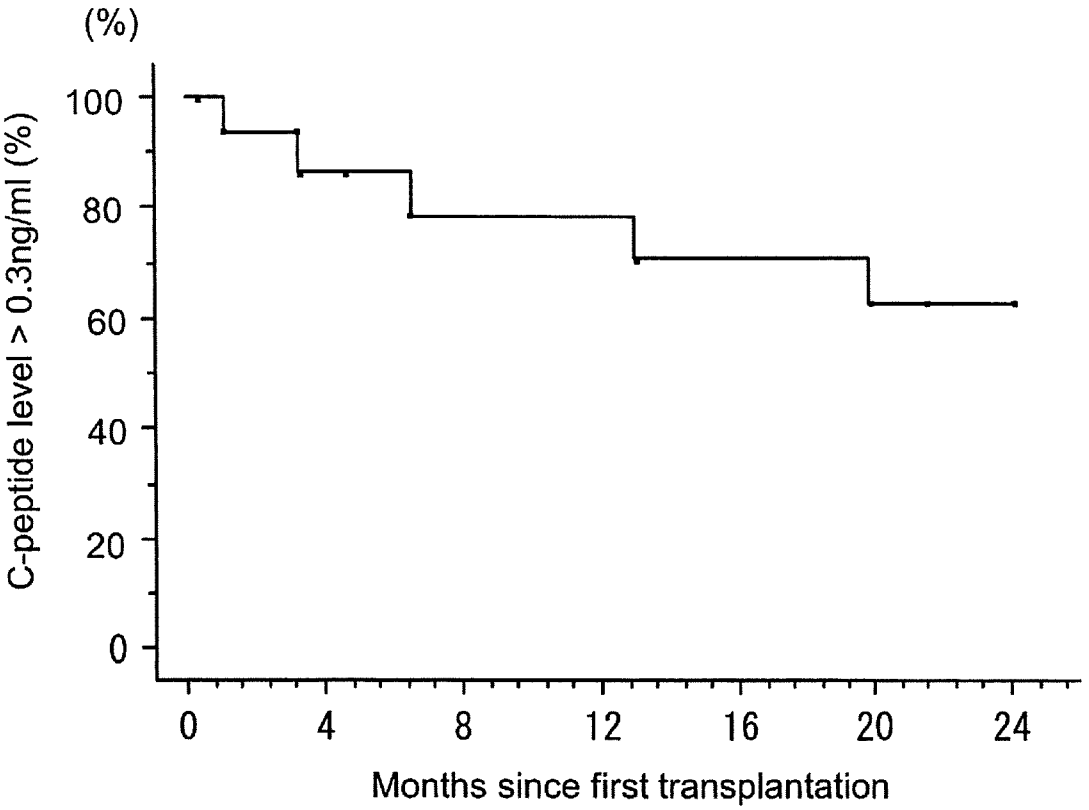

(文献(9)より引用)

図2. 膵島生着率（日本）

2007年3月までに17名に33回の移植が施行された。内訳は1回7名，2回4名，3回6名であった。それぞれの移植後1カ月におけるインスリン必要量は術前39.7±18.0 U/dayに対して，1回移植後24.2±11.0 U/day，2回移植後21.4±11.5 U/day，3回移植後21.0±7.7 U/dayと減少した。HbA1c値についても，術前8.8±1.8%に対して，1回移植後7.5±1.4%，2回移植後6.5±1.4%，3回移植後6.2±1.2%と低下した。初回移植後6ヵ月，1年，2年時における累積膵島生着率（C-ペプチド0.3ng/ml以上で判定）はそれぞれ86.5%，78.7%，62.9%であった[9]（図2）。

4．当院における膵島分離

膵島移植は，ドナーから膵臓を摘出し分離施設に運搬して（膵臓保存工程），GMP基準に則ったクリーンルーム内で膵臓から膵島を分離する必要がある。膵島分離法はRicordi法[1]が一般的に用いられている。まず，酵素（コラゲナーゼ）を膵管から注入し，温度を37℃近くまで上げることによって酵素を活性化させて膵臓を消化する（膵臓消化工程）。消化によって細かくなった膵組織から，比重勾配を利用して膵島だけを集める（膵島純化工程）。こうして分離された膵島を直ちにあるいは1，2日間培養後に，レシピエントに局所麻酔下で門脈を通して肝臓内に移植する。

膵島分離は脳死ドナー膵を用いても十分な膵島収量を安定して得ることが困難であるが，温阻血障害のかかる心停止ドナーを用いた場合，さらに難しくなることが予想された。そこで当院では心停止ドナー膵島移植を始めるに当たって，上記の作業行程のひとつひとつを細かく検討し改良を加えて心停止ドナーに特化した膵島分離法を開発した（図3）。

