(*Transplantation* 2011;92: 1231–1236)

Cell-based therapies using pancreatic islets have emerged as a promising new approach for the treatment of insulin-dependent diabetes mellitus. Currently, the preferred organ for the transplantation of islet cells is the liver. The majority of the clinical trials treating type 1 diabetes mellitus are designed for transplantation of islets into the liver through the portal vascular system (*1, 2*), but this approach is limited. There is a gradual loss of the transplanted islets rendering the majority of the recipients to switch back to insulin-dependent from their independent status (*3*). It has been suggested that portal infusion of islets is associated with a number of complications, which include instant blood-mediated inflammatory reactions (*2, 4*), complement cascade activation (*5*), and leukocyte infiltration (*6*). Ultimately, these immune-related problems result in graft failure, and the majority of the recipients switch back to insulin-dependent from their independent status (*3*).

To prolong the longevity of the transplanted islet cells, there are emerging new approaches designed to bioengineer functional islet systems at extra-hepatic sites, such as subcutaneous, subrenal, and abdominal spaces. Of these candidate sites, the subcutaneous site remains the most attractive, because the transplantation of islet cells and tissue systems to this particular area can be performed with minimal invasive-

This work was supported, in part, by Special Coordination Funds for Promoting Science and Technology (K.O., M.Y., and T.O.), Global Center of Excellence Program (K.O. and M.Y.), and Grant-in-Aid (No. 21300180 to K.O. and No. 20591227 to M.G.) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

M.Y. serves as consultant and shareholder for CellSeed, Inc. (Japan). T.O. is an investor in CellSeed, Inc. (Japan) and an inventor/developer designated on the patent for the temperature-responsive culture surfaces (patent nos. JP1972502, US5284766, FR0382214, NL0382214, DE0382214, GB0382214, SE0382214, and CH0382214).

[1] Department of Surgery, Fukushima Medical University, Fukushima, Japan.

[2] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Shinjuku, Tokyo, Japan.

[3] Address correspondence to: Kazuo Ohashi, M.D., Ph.D., Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan.

E-mail: ohashi@abmes.twmu.ac.jp

T.S., K.O., R.U., H.Sh., K.I., and M.G. participated in research design; T.S., R.U., H.Su., K.I., and M.Y. participated in the performance of the research; T.S., R.U., and H.Sh. participated in data analysis.; K.O., K.I., T.O., and M.G. participated in interpreting the data; and T.S., K.O., R.U., and K.I. participated in writing the manuscript.

Received 1 February 2011. Revision requested 22 February 2011.

Accepted 12 September 2011.

ISSN 0041-1337/11/9211-1231

DOI: 10.1097/TP.0b013e3182375835

ness (*7–13*). Dispersed islet cells infused into the subcutaneous space have been shown to survive, but only using approaches that either prevascularize the transplantation site (*8, 14*) or use a synthetic polymer scaffold to allow for cell attachment to the ectopic site (*15–17*). In the absence of these additional modifications, the success rate of the engraftment of the transplanted islet cells remains poor. Moreover, it is not clear whether these additional steps required to promote islet cell engraftment and survival are readily amenable to the clinical setting.

For these reasons, our laboratory developed a novel cell sheet technology in which individually dispersed cells are allowed to form a thin, contiguous monolayer. The cells comprised in this cell sheet format are able to communicate among themselves and act as an intrinsic biological system that can recognize and sense changes in physiological parameters after transplantation. In our previous investigations, cell sheets were engineered from a number of sources including oral mucosal cells (*18*), cardiomyocytes (*19*), liver cells (*11*), and recently islet cells (*20*). These newly engineered cell sheets have the advantage over individually dispersed cell clusters by allowing for multilayer approach leading to the creation of three-dimensional tissues (*11, 19*), which has been shown to prolong the viability and functionality of these cell sheets for therapeutic applications.

This study was designed to engineer functional islet tissues in a subcutaneous site using our novel cell sheet technology. Our results demonstrate that the islet cell sheets are capable of engraftment in the subcutaneous space and continually function as a newly bioengineered islet tissue to normal the glycemic index in the diabetic mice. In all, the islet tissue engineering approach described in this study is a unique and effective tissue engineering procedure as a potential treatment modality for diabetes mellitus and has many benefits, including its longevity of function, over previously published approaches using individually dispersed cell systems.

RESULTS

Therapeutic Potential of Islet Tissue Engineering Using Islet Cell Sheet

As shown in Figure 1A, nonfasting blood glucose (NFBG) levels in all the recipient diabetic severe combined immunodeficiency (SCID) mice (n=7) returned to a state of normoglycemia within 1 week after the transplantation of islet cell sheets, whereas all the mice in the sham-operated control group (n=6) remained hyperglycemic. In some diabetic SCID mice, we performed the injection of dispersed islet cells (equivalent cell number of two islet cell sheets) into the subcutaneous site and found that there were minimal decreases in their NFBG levels, but all the mice showed persistent hyperglycemic status after the cell injection (Fig. 1A). In another set of experiments, stable and long-term (>110 days) therapeutic effects were confirmed (Fig. 1B). After diabetic mice achieved a state of normoglycemia, two mice were chosen for graft removal. Immediately after the graft removal, a steep rise in the NFBG levels of both mice was detected as it reattained a hyperglycemic state (Fig. 1B). The recipient SCID mice showed improved clinical conditions with a