膵臓摘出

本邦における膵島移植のための膵臓摘出は今の

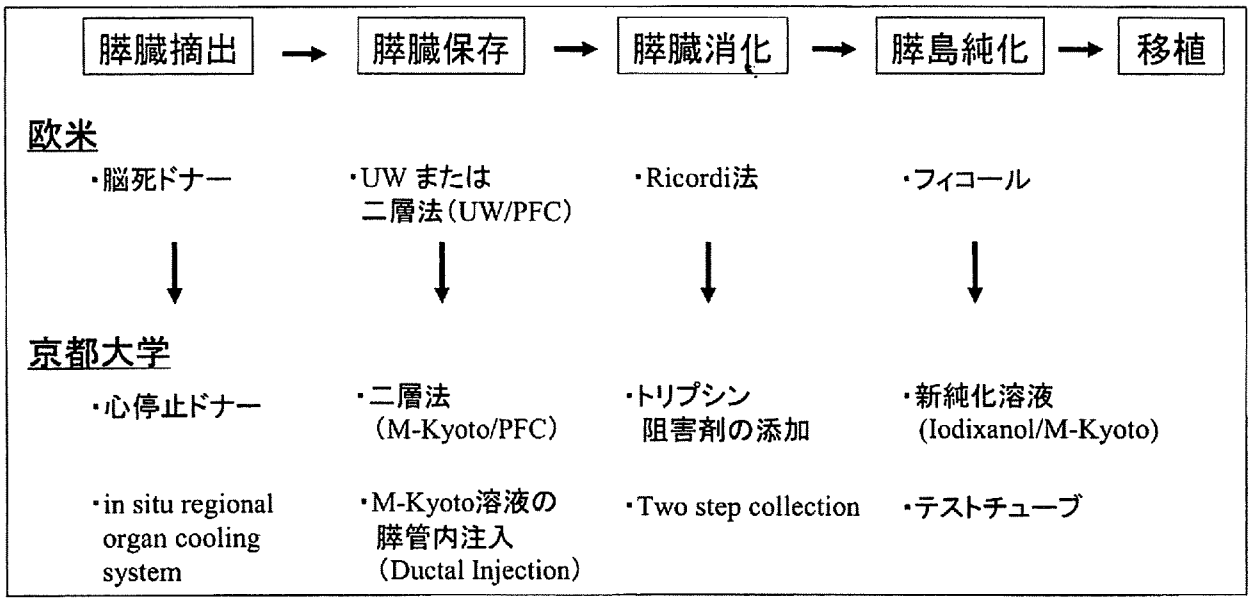

図3. 京都大学における膵島分離法の改良

ところ全例腎移植のための腎臓摘出と同時に行なっている。腎臓摘出の際には，同意が得られた場合，ドナーの臨床的脳死が確認された後にダブルバルーンカテーテルを大腿動脈から挿入し，心停止後できるだけ早く冷却液を灌流している（in situ regional organ cooling system)。膵島移植のために膵臓も同時に摘出する際にはダブルバルーンが腹腔動脈もカバーするように留置する。それによって心停止後，膵臓も速やかに冷却され温阻血時間を短縮している[10]。手術室では腎摘出が最初に行われるが，開腹直後に大網を開放し膵前面に細胞外液のアイススラッシュを入れて膵臓を冷却する。そして腎摘出終了後，膵臓を摘出する。

膵臓保存工程

膵島移植のための膵臓保存には膵臓移植と同様にUW（University of Wisconsin）液が一般的に使用されていたが，最近は黒田らが開発した二層法[11]（UW/Perfluorocarbon（PFC））が欧米でも標準的に用いられている[12]。しかしながら，UW液は①コラゲナーゼ活性を阻害，②カリウム濃度が高い細胞内液型組成である（高カリウムは膵β細胞のインスリン脱顆粒を促進し膵島障害に関係するといわれている）ことから，膵島分離前の膵臓保存にはもっと適した保存溶液があると考えられた。そこで私達は臓器保存液として開発されたET-Kyoto溶液にトリプシン阻害剤（Ulinastatin）を加えたM-Kyoto溶液という膵島移植に特化した溶液を開発した。ET-Kyoto溶液はコラゲナーゼ活性をほとんど阻害せず，カリウム濃度が低い細胞外液型組成である。Ulinastatinは膵外分泌腺から放出されるトリプシンによる自己融解を抑制し膵島を保護する[13]。まず，膵臓摘出直後にバックテーブルでトリミングの後，膵管内に膵管カニューレを挿入し，そこから膵重量1g当たり1mlの量のM-Kyoto溶液を注入し，膵管保護を行なう（ductal injection）[14]。それから膵臓をM- Kyoto / Perfluorocarbon（PFC）の二層法で保存し，当院まで搬送する。

膵臓消化工程

コラゲナーゼを膵管から注入した後，膵臓をRicordi Chamberに入れて消化を開始する。2分毎に組織をサンプリングして消化具合をモニターしている。そのサンプルの中に一つでもフリーの膵島が見られたら，灌流液中の組織の回収を始める（一次回収）。この際，遠心沈降の後にコラゲナーゼを含んだ上清は消化回路の中に戻す。この操作によって，早くにフリーとなった膵島の過消化を防ぐことができる[15]。消化組織サンプル中に

多くのフリーの膵島が見られ出したら，希釈と冷却で消化を止めながら全組織の回収を始める（二次回収）。このように二段階の組織回収を行っている（Two step collection）。

また，消化後の組織洗浄液にもトリプシン阻害剤（Ulinastatin）を加えて，膵島保護に努めている。

膵島純化工程

通常，膵島純化に使う比重勾配はフィコールで作られている。しかし，フィコールには組織障害性があるため，私達は比重勾配に造影剤であるIodixanol[16]を用いたM-Kyoto溶液ベースの純化溶液を開発した。この溶液は，エンドトキシンレベルが低いこと，また，従来の純化溶液に比べて粘度が格段に低いため遠心中に膵島組織が受ける物理的な力（せん断力）が小さくてすむことが有利な点である。

心停止ドナー膵の場合は温阻血によって外分泌組織の比重が軽くなる。そこで，6通りに比重を振ったテストチューブに少量のサンプル組織をそれぞれ入れて遠沈し，外分泌組織の沈み具合をみて高比重溶液の最適な値を決定している。

これらの方法を用いて，当院では2004年4月から2007年3月末までに25例の心停止ドナー膵を用いた膵島分離を行なった。そのうち20例（80%）が移植基準を満たした。北米の代表的な膵島移植施設での膵島分離成功率（移植率）は，脳死ドナーを用いても50%を超えていないことを考えると，分離の成功率を飛躍的に向上させたことになる。

5．当院における移植成績の検討

当院ではこれまでインスリン依存状態糖尿病患者9名に対して計19回（ドナー20名分）の移植を施行した。内因性インスリンの分泌には，24時間ほぼ一定量が出続ける基礎分泌と食事などの血糖値の上昇に対応してタイミングよく出る追加分泌があるが，移植後1ヵ月目の解析で，1回の膵島移植でインスリンの基礎分泌補充量は有意に減少することが分かった。また，血糖値の不安定性の指標であるM値（日内の血糖値が100あるいは120 mg/dlの基準値からどれぐらいかけ離れているかを示す値）とMAGE（日内の血糖値の変動幅の大きさを示す値）については有意に低下し，重症低血糖が消失した[17]。

インスリン離脱については，複数回移植を行なった6人のうち3人で達成することができた。ただし，その期間はそれぞれ215，79，14日とアルバータ大学から発表された脳死ドナーを用いた場合の長期成績と比べると非常に劣っている。

また，複数回移植症例6人中2人において，初回移植からそれぞれ30ヵ月後と50ヵ月後に移植膵島の廃絶を確認し，免疫抑制剤を中止した。1例は自己抗体がdouble positiveで，特に抗GAD抗体は大変高値であった。もう1例は，移植前のインスリン使用量が0.7U/kg以上とインスリン抵抗性が高いと考えられる症例であった。複数回移植を行なった膵島単独症例5人のうち2人において現在でもCペプチドは陽性（basal c-peptide level≧0.3ng/ml）である（それぞれ初回移植後37，39ヵ月経過）。

6．おわりに

当院におけるインスリン離脱率及び2007年に膵・膵島移植研究会 膵島移植班から報告された本邦における膵島生着率はともにアルバータ大学から発表された成績に比べると劣っている。その原因として，エドモントンプロトコールでは治験としてレシピエント適応基準が厳しく設けられていること，欧米のドナーのBMI（Body Mass Index）が大きいこと，アジア人は欧米人に比べてインスリン分泌能が低いこと，そして，心停止ドナーを用いているため膵臓が温阻血で障害されていることなどが考えられる。

移植効果については，膵島移植後1ヵ月におけるインスリン必要量とHbA1c値は，移植前に比べると明らかに減少した。従って，マージナルドナーである心停止ドナーを用いた膵島移植で重症インスリン依存状態糖尿病に対して治療効果を示

すことができたと言える。深刻なドナー不足の問題を考えると，本邦における結果は大きな意義がある。

当院では，膵臓摘出から膵島分離までの各ステップを詳細に検討し改良を加えて，心停止ドナーに特化した膵島分離法を開発した。その結果，心停止ドナーの膵臓でも高い分離成功率を得ることができた。通常膵島分離では，脳死ドナー膵を用いても十分な収量を安定して得ることが困難である。ドナー膵の有効利用の観点から，当院で開発した膵島分離法は非常に有用で、世界に発信できる技術と考えている。

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Regular Article

Larger Dosage Required for Everolimus than Sirolimus to Maintain Same Blood Concentration in Two Pancreatic Islet Transplant Patients with Tacrolimus