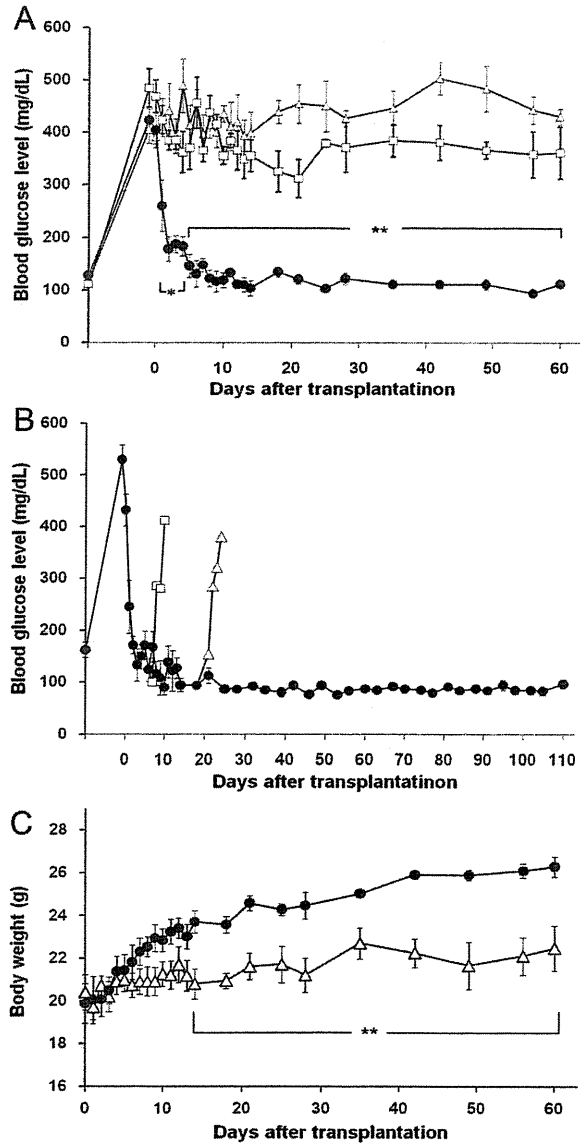

FIGURE 1. Nonfasting blood glucose levels (A,B) and body weights (C) of diabetic SCID mice with or without transplantation of islet cell sheets. (A) At day 0, diabetic SCID mice were transplanted with two islet cell sheets (*circle*; n=7) or the same number of dispersed islet cells (*square*; n=6) into the subcutaneous space on the dorsal site of diabetic SCID mice. As a control, diabetic SCID mice were sham operated (*triangle*; n=6). Mice that received sham operation or dispersed islet cells remained hyperglycemic. (B) At day 0, diabetic SCID mice were transplanted with two islet cell sheets (*circle*; n=7). All recipient mice transplanted with islet cell sheets returned toward normoglycemia (<200 mg/dL) by day 7 and remained at this level for over 110 days (n=5). Subcutaneous neo-islet tissues were surgically removed at day 7 (*square*; n=1) and day 21 (*triangle*; n=1). (C) Body weight changes of the recipient SCID mice with two islet cell sheets (*circle*; n=7) and sham-operated diabetic mice (*triangle*; n=6). **P* less than 0.05, ***P* less than 0.01 vs. the other two groups analyzed by ANOVA followed by Games-Howell post hoc test in (A) and ***P* less than 0.01 vs. the other group analyzed by Student's *t* test or Mann-Whitney *U* test in (C). ANOVA, analysis of variance; SCID, severe combined immunodeficiency.

steady increase in body weight after the transplantation procedure (Fig. 1C).

Histological assessments of tissue samples were made using tissues harvested at days 4 (Fig. 2A) and 60 (Fig. 2B–F). The histology showed that clusters of islet tissues were formed at the transplantation site. The cells in the neo-islet tissues retained structural morphology characteristic of pancreas islets. Strong cytoplasmic expression of rat insulin and glucagon was observed following immunohistochemistry, which confirmed the islet-specific phenotypes of the neo-engineered islet tissues in vivo (Fig. 2B–D). We observed that numerous platelet-endothelial cell adhesion molecule (PECAM)-1-positive cells were found at close proximity to the insulin-positive grafts at day 4 (Fig. 2A). Furthermore, intense vascular networks composed of PECAM-1-positive vascular endothelial cells were formed within and surrounding the neo-engineered islet tissues at day 60 (Fig. 2E,F). These findings also demonstrated the ability to form blood vascular network at an early stage after cell sheet transplantation and also to synthesize and store insulin and glucagon. Species specificity of these histological staining was confirmed by negative immune complex signals detected in the normal mouse pancreas samples (data not shown).

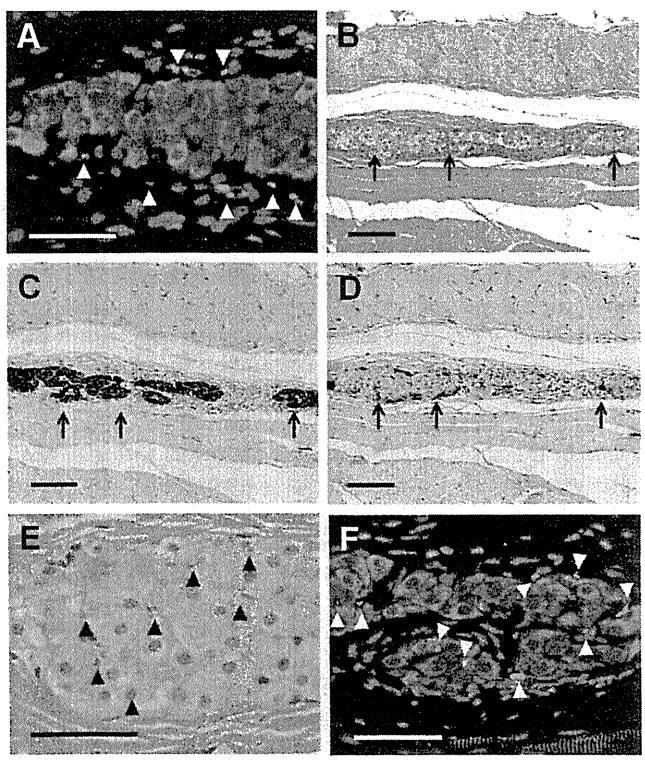

FIGURE 2. Histological and immunohistochemical analyses of the neo-islet tissues engineered in the subcutaneous site at day 4 (A) and day 60 (B–F). Sections of the subcutaneous tissues containing the transplanted islet cell sheets were processed for hematoxylin-eosin staining (B,E), immunofluorescence staining for insulin (*red*) and PECAM-1 (*green*) (A,F), and immunohistochemical staining for insulin (C) and glucagon (D). Arrows indicated neo-islet tissue in the subcutaneous site. Intense vascular networks were recognized within and around the neo-islet tissues (A, E, and F, *arrowheads*). Scale bars=50 μm (A, E, and F) and 100 μm (B–D). PECAM-1, platelet-endothelial cell adhesion molecule-1.

Functional Confirmation of the Engineered Neo-Islet Tissues

To further confirm the functionality of the engineered neo-islet tissues in vivo, intraperitoneal glucose tolerance tests (IPGTTs) were assessed at day 30. After administration of the glucose into the peritoneal space, the blood glucose levels of the control diabetic SCID mice were immediately elevated at over 500 mg/dL and remained above 350 mg/dL at the end of the experiment (150 min; Fig. 3). In contrast, the blood glucose levels of the recipient SCID mice and nondiabetic naive SCID mice showed temporal elevations at 15 and 30 min and thereafter showed sharp declines and returned to the normal levels (Fig. 3).