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: We attempted a switch of mammalian target of rapamycin (mTOR) inhibitors from sirolimus to everolimus, a derivative of sirolimus and now on the market in Japan, in two pancreatic islet transplant patients. Both patients were administered tacrolimus with sirolimus or everolimus. They had been administered 5 or 9 mg sirolimus once a day and had maintained a trough concentration of about 15 ng/mL as measured by high performance liquid chromatography with ultraviolet detection. After the switch from sirolimus to everolimus, they were given 10 or 12 mg/day of everolimus twice a day to maintain a trough concentration of 12-15 ng/mL as measured by a fluorescence polarization immunoassay (FPIA) method. Afterward, the blood concentrations of everolimus and sirolimus after the conversion were measured by high performance liquid chromatography with mass spectrometry and everolimus concentrations were found to be 5-10 ng/mL. These data show that a larger dosage is needed for everolimus than sirolimus to maintain the same trough blood concentration. Data obtained by the FPIA for everolimus should be carefully evaluated after switching from sirolimus to everolimus because of the cross-reactivity of the antibody with sirolimus.

Keywords: everolimus; sirolimus; tacrolimus; pancreatic islet transplantation

Introduction

Pancreatic islet transplantation is a critical treatment for type 1 diabetes when it is difficult to control blood glucose levels despite an optimal insulin regimen and less invasive than pancreatic transplantation. With the Edmonton protocol,[1)] results of pancreatic islet transplantation improved markedly. According to the Edmonton protocol, Kyoto University Hospital performed 17 transplantations from non-heart-beating donors for 9 patients as of the end of 2006. The first successful living-donor islet transplantation was carried out on January 19, 2005.[2)]

The Edmonton protocol consists of high-dose sirolimus (rapamycin) and low-dose tacrolimus for immunosuppression.[1)] Sirolimus suppresses the proliferation of lymphocytes by blocking growth factor-driven signal transduction through the inhibition of mammalian target of rapamycin (mTOR).[3)] In Japan, however, sirolimus is not approved by the Japanese government as an immunosuppressant. Everolimus, a derivative of sirolimus, has a shorter elimination half-life than sirolimus,[4–6)] and is expected to achieve a steady-state more quickly and adjust blood concentrations more easily. Everolimus has already been approved as an immunosuppressant in Europe and in March 2007, was approved as an immunosuppressant for heart transplant patients in Japan. Hence, we conducted a switch of mTOR inhibitors from sirolimus to everolimus in pancreatic islet transplant patients. Generally, clinical studies on everolimus in organ transplant patients have been performed with the concomitant administration of cyclosporine and steroids. There are a few reports on everolimus using tacrolimus.

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Since everolimus as well as cyclosporine and tacrolimus are metabolized by cytochrome P450 (CYP) 3A and also transported via P-glycoprotein,[7-9] pharmacokinetic interactions may vary between everolimus and tacrolimus or cyclosporine.

Here, we report pharmacokinetic differences between sirolimus and everolimus in two pancreatic islet transplant patients concomitantly administered tacrolimus. The blood concentration of everolimus was measured by fluorescence polarization immunoassay (FPIA) method as well as high performance liquid chromatography with mass spectrometry (LC/MS).

Methods

Ethics: These studies were conducted in accordance with the Declaration of Helsinki and its amendments and were approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee. Written informed consent was obtained from each patient.

Monitoring of blood concentrations for immunosuppressants: Whole blood concentrations of sirolimus (Rapamune®, Wyeth, Madison, NJ) were measured by high performance liquid chromatography with ultraviolet detection (HPLC-UV) as described previously.[10] The whole blood concentration of everolimus (Certican®, Novartis Pharma AG, Basel, Switzerland) was determined by a FPIA (Innofluor® Certican® Assay, Seradyn, Inc., Indianapolis, IN) using a TDxFLx® analyzer (Abbott Japan Co. Ltd., Tokyo, Japan).

Remnant blood samples after measurement of everolimus by FPIA were stored at −80°C. Everolimus and sirolimus whole blood concentrations were determined by a liquid-liquid extraction procedure and analysis of the extract by LC/MS in selected ion monitoring mode using atmospheric pressure chemical ionization as an interface at the laboratory of Novartis Pharma S. A. S. (Rueil Malmaison, France). Assay quantification limits were 0.3 ng/mL for everolimus and 0.5 ng/mL for sirolimus.

Cross-reactivity of sirolimus with the antibody for everolimus: To evaluate the cross-reactivity of sirolimus with the antibody for everolimus used in the assay, sirolimus was spiked in control human whole blood and sirolimus concentration was measured using FPIA for everolimus. Sirolimus concentrations were prepared at 5, 10, 20 and 50 ng/mL and tested in triplicate.

Time course study of everolimus in islet transplant patients: On the day immediately before the discharge of each patient, a time course study of everolimus was conducted. Blood samples were collected just before and 1, 2, 4, and 8 hrs after the morning administration. Whole blood concentrations of everolimus were determined using LC/MS at the laboratory of Novartis.