Detection of Rat-Specific C-Peptide in the Recipient Mice

Because C-peptide is species-specific and is produced during the cleavage step from the catalysis of proinsulin, we measured the serum levels of rat-specific C-peptide. As shown in Figure 4A, significant amount of rat C-peptide was detected only in the islet cell sheet-transplanted SCID mice. Accordingly, significantly high insulin levels were detected in the blood samples of recipient SCID mice compared with those of sham-operated control diabetic mice. There was no statistical significance in the blood insulin levels between the graft recipient SCID mice versus normal SCID mice (Fig. 4B). These findings confirmed that the phenotypic correction of the diabetic status was due to the de novo production of rat insulin from the engineered neo-islet tissues.

DISCUSSION

This study describes a novel tissue engineering approach that uses dispersed islet cells to form a contiguous

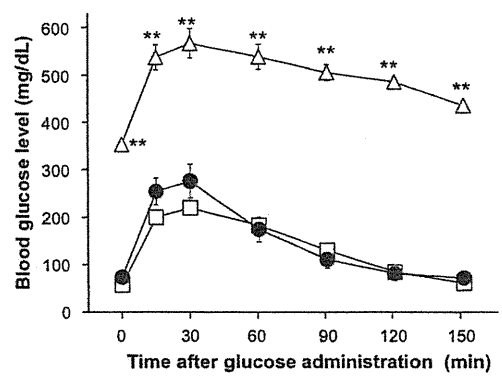

FIGURE 3. IPGTT was performed at day 30 of the experiment in the diabetic SCID mice. Diabetic SCID mice were either sham-operated (*triangle*; n=5) or transplanted with islet cell sheets (*circle*; n=8) and at day 30 after the procedure, IPGTT (2 mg glucose/g body weight) was performed. As a control, the effects on the IPGTT in the diabetic SCID mice were compared with normal SCID mice (*square*; n=5). ***P* less than 0.01 vs. the other two groups analyzed by ANOVA followed by Games-Howell post hoc test. IPGTT, intraperitoneal glucose tolerance test; ANOVA, analysis of variance; SCID, severe combined immunodeficiency.

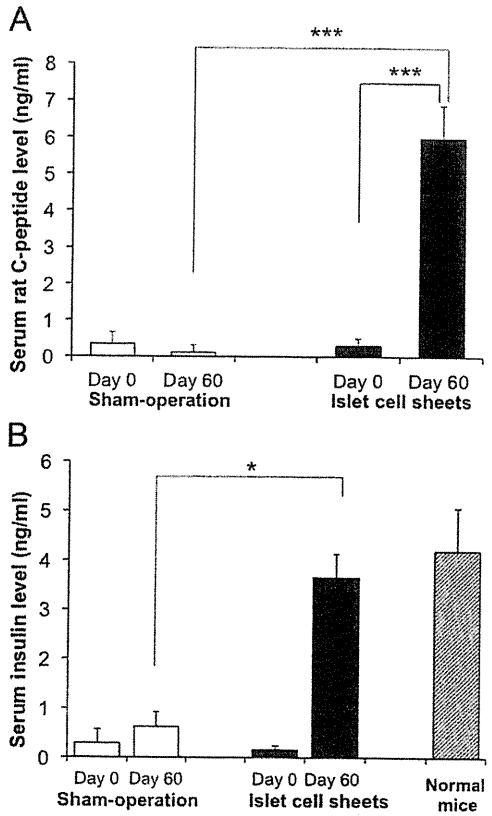

FIGURE 4. Serum levels of rat-specific C-peptide (A) and insulin (B) in the islet cell sheet-transplanted SCID mice. Serum samples were obtained from the normal (*hatched bar*; n=8) and diabetic SCID mice either 2 days before the transplantation of the islet sheets (*black bar*; n=13) or sham operation (day 0; *white bar*; n=6) and 60 days after the surgical procedure (day 60). **P* less than 0.05 and ****P* less than 0.001 vs. all the other groups except for the normal SCID mouse group using ANOVA followed by Games-Howell post-hoc test. ANOVA, analysis of variance; SCID, severe combined immunodeficiency.

monolayer sheet that can be readily used for transplantation into ectopic sites for the production of therapeutic proteins necessary for the treatment of diabetes mellitus. In recipient diabetic SCID mice receiving the transplanted islet cell sheets, normal levels of blood glucose were restored, and high serum levels of rat-specific C-peptide were detected. This strongly demonstrates that engineered neo-islet tissues were capable of producing and secreting insulin into the systemic circulation. The functionality of the engineered neo-islet tissues was further confirmed by the IPGTTs. The ability of the de novo engineered neo-islet tissues to sense and release insulin was likely attributed to the formation of a highly vascular network within and surrounding the transplanted tissues.

An important feature of our approach to engineer islet cell sheet in vitro is the use of a temperature-responsive poly(*N*-isopropylacrylamide) (PIPAAm)-grafted dishes (*21, 22*). A simple lowering of the incubator temperature allows for easy detachment of the cultured cell sheet without the use of harmful proteolytic enzymes. This enables us to harvest the cell sheets as a contiguous monolayer that retains its native intercellular communications and intracellular microstructures, which are essential for normal cellular function (*11, 19, 21*). These interwoven cells exhibited the formation and maintenance of desmosome structures during the 2-day culture period (*20*). In addition, numerous secretion granules were observed throughout the cytoplasm of the islet cells within the sheets (*20*). The detection of structural elements found in normal intact islet tissue is promising for the use of this approach in producing insulin in vivo.

It is interesting, however, the differences in the survivability between individually dispersed islet cells compared with our engineering cell sheet format. Although both the dispersed cells and our newly formed tissue sheets are capable of engrafting and functioning within the transplantation site, our data would suggest that the tissue sheet format can exhibit prolonged survivability compared with individually dispersed islet cells and/or clusters. One possible explanation is that islet cells are prone to progressing toward apoptotic cell death pathways once they are dispersed into single cells (*19, 23–26*), so it may be plausible that the islet cells within the cell sheet format may be protected from entering the cell death pathway and allow for the prolonged survival upon transplantation.

Another possibility may be the close proximity of the islet cells to the vascular network that is formed during the tissue engineering process. Researchers have reported that the diffusion-based oxygen supply is limited to 50 to 100 μm distance from the vascular channel (*27*). As islets are a clustered mass of cells made of approximately 3000 cells with a diameter of 50 to 400 μm, it is possible that the diffusion of oxygen is severely limited to only a small proportion of cells that are located at a proximal surface to the active vascular network. Considering the fact that the subcutaneous space is not actively vascularized, individual cells clustered in a ball may not be adequately perfused with nutrients. A number of researchers have designed vascularized platforms within the subcutaneous space in hopes of enhancing the islet survival time (*28, 29*). On the other hand, our monolayered cell sheet array may not be limited by the diffusion of gases for their survival in the absence of vascularized networks. It may also be possible that islet cell sheet has a high ability to recruit vascular endothelial cells, which results in the creation of an active vascular network. We speculate this latter possibility because of our findings that numerous PECAM-1-positive cells were detected around the neo-islet tissues at an early phase (day 4 after transplantation). It is, therefore, important to note that the present islet cell sheet-based approach does not require such preparation of vascularized platform but results in engineering functional islet tissues.