Results

Case report: Patient 1, a 48-year-old Japanese woman, had been treated with sirolimus and tacrolimus (Prograf®, Astellas Pharma Inc., Tokyo, Japan) after islet transplantation, according to the Edmonton protocol.[1] Thirty-six days after the transplantation, the mTOR inhibitor was converted. We called the day of conversion day 0. Both everolimus and sirolimus were administered on day 0 and only everolimus was administered after that. She kept taking tacrolimus as before (3–4 mg/day). Sirolimus was administered once a day. Everolimus and tacrolimus were administered twice daily. Blood sampling was performed once a day in the morning before the next administration of drugs. Before day 0, the whole blood concentration of sirolimus was quantified by HPLC-UV to adjust the trough concentration of sirolimus to 12–15 ng/mL. After day 0, the dosage of everolimus was adjusted to achieve a target trough blood concentration of 12–15 ng/mL as determined by FPIA. On day 0, the administration of everolimus was started at 4 mg/day, which was less than the dosage of sirolimus on day −1 (5 mg/day). Since the trough concentration of everolimus gradually decreased, the everolimus dosage was increased to 10 mg/day and the blood concentration reached the target level (**Fig. 1**, upper panel).

Patient 2, a 41-year-old Japanese woman, started the administration of everolimus 63 days after transplantation. Based on experience with patient 1, from the start, she was administered 12 mg/day of everolimus, this being greater than the dosage of sirolimus on day −1 (9 mg/day). As a result she did not experience a remarkable fall in the trough concentration of everolimus (**Fig. 1**, lower panel). During the switch from sirolimus to everolimus, she was concomitantly administered 4–6 mg/day of tacrolimus.

Neither patient showed remarkable change in tacrolimus trough concentration, which remained at 3–6 ng/mL, or had clinical complications during the study period. Neither patient was treated with potent inducers or inhibitors of CYP3A and P-glycoprotein.

Pharmacokinetic analysis: Whole blood concentrations of everolimus and sirolimus after the conversion were determined using LC/MS. After discontinuance of administration, sirolimus remained in the blood for several days (**Fig. 1**). The concentration of everolimus measured by FPIA was greater than that obtained by LC/MS, especially immediately after the conversion. To evaluate the cross-reactivity of sirolimus with the antibody for everolimus in the assay, we measured concentrations of sirolimus spiked in control human whole blood using FPIA for everolimus. As shown in **Figure 2**, the antibody for everolimus showed extensive cross-reactivity with sirolimus ([Detected as everolimus] = 1.43 + 0.47 × [Sirolimus concentration], $r^2 = 0.992$).

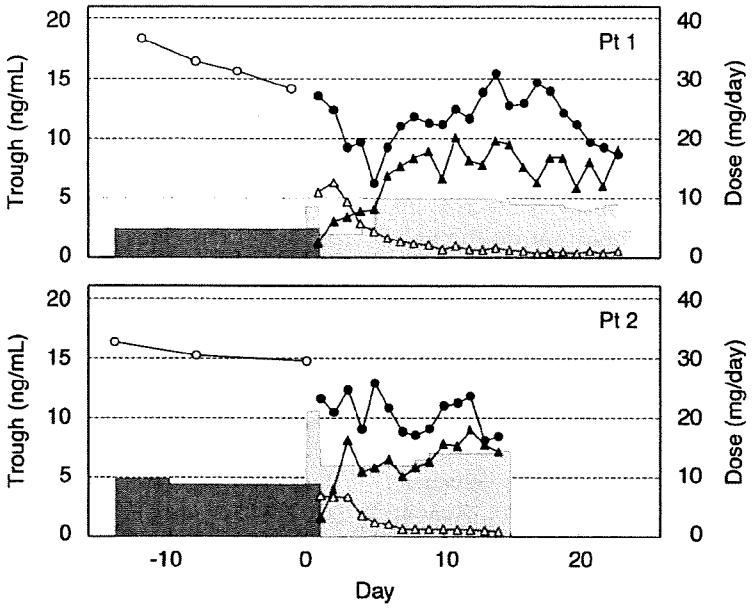

Fig. 1. Trough blood concentrations of sirolimus measured by HPLC-UV (open circles) and LC-MS (open triangles) and those of everolimus measured by FPIA (closed circles) and LC-MS (closed triangles) are plotted for each patient
Dark and light shaded areas show daily dosages of sirolimus and everolimus, respectively.

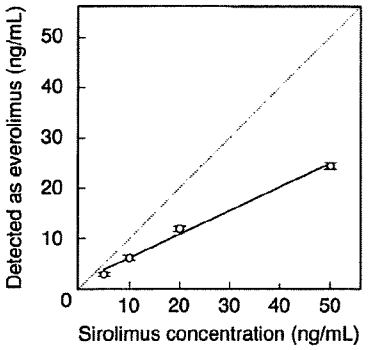

Fig. 2. Sirolimus blood concentrations measured by the FPIA method for everolimus. Each point represents the mean ± SD (n = 3)
The solid line shows the fitting line. The dotted line represents the line of identity (*i.e.*, slope = 1).