Another key benefit in the development of the islet cell sheet technology for transplantation into ectopic subcutaneous sites is its relatively minimal invasiveness to the patient and can be performed under local anesthesia. Moreover, accessibility to the transplanted site would be a simple procedure in cases where: (1) subsequent biopsies are needed to examine the engraft and differentiation of the islet cell sheets or (2) additional transplantation procedures can be performed to increase the therapeutic efficacy. However, to advance our current methodology toward the clinics, there are several remaining issues that need to be addressed. One of the major ones is the prevention of the transplanted cells from host immunologic allosensitization. Recently, cotransplanta-

tion of islets with Sertoli cells has shown to have immunoprotective effects on the islets from allogenic immune responses (*30, 31*). In response to this issue and these recent findings, we have attempted to engineer a temperature-responsive culture surface that would enable us to pattern a monolayer structure using multiple types of cells (*32, 33*). Using this type of culture system may facilitate the production of a monolayer islet cell sheet interwoven with Sertoli cells. Another important factor to consider is the size of the transplant area in the human patients. Considering the fact that there is a big difference in the body size between mice and human beings, a larger transplant area of neo-islet may be required to achieve therapeutic effects. One potential resolution for this issue would be to create more complex and multilayered stratified tissues within a confined space. Toward this goal, our group has attempted to create a stamp manipulator system that allows us to precisely stratify multiple cell sheets within a single confined site (*32, 34*). By integrating the cellular biology with the new tissue culture technologies, therapeutic targets may be treatable in the foreseeable future by using this multilayering approach.

As this methodology advances toward clinical consideration as a viable methodology to treat human disease, we have to be cognizant that the availability of islet cells will remain a limited resource. We found that a relatively small number of cells ($\sim 3.3 \times 10^6$ islet cells) were needed to provide a state of persistent normoglycemia in mice, but further studies are needed to determine what the minimal number of cells are needed to maintain therapeutic efficacy. However, islet cell can be lost during the islet dispersion process and cell attachment process (*35*). For these reasons, further refinements in the cell culture methodologies are needed to optimize islet cell sheet engineering with the minimal number of cells isolated from donor samples.

In conclusion, we have experimentally succeeded in reverting the hyperglycemic state of a mouse model of diabetes mellitus by de novo engineering islet tissues in an ectopic subcutaneous space. In recent years, considerable efforts had been made in generating insulin-producing cells from other cellular sources, including embryonic stem cells (*36, 37*), or induced pluripotent stem cells (*38*). Because these cell-generation processes are normally conducted under the culture condition, our cell sheet engineering approach could contribute in advancing the regenerative medicine using these newly generated cells.

MATERIALS AND METHODS

Animals

Male Lewis rats (LEW/CrlCrlj, 8 to 12 weeks old; Charles River, Yokohama, Japan) were used as islet donors. Male SCID mice (C.B-17/Icr-scid/scidJcl, 7–10 weeks old; CLEA, Tokyo, Japan) were used as graft recipients. All animal studies were performed in accordance with the institutional guidelines.

Islet Isolation and Single Cell Purification

Pancreatic islets were isolated from Lewis rats as described elsewhere (*20, 39, 40*). Islets were subsequently cultured in Roswell Park Memorial Institute 1640 medium (Sigma, St. Louis, MO). The next day, islets were dispersed using Trypsin–EDTA (Invitrogen, Carlsbad, CA) to obtain single cells (*41*).

Islet Cell Culture on the Temperature-Responsive Culture Dish and Recovery of Islet Cell Sheet

Dispersed islet cells were cultured to engineer monolayered islet cell sheet as described previously (*20*). In brief, temperature-responsive culture dishes were created by covalently grafting PIPAAm by electron beam irradiation, and this surface was subsequently coated with rat laminin-5 (Millipore, Billerica, MA). A previous study confirmed that the grafted PIPAAm remained on the dish side during cell sheet harvesting process, and thus PIPAAm does not attach to the cell sheet side (*42*). Dispersed islet cells were plated at a density of 0.57×10^6 cells/cm^2 on 35-mm dishes. When the cultured islet cells reached confluency at day 2, the cultured cells were detached from the PIPAAm dish as a uniformly connected tissue sheet by lowering the culture temperature to 20°C for 30 min.

Induction of Diabetic Status and Neo-Islet Tissue Engineering Procedures

SCID mice were rendered diabetic by intraperitoneal injection of streptozotocin (Sigma; 0.22 mg/gram body weight). Only SCID mice that exhibited NFBG values more than 350 mg/dL for 2 consecutive days were categorized as diabetic mice. Islet cell sheets were recovered with the support membrane for transplantation into the subcutaneous site as previously described (*11, 20*). To transplant the islet cell sheets, an L-shaped skin incision in the left dorsal skin region was exposed. After a 5-min attachment period, the support membrane was carefully removed. An additional layer of islet cell sheet was then transplanted on top of the first sheet. Cell counting evaluation revealed that two layers of islet cell sheet were made up of $3.3 \pm 0.1 \times 10^6$ islet cells (n=5).

Validation of Therapeutic Effects of Engineering Neo-Islet Tissues

Blood samples were periodically obtained by tail snipping to assess NFBG. At day 60, serum samples were obtained to measure rat-specific C-peptide levels and rat nonspecific insulin levels using enzyme-linked immunosorbent assay kits from Wako (Osaka, Japan) and Shibayagi (Gunma, Japan), respectively. For two recipient mice at either day 7 or 21, subcutaneous neo-islet tissues were excised by removing the portion of the surrounding abdominal wall and adjoining skin areas.

Intraperitoneal Glucose Tolerance Tests

The functionality of the newly engineered neo-islet tissues was evaluated in vivo by performing IPGTTs at day 30. After 18 hr of fasting, the mice received intraperitoneal inoculation of a glucose solution (2 mg/g body weight).

Histological and Immunohistochemical Analyses

At day 60, subcutaneous tissues were harvested and fixed in 10% buffered formalin. Specimens were paraffin-embedded and sectioned (5 μm thick) for hematoxylin-eosin staining and immunohistochemical staining. For immunohistochemical analyses, sections were incubated overnight at 4°C with either anti-rat insulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-rat glucagon (Progen, Heidelberg, Germany) followed by secondary antibody labeling. Visualization of the immune complexes was performed by incubating with 3,3′-diaminobenzidine. Sections were also used for immunofluorescence analysis of rat insulin and PECAM-1 by incubating with rabbit anti-rat insulin antibody (Santa Cruz Biotechnology) and goat anti-rat PECAM-1 antibody (Santa Cruz Biotechnology). Secondary antibody labeling was performed using Alexa-Fluor-594-conjugated anti-rabbit immunoglobulin (Invitrogen) and Alexa-Fluor-488-conjugated anti-goat immunoglobulin. The slides were mounted with mounting media containing 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen).

Statistical Analyses

All of the values calculated were provided as mean±standard error. The Student's *t* test was used for comparison between two groups. When the data set did not have equal variance, Mann-Whitney *U* test was used. When more

than two groups were compared, an analysis of variance was performed followed by Games-Howell post hoc test. A probability value of P less than 0.05 was considered statistically significant.

ACKNOWLEDGMENTS

The authors thank Ms. K. Kanegae (Tokyo Women's Medical University), Ms. H. I and Ms. Y. Kikuta (Fukushima Medical University) for technical assistance, Mr. H. Watanabe (CellSeed) for providing the modified temperature-responsive culture surfaces, and Dr. F. Park (Medical College of Wisconsin) for his editorial assistance of this manuscript.

REFERENCES

1. Merani S, Toso C, Emamaullee J, et al. Optimal implantation site for pancreatic islet transplantation. *Br J Surg* 2008; 95: 1449.
2. Bennet W, Groth CG, Larsson R, et al. Isolated human islets trigger an instant blood mediated inflammatory reaction: Implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Ups J Med Sci* 2000; 105: 125.
3. Shapiro AM, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* 2006; 355: 1318.
4. van der Windt DJ, Bottino R, Casu A, et al. Rapid loss of intraportally transplanted islets: An overview of pathophysiology and preventive strategies. *Xenotransplantation* 2007; 14: 228.
5. Balamurugan AN, Bottino R, Giannoukakis N, et al. Prospective and challenges of islet transplantation for the therapy of autoimmune diabetes. *Pancreas* 2006; 32: 231.
6. Roep BO, Stobbe I, Duinkerken G, et al. Auto- and alloimmune reactivity to human islet allografts transplanted into type 1 diabetic patients. *Diabetes* 1999; 48: 484.
7. Fox IJ, Schafer DF, Yannam GR. Finding a home for cell transplants: Location, location, location. *Am J Transplant* 2006; 6: 5.
8. Pileggi A, Molano RD, Ricordi C, et al. Reversal of diabetes by pancreatic islet transplantation into a subcutaneous, neovascularized device. *Transplantation* 2006; 81: 1318.
9. Kawakami Y, Iwata H, Gu Y, et al. Modified subcutaneous tissue with neovascularization is useful as the site for pancreatic islet transplantation. *Cell Transplant* 2000; 9: 729.
10. Hussey AJ, Winardi M, Wilson J, et al. Pancreatic islet transplantation using vascularised chambers containing nerve growth factor ameliorates hyperglycaemia in diabetic mice. *Cells Tissues Organs* 2010; 191: 382.
11. Ohashi K, Yokoyama T, Yamato M, et al. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. *Nat Med* 2007; 13: 880.
12. Yokoyama T, Ohashi K, Kuge H, et al. In vivo engineering of metabolically active hepatic tissues in a neovascularized subcutaneous cavity. *Am J Transplant* 2006; 6: 50.
13. Ohashi K, Waugh JM, Dake MD, et al. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. *Hepatology* 2005; 41: 132.
14. Kawakami Y, Iwata H, Gu YJ, et al. Successful subcutaneous pancreatic islet transplantation using an angiogenic growth factor-releasing device. *Pancreas* 2001; 23: 375.
15. Dufour JM, Rajotte RV, Zimmerman M, et al. Development of an ectopic site for islet transplantation, using biodegradable scaffolds. *Tissue Eng* 2005; 11: 1323.
16. Blomeier H, Zhang X, Rives C, et al. Polymer scaffolds as synthetic microenvironments for extrahepatic islet transplantation. *Transplantation* 2006; 82: 452.
17. Vériter S, Mergen J, Goebbels RM, et al. In vivo selection of biocompatible alginates for islet encapsulation and subcutaneous transplantation. *Tissue Eng Part A* 2010; 16: 1503.
18. Ohki T, Yamato M, Murakami D, et al. Treatment of oesophageal ulcerations using endoscopic transplantation of tissue-engineered autologous oral mucosal epithelial cell sheets in a canine model. *Gut* 2006; 55: 1704.
19. Shimizu T, Sekine H, Isoi Y, et al. Long-term survival and growth of pulsatile myocardial tissue grafts engineered by the layering of cardiomyocyte sheets. *Tissue Eng* 2006; 12: 499.
20. Shimizu H, Ohashi K, Utoh R, et al. Bioengineering of a functional sheet of islet cells for the treatment of diabetes mellitus. *Biomaterials* 2009; 30: 5943.
21. Yang J, Yamato M, Sekine H, et al. Tissue engineering using laminar cellular assemblies. *Adv Mater* 2009; 21: 1.
22. Yang J, Yamato M, Shimizu T, et al. Reconstruction of functional tissues with cell sheet engineering. *Biomaterials* 2007; 28: 5033.
23. Grossmann J. Molecular mechanisms of "detachment-induced apoptosis—Anoikis." *Apoptosis* 2002; 7: 247.
24. Thomas F, Wu J, Contreras JL, et al. A tripartite anoikis-like mechanism causes early isolated islet apoptosis. *Surgery* 2001; 130: 333.
25. Thomas FT, Contreras JL, Bilbao G, et al. Anoikis, extracellular matrix, and apoptosis factors in isolated cell transplantation. *Surgery* 1999; 126: 299.
26. Wang RN, Rosenberg L. Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *J Endocrinol* 1999; 163: 181.
27. Tsai AG, Friesenecker B, Mazzoni MC, et al. Microvascular and tissue oxygen gradients in the rat mesentery. *Proc Natl Acad Sci U S A* 1998; 95: 6590.
28. Balamurugan AN, Gu Y, Tabata Y, et al. Bioartificial pancreas transplantation at prevascularized intermuscular space: Effect of angiogenesis induction on islet survival. *Pancreas* 2003; 26: 279.
29. Fumimoto Y, Matsuyama A, Komoda H, et al. Creation of a rich subcutaneous vascular network with implanted adipose tissue-derived stromal cells and adipose tissue enhances subcutaneous grafting of islets in diabetic mice. *Tissue Eng Part C Methods* 2009; 15: 437.
30. Dufour JM, Rajotte RV, Kin T, et al. Immunoprotection of rat islet xenografts by cotransplantation with sertoli cells and a single injection of antilymphocyte serum. *Transplantation* 2003; 75: 1594.
31. Kin T, Rajotte RV, Dufour JM, et al. Development of an immunoprivileged site to prolong islet allograft survival. *Cell Transplant* 2002; 11: 547.
32. Elloumi Hannachi I, Itoga K, Kumashiro Y, et al. Fabrication of transferable micropatterned-co-cultured cell sheets with microcontact printing. *Biomaterials* 2009; 30: 5427.
33. Itoga K, Yamato M, Kobayashi J, et al. Cell micropatterning using photopolymerization with a liquid crystal device commercial projector. *Biomaterials* 2004; 25: 2047.
34. Tsuda Y, Shimizu T, Yamato M, et al. Cellular control of tissue architectures using a three-dimensional tissue fabrication technique. *Biomaterials* 2007; 28: 4939.
35. Callewaert H, Gysemans C, Cardozo AK, et al. Cell loss during pseudoislet formation hampers profound improvements in islet lentiviral transduction efficacy for transplantation purposes. *Cell Transplant* 2007; 16: 527.
36. Assady S, Maor G, Amit M, et al. Insulin production by human embryonic stem cells. *Diabetes* 2001; 50: 1691.
37. Shi Y. Generation of functional insulin-producing cells from human embryonic stem cells in vitro. *Methods Mol Biol* 2010; 636: 79.
38. Tateishi K, He J, Taranova O, et al. Generation of insulin-secreting islet-like clusters from human skin fibroblasts. *J Biol Chem* 2008; 283: 31601.
39. Carter JD, Dula SB, Corbin KL, et al. A practical guide to rodent islet isolation and assessment. *Biol Proced Online* 2009; 11: 3.
40. Sutton R, Peters M, McShane P, et al. Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation* 1986; 42: 689.
41. Peakman M, McNab GL, Heaton ND, et al. Development of techniques for obtaining monodispersed human islet cells. *Transplantation* 1994; 57: 384.
42. Akiyama Y, Kushida A, Yamato M, et al. Surface characterization of poly(N-isopropylacrylamide) grafted tissue culture polystyrene by electron beam irradiation, using atomic force microscopy, and X-ray photoelectron spectroscopy. *J Nanosci Nanotechnol* 2007; 7: 796.