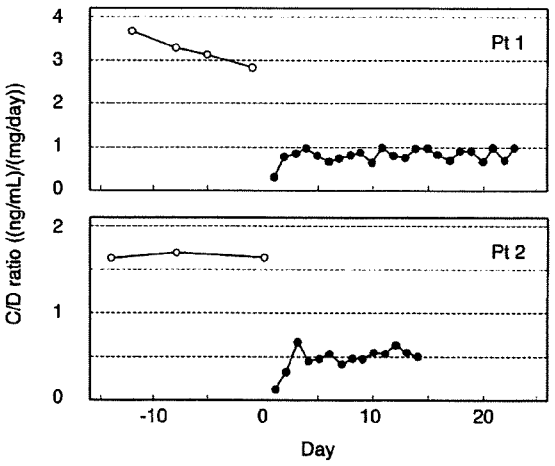

Fig. 3. The trough concentration per dose (C/D) ratios of sirolimus (open circles) and everolimus (closed circles) were plotted for each patient

Figure 3 shows the trough concentration per dose (C/D) ratio profiles of sirolimus and everolimus. C/D ratios of everolimus were calculated from concentrations determined by LC/MS and the dosage administered on the previous day. In patient 1, C/D ratios of sirolimus and everolimus were 3.26 ± 0.35 (ng/mL)/(mg/day) (mean ± standard deviation, n = 4) and 0.87 ± 0.12 (n = 22, except day 1), respectively. In patient 2, the ratios were 1.67 ± 0.03 (n = 3) and 0.52 ± 0.09 (n = 13, except day 1), respectively. In each patient, the C/D ratio of everolimus was approximately three times less that of sirolimus. C/D ratios of everolimus and sirolimus in patient 1 were twice those in patient 2.

We performed a time course study on everolimus. On day 23 for patient 1 and day 13 for patient 2. Everolimus concentration profiles measured by LC/MS are shown in **Figure 4**. Patient 1 was administered 4.5 mg everolimus and the peak concentration (17.1 ng/mL) was obtained at

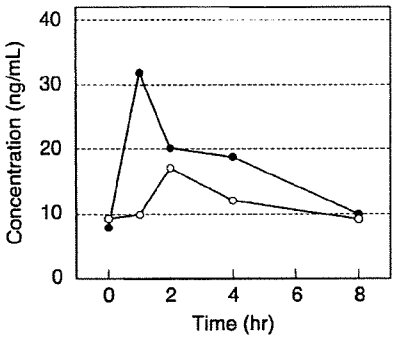

Fig. 4. Everolimus blood concentration profiles after oral administration in the two patients
Open and closed circles show everolimus concentration of patient 1 and patient 2, respectively.

2 h after the administration. Patient 2 was administered 7 mg everolimus and the peak concentration (31.8 ng/mL) was obtained at 1 h. The areas under the concentratioin-time curve from 0 to 8 h (AUC_{0-8}) calculated by the trapezoidal method were 94 and 142 ng·hr/mL in patient 1 and patient 2, respectively, while the concentrations at pre-dose and 8 h in patient 1 were nearly the same as those in patient 2, respectively.

Discussion

As shown in **Figure 1**, our patients were administered 8–14 mg/day of everolimus (with tacrolimus), and achieved trough concentrations of 5–10 ng/mL as measured by the LC/MS. Compared with other reports in which 1.5 or 3 mg/day of everolimus with cyclosporine were administered to renal transplant patients to maintain trough concentrations in a similar range,[11,12] our doses were quite large. We consider that this discrepancy mainly resulted from the difference in calcineurin inhibitor used, namely tacrolimus or cyclosporine. Everolimus as well as tacrolimus and cyclosporine are substrates of CYP3A and P-glycoprotein,[7-9] but lower blood concentrations of tacrolimus than cyclosporine in the clinical situation compared with each affinity value may have little influence on the pharmacokinetics of everolimus. Recently, Kovarik *et al.*[13] reported that the level of exposure to everolimus was 2.5 fold higher with cyclosporine than tacrolimus. It has been reported that average everolimus predose blood concentrations were significantly lower by 2.9 fold in the absence compared with the presence of cyclosporine.[12] The trough concentrations of sirolimus with cyclosporine are reported to be 1.42 times higher than those with tacrolimus.[14] Taking these findings into consideration, cyclosporine has a more profound effect on everolimus than sirolimus pharmacokinetics and our patients may need a considerably larger dosage of everolimus due to the lack of pharmacokinetic interaction with tacrolimus.

Interestingly, the C/D ratio of everolimus was three times smaller than that of sirolimus in the same patients (**Fig. 3**). Coadministration of inhibitors or inducers of CYP3A or P-glycoprorein would be expected to alter sirolimus or everolimus pharmacokinetics, but comedications in the two patients did not change during the study period. Hepatic impairment would decrease the oral clearance of sirolimus,[15] but neither patient had clinical complications such as hepatic dysfunction. Actually, the trough concentrations of tacrolimus, also metabolized by CYP3A and transported via P-glycoprotein, remained in a similar range during the conversion from sirolimus to everolimus in these patients. Therefore, we consider that a larger dosage is needed for everolimus than sirolimus to maintain the same trough blood concentration in the same patients with tacrolimus. As discussed in the previous paragraph, in the case of concomitant administration of cyclosporine, dosage of everolimus might not be so different from that of sirolimus, because of the more profound pharmacokinetic interaction of cyclosporine with everolimus compared to sirolimus. Pharmacokinetic differences between sirolimus and everolimus with cyclosporine in the same patient should be clarified in future study.

Everolimus has been reported to have a large inter-individual variability in the pharmacokinetics,[16] as also found in our cases. In the time course study, the trough concentrations of everolimus in patients 1 and 2 were similar and peak concentrations and AUC_{0-8} in patient 2 were approximately twice those in patient 1 at dosage of 7 mg and 4.5 mg, respectively (**Fig. 4**). Apparent clearance of everolimus approximately estimated by the dose-normalized AUC_{0-8} seems similar in these patients. In contrast, dose-normalized trough concentrations for everolimus and sirolimus were different as also shown in **Figure 3**. One possible reason for these findings is that the patients had different absorption profiles. In general, the recommended therapeutic range for everolimus is reported as a trough concentration of 3 to 8 ng/mL[17] and the clinical significance of AUC monitoring for everolimus remains to be elucidated.

FPIA is easy and convenient to determine whole blood concentrations of everolimus, but it is known to overestimate everolimus concentrations due to cross-reactivity of the antibody with metabolites of everolimus.[18] Actually, the everolimus concentration measured by FPIA was greater than that obtained by LC/MS over the study period (**Fig. 1**). This finding is consistent with a report using samples from renal transplant recipients.[19] In a recent report,[20] FPIA gave a positive bias of 1.2 ng/mL compared with HPLC-UV. The antibody for everolimus may cross-react with sirolimus because of the similarity in chemical structure between everolimus and sirolimus. Immediately after switching of the mTOR inhibitors, it was considered that few metabolites of everolimus were present in blood, but the values obtained were greater

with FPIA than LC/MS (**Fig. 1**). We consider the difference between the two methods to be caused by cross-reactivity with sirolimus and clarified the cross-reactivity of sirolimus with the antibody used in FPIA for everolimus (**Fig. 2**), as consistent with recent reports.[19,20] However, since the values measured by FPIA exceeded the sum of everolimus and sirolimus concentrations measured by LC/MS immediately after the conversion (**Fig. 1**), we consider that metabolites of sirolimus may also cross-react with the antibody of FPIA. These results indicate that the values of everolimus by the FPIA method should be carefully evaluated especially when transplant patients are switched from sirolimus to everolimus.

In conclusion, we report two cases of changing mTOR inhibitors from sirolimus to everolimus with tacrolimus after pancreatic islet transplantation. Each patient needed a considerably larger dosage of everolimus compared to sirolimus to maintain the same trough blood concentrations, which may be explained by lack of pharmacokinetic interaction between tacrolimus and mTOR inhibitors. The concentrations of everolimus measured by FPIA were considerably greater than those by LC/MS. These findings should provide useful information regarding the replacement of sirolimus with everolimus in transplant patients.

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Comparison of Trypsin Inhibitors in Preservation Solution for Islet Isolation

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Islet transplantation has recently emerged as an effective therapy and potential cure for type 1 diabetes mellitus. Recent reports show that the two-layer method (TLM), which employs oxygenated perfluorochemical (PFC) and University of Wisconsin (UW) solution, is superior to simple cold storage in UW for pancreas preservation in islet transplantation. Moreover, we recently reported that islet yield was significantly higher in the ET-Kyoto solution with ulinastatin (MK)/PFC preservation solution compared with the UW/PFC preservation solution in the porcine model and that the advantages of MK solution are trypsin inhibition and less collagenase inhibition. In this study, we compared ulinastatin with another trypsin inhibitor, Pefabloc, in preservation solution for islet isolation. Islet yield before purification was higher in the MK/PFC group compared with the ET-Kyoto with Pefabloc (PK)/PFC group. The stimulation index was higher for the MK/PFC group than for the PK/PFC group. These data suggest that ET-Kyoto with ulinastatin was the better combination for pancreas preservation than ET-Kyoto with Pefabloc. Based on these data, we now use ET-Kyoto solution with ulinastatin for clinical islet transplantation.