Production of Islet Cell Sheets Using Cryopreserved Islet Cells

K. Ohashi, S. Mukobata, R. Utoh, S. Yamashita, T. Masuda, H. Sakai, and T. Okano

ABSTRACT

Background. To establish novel islet-based therapies, our group has recently developed technologies to create a contiguous, monolayered sheet made from freshly dispersed islet cells. Islet cell sheets generated from freshly isolated cells are easily transplantable for engraftment into subcutaneous sites in rodents. The use of a temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), grafted culture dishes with laminin-5 coating is an important feature of this process. To expand the utility of this protocol, the present study was performed to assess whether sheets generated using cryopreserved islet cells maintained viability and normal cellular phenotypes.

Methods. Dispersed islet cells obtained from Lewis rats were, cryopreserved using University of Wisconsin solution and 10% dimethyl sulfoxide. Specially coated plastic dishes were prepared by covalently immobilizing PIPAAm onto the culture plastic, followed by a coating of rat laminin-5. After 1 month of cryopreservation, the thawed cells were plated onto the PIPAAm-coated dishes.

Results. Viability of the thawed islet cells as assessed by trypan blue exclusion test was 86% ± 5%. Thawed dispersed islet cells favorably attached to PIPAAm dishes could be harvested as a contiguous cell sheet using a simple change in culture temperature conditions. Electron microscopy showed the harvested islet cell sheet to retain cell–cell connections and numerous secretion granules.

Conclusions. The present data indicated that dispersed islet cells, which were appropriately frozen and thawed, represent another viable cells source to create functional islet sheets for tissue engineering and potential clinical applications.

RECENT CLINICAL SUCCESSES in islet and hepatocyte transplantation have encouraged us to establish new cell-based approaches by bioengineering functional tissues at extrahepatic sites in vivo.[1–7] Toward this goal, our group developed a novel culture technology to bioengineer a contiguous monolayer tissue structure comprised of islet cells or hepatocytes, a cell sheet.[2,4] An important feature of this technology is the use of a temperature-responsive polymer, poly(*N*-isopropylacrylamide; PIPAAm), which is covalently immobilized onto culture dish surfaces.[5] PIPAAm has hydrophobic properties above a lower critical solution temperature (LCST) threshold of 32°C; it can become hydrophilic at temperatures below the LCST. Islet cell attachment is further improved with the additional coating of the PIPAAm surface with laminin-5.[4] At normal culture temperatures of 37°C, cells adhere to the coated surfaces, spreading as a monolayer. Reduction in temperature below the LCST results in hydration of PIPAAm allowing cells to detach from the culture dishes as an intact sheet. It is important to note that harvested islet cell sheets retain cell-to-cell intercommunications formed during the culture.[2,5] Our previous study showed that this islet cell

From the Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University (K.O., S.M., R.U., S.Y., T.M., T.O.), Shinjyuku, Tokyo, Japan, and CellSeed Inc (S.M., T.M., H.S.), Shinjyuku, Tokyo, Japan.

This work was supported in part by Special Coordination Funds for Promoting Science and Technology (K.O., T.O.), Global Center of Excellence Program (K.O.), and Grant-in-Aid (No. 21300180 to K.O.) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Address reprint requests to Kazuo Ohashi, MD, PhD, Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. E-mail: ohashi@abmes.twmu.ac.jp

0041-1345/11/$–see front matter
doi:10.1016/j.transproceed.2011.10.027

sheet is amenable for transplantation and engraftment into the subcutaneous space of rodents.[4]

Presently, clinical procedures using islet transplantation have been performed with freshly isolated islets. Considering that large purified islet masses are not routinely isolated from every pancreatic donor, it is important to establish a cryopreservation pool of islets.[8] The present study was performed to determine whether dispersed rat islet cells were efficiently cryopreserved and thawed for cultures of cell sheets as a potential therapeutic protocol.

METHODS

Islet Isolation and Single Cell Purification

Isolated pancreatic islets were purified from 8 to 12-week old male Lewis rats (Charles River, Yokohama, Japan) by static digestion of the pancreas with collagenase (Collagenase V, Sigma, St Louis, Mo, USA) followed by Histopaque (Sigma-Aldrich, St Louis, Mo, USA) density gradient centrifugation. Islets were subsequently cultured in RPMI 1640 medium (Invitrogen, Carlsbad, Calif, USA) containing 10% fetal bovine serum (FBS) overnight. The following day, islets were dissociated using Trypsin-EDTA (Invitrogen) to disperse single islet cells as described previously.[3]

Cryopreservation of Dispersed Islet Cells

For freezing, dispersed islet cells were resuspended in University of Wisconsin (UW) solution (ViaSpan, Fujisawa Pharmaceutical, Osaka, Japan) containing 10% dimethyl sulfoxide (DMSO) at a ratio of 3×10^6 cells/mL. Cells placed into 1.5-mL cryovials were placed into a Nalgene freezing apparatus (Nalge Nunc, Rochester, NY, USA) for slow freezing to −80°C. Thereafter they were stored in the vapor phase of a liquid nitrogen tank. After 4 weeks of cryopreservation, the cells were thawed by rapid immersion in a 37°C water bath. We assessed recovered cell numbers and their viability by trypan blue exclusion tests.

Islet Cell Culturing on the Temperature-Responsive Culture Dish and Recovery of the Islet Cell Sheet

Culture dishes especially created for fabrication of islet cell sheets were prepared as described previously.[4] In brief, 24-well or 35-mm culture dishes were covalently grafted with the temperature-responsive polymer PIPAAm followed by coating with rat laminin-5 (Millipore, Billerica, Mass, USA). Thawed rat islet cells were resuspended in RPMI 1640 medium containing 10% FBS for plating onto the PIPAAm dishes at a density of 0.5×10^6 cells/cm^2 for culture at 37°C. On day 1, we determined plating efficiency by counting attached versus nonattached cells. At day 3, we assessed confluency, which was expressed as the percentage of attached cells per culture surface area. On day 3, we conducted an insulin secretion assay as described previously.[4] Subsequently, the culture temperature was lowered from 37°C to 20°C for 30 minutes to harvest the cultured islet cells as a cell sheet. The temperature change initiated a natural alteration of PIPAAm resulting in spontaneous detachment of the islet cells from the culture dish surface. The supernate was removed, and the top of the cells was covered a prewet support membrane (CellSeed, Tokyo, Japan) using culture media. Together with the support membrane, islet cells were harvested as a uniformly connected cell sheet.

Electron Microscopy

Harvested islet cell sheets were fixed in 2% glutaraldehyde in 0.1 mmol/L phosphate buffer. The ultrathin sections (80-nm thickness) of the islet cell sheets were examined by transmission electron microscopy (JEOL JEM1200EX, at 80 kV).

RESULTS

For each tube, 3×10^6 dispersed islet cells were frozen. An average of $2.0 \pm 0.7 \times 10^6$ cells (average ± standard deviation) were recovered at an efficiency of 68% after cryopreservation of the cells for 4 weeks. Cellular viability of the prefrozen dispersed islet cells ranged from 82% to 94% (86.3% ± 5.3%), which was slightly higher than that of the thawed islet cells (74%–90%; mean = 80.5% ± 6.8%; Table 1). The thawed cells exhibited minimal reduction in viability (6.8% ± 4.2% reduction) during the cryopreservation and thawing processes (Table 1).

We then determined whether the dispersed, cryopreserved islet cells attached onto laminin-5-coated PIPAAm culture dishes. Plating efficiency at day 1 ranged from 78% to 91% (mean = 85.6% ± 5.5%). At day 3, the cell confluency reached >90% in all four lots (95.6% ± 3.2%; Fig 1). In marked contrast, islet cells were not able to favorably attach and expand on noncoated PIPAAm culture surfaces (Fig 1D), indicating the importance of laminin-5 for cell attachment and monolayer islet cell expansion. Insulin secretion assay at day 3 showed an increased response to glucose concentrations (Fig 1B).

At day 3 of the culture protocol, the temperature was temporarily reduced from 37°C to 20°C for 20 minutes to initiate cell detachment from the PIPAAm culture surfaces. In all four islet lots this temperature change resulted in harvest of the islet cells as an intact sheet format. Transmission electron microscopy showed that the harvested islet cell sheet was a uniformly spread monolayer with a two-dimensional tissue structure (Fig 2A). Numerous secretion granules and cell-to-cell connections (eg, desmosome junctions and gap junctions) were observed within the islet cell sheet demonstrating functional and structural characteristics as a two-dimensional neo-islet tissue (Fig 2B, 2C).

Table 1. Cellular Viability of Dispersed and Cryopreserved Rat Islet Cells

Isolation Lot No.	1	2	3	4	Average ± SD
Viability (fresh/thawed)	84/80	85/74	94/90	82/78	86.3 ± 5.3/80.5 ± 6.8
% reduction in viability	4.8	12.9	4.3	4.9	6.8 ± 4.2

Dispersed rat islet cells were cryopreserved in University of Wisconsin solution with 10% demithyl sulfoxide for 4 wk. Cellular viability was determined by trypan blue exclusion test. SD, standard deviation.

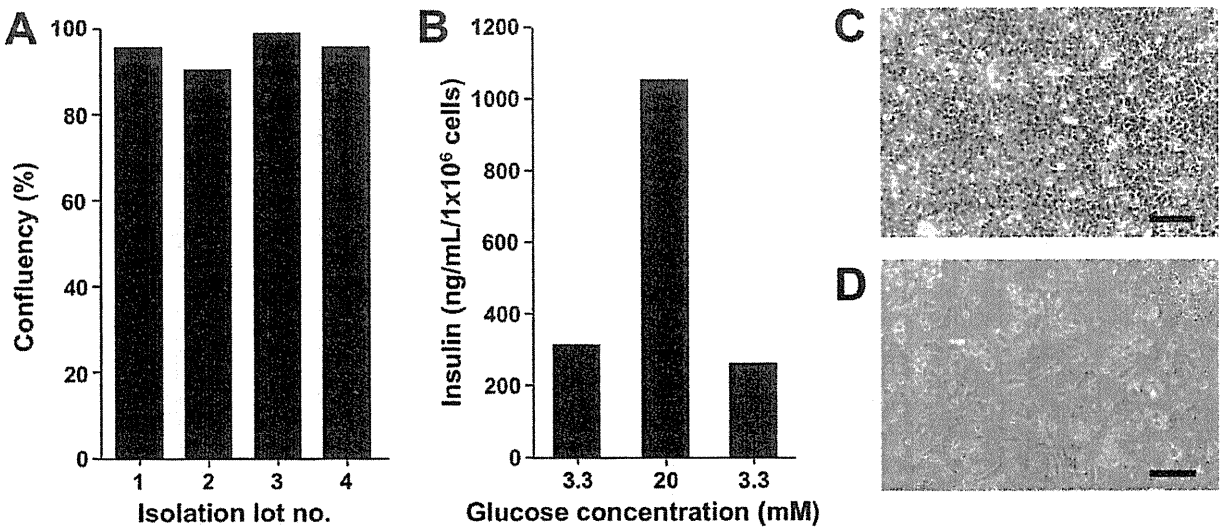

Fig 1. Cryopreserved islet cells. (**A**) Recovered islet cells were plated (0.5 × 10^6 cells/cm^2) on laminin-5-coated poly (*N*-isopropylacrylamide) (PIPAAm) dish, and cell confluency was determined at day 3. (**B**) Representative data of insulin secretion assay using cryopreserved islet cells from lot No. 3. (**C, D**) Morphology of islet cells at day 3 cultured on PIPAAm dishes coated with (**C**) or without (**D**) rat laminin-5. Scale bars = 100 μm.