Key words: Islet transplantation; Islet isolation; MK solution; Trypsin inhibitor; Preservation solution

INTRODUCTION

Since the report of the Edmonton protocol (37), islet transplantation has advanced significantly and more than 600 type 1 diabetes patients in more than 50 institutions have undergone islet transplantation to cure their disease. The cadaveric pancreas is injured due to brain death, hypotension, and vasopressor therapy, and subsequently from warm ischemia after donor cross-clamping and cold ischemic storage. There is a clear relationship between these injuries and the reduced success of subsequent islet isolation (4,12). In Japan, pancreatic islets are isolated from non-heart-beating donors (NHBDs) for clinical islet transplantation because donations from heart-beating brain-dead donors are only two to five cases per year and most of their pancreata are used for pancreas organ transplantation. We therefore need to develop an efficient isolation technique for NHBD pancreata.

We have recently demonstrated that islet isolation and transplantation with NHBDs using the modified Ricordi method (Kyoto islet isolation method) effectively cures type 1 diabetes (23). The transplantation rate (transplantation number/isolation number) is more than 80%, higher than recently published data using brain-dead heart-beating donors (14,21,31). The isolation method includes an in situ cooling system for pancreas procurement (19), ductal injection (28), the modified two-layer method (MK/PFC) (27,30), and iodixanol-based purification (14). We previously showed that MK/PFC preservation significantly improved islet yields, compared with UW/PFC preservation (30). MK solution includes a trypsin inhibitor (ulinastatin), which is one of the advantages of this solution. Indeed, pancreas preservation using MK solution was superior to preservation with ET-Kyoto solution without the trypsin inhibitor in a rat model (30).

In this study, we compared ulinastatin with another trypsin inhibitor, Pefabloc, in preservation solution for islet isolation.

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MATERIALS AND METHODS

Preservation Solution

We used ET-Kyoto solution (5,32) with ulinastatin (Miraclid®, Mochida Pharmaceutical, Tokyo, Japan; ET-Kyoto + ulinastatin = "MK solution") or Pefabloc (Roche Applied Science, Germany; ET-Kyoto + Pefa bloc = "PK solution"). The components of the solutions are shown in Table 1.

Measurement of Trypsin Inhibition Ability of Solutions

In order to assess the trypsin inhibition of MK solution, PK solution, and ET-Kyoto solution without trypsin inhibitors (control), 3 ml of 0.3 mM *N*-benzoyl-L-arginine ethylester reagent (BAEE; Sigma, Tokyo, Japan) were incubated for 5 min at 25°C and then 5 μl of 1 mg/ml trypsin and 45 μl of each solution were added. Trypsin activity was measured by absorption spectrophotometry (λ253 nm) using BAEE for the trypsin substrate, according to a previous report (17). Absorbance was measured every minute for 6 min. A BAEE unit was defined as a change in optical density of 0.001/min.

Porcine Islet Isolation

Porcine pancreata were obtained at a local slaughterhouse. The operation was started about 10 min after the cessation of heart beating. After removing the pancreas, we immediately inserted a cannula into the main pancreatic duct, infused each preservation solution for ductal protection, and put the pancreas into a two-layer preservation container that had one of the preservation solutions (preservation solution/PFC). Operation time was defined as the time elapsed between the start of operation and removal of the pancreas. Warm ischemic time (WIT) was defined as the time elapsed between cessation of heart beating and placement of the pancreas into the preservation solution. Cold ischemic time (CIT) was defined as the time elapsed between placement of the pancreas into the preservation solution and the start of islet isolation.

Islet isolation was conducted in accordance with the method described in the Edmonton protocol (15,16,33, 34,37). In brief, after decontamination of the pancreas, the ducts were perfused in a controlled fashion with a cold enzyme blend of Liberase HI (1.4 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN). The distended pancreas was then cut into nine pieces, placed in a sterilized Ricordi chamber, and shaken gently. While the pancreas was being digested by recirculating the enzyme solution through the Ricordi chamber at 37°C, we monitored the extent of digestion with dithizone staining by taking small samples from the system. Once digestion was completed, RPMI-1640 medium (Gibco, Carlsbad, CA) was introduced into the system, and the system was cooled to stop further digestive activity. The digested tissue was collected and washed with fresh medium to remove the enzyme. The phase I period was defined as the time between placement of the pancreas in the Ricordi chamber and the start of collecting the digested pancreas. The phase II period was defined as the time between the start and end of collection.

Islets were purified with a continuous density gradient with Iodixanol-Kyoto solution in an apheresis system (COBE 2991 cell processor, Gambro Laboratories, Denver, CO). Because Iodixanol has a lower viscosity than Ficoll, it needs less force during centrifugation, which causes less damage to islets. For the solution, low-density (density: 1.077) and high-density (density: 1.100–1.125) Iodixanol-Kyoto solutions were produced by changing the volumetric ratio of Iodixanol and Kyoto solution.

Table 1. Composition of Each Preservation Solution

	MK	PK
Na (mmol