DISCUSSION

The present study describes highly viable islet cells recovered following cryopreservation with minimal loss of cellular viability after being frozen, stored, and thawed. Thawed islet cells were favorably cultured on laminin-5-coated PIPAAm dishes. Furthermore, a monolayered contiguous islet cell sheet format was successfully bioengineered by a simple temperature change from 37°C to 20°C in the incubator. Similar to our previous study using freshly dispersed islet cells,[4] cryopreserved islet cells were able to form desmosomes, secretion granules, and gap junction structures within the cell sheet, suggesting that de novo formed islet sheet exhibited phenotypes consistent with biological functionality.

Unlike freshly isolated islets, the use of cryopreserved islet cells may be more attractive for eventual clinical application for a number of reasons.[8] First, a therapeutic protocol for each individual can be more flexible and not regimented to a particular schedule. Second, contamination of the islets with bacteria and fungi can be identified prior to use. Third, the number of islet cells would not be a limiting factor; it could be grown to amounts needed for each application. Last, transportation of these islet cells without any loss in viability to an institution throughout the world is possible as frozen material.

To improve islet engraftment, considerable attention has recently been paid to developing alternative approaches to conventional intraportal islet transplantation.[3,6] To support islet engraftment at extrahepatic sites, various biodegradable polymers have been applied as tissue scaffolds. However, biocompatibility needs to be

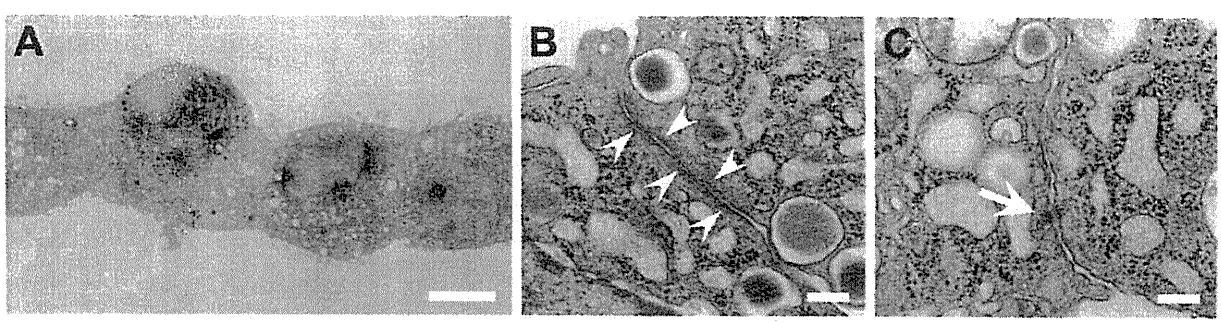

Fig 2. Transmission electron microscopy of an islet cell sheet comprised of cryopreserved rat islet cells. At day 3, cultured islet cells were harvested as a cell sheet by reducing culture temperature to 20°C for 30 minutes. (**A**) Harvested islet cell sheet was found to be a uniformly connected monolayered configuration. (**B**) Gap junctions (arrowheads) and (**C**) desmosome junctions (arrow) formed between the islet cells in the harvested cell sheet. Numerous mature dense-cored secretion granules and immature gray granules observed in the cytoplasm. Scale bars = 5 μm (**A**), 200 nm (**B, C**).

enhanced in the use of biodegradable polymers, since the degradation of polymer materials is occasionally associated with fibrosis or inflammatory responses.[5] In marked contrast, islet cell sheets bioengineered using PIPAAm dishes have been shown to be free from biodegradable polymers, since covalently grafted PIPAAm remains on the dish at the time of cell harvest. Our previous study showed that individual cell sheets could be stratified to create cell-dense three-dimensional architectures.[4,5] The therapeutic potential of the islet tissue engineering using the cryopreserved islet cells is currently under investigation, but previous studies using hepatocytes[2,7] have suggested that this type of cell sheet format will prove to have similar potential as a treatment modality.

The present study examined the efficacy of UW solution as a freezing media with 10% DMSO as a cryoprotectant for freshly dispersed islet cells, since this solution recipe has been reported to be effective for hepatocyte cryopreservation.[9] Our cryopreservation protocol has confirmed minimal loss of cell viability during the freeze/thaw process. Thawed islet cells showed attachment onto the surface of culture dishes; these cells could be bioengineered into sheets. Since our current and previous studies have shown that the cell sheet format is more effective,[2,4,5,7] we believe that the cryopreservation process to bioengineer islet cell sheets represents attractive feature for future clinical applications.

ACKNOWLEDGMENT

The authors would like to thank Dr F. Park (Medical College of Wisconsin) for his editorial assistance in the preparation of this manuscript.

REFERENCES

1. Puppi J, Strom SC, Hughes RD, et al: Improving the techniques for human hepatocyte transplantation: report from a consensus meeting in London. Cell Transplant 2011; e-pub
2. Ohashi K, Yokoyama T, Yamato M, et al: Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. Nat Med 13:880, 2007
3. Shapiro AM, Lakey JR, Paty BW, et al: Strategic opportunities in clinical islet transplantation. Transplantation 79:1304, 2005
4. Shimizu H, Ohashi K, Utoh R, et al: Bioengineering of a functional sheet of islet cells for the treatment of diabetes mellitus. Biomaterials 30:5943, 2009
5. Yang J, Yamato M, Sekine H, et al: Tissue engineering using laminar cellular assemblies. Adv Mater 21:1, 2009
6. van der Windt DJ, Echeverri GJ, Ijzermans JN, et al: The choice of anatomical site for islet transplantation. Cell Transplant 17:1005, 2008
7. Ohashi K, Tatsumi K, Tateno C, et al: Liver tissue engineering utilizing hepatocyte propagated in mouse livers in vivo. Cell Transplant 2011, in press
8. Taylor MJ, Baicu S: Review of vitreous islet cryopreservation. Organogenesis 5:155, 2009
9. Terry C, Dhawan A, Mitry RR, et al: Optimization of the cryopreservation and thawing protocol for human hepatocytes for use in cell transplantation. Liver Transpl 16:229, 2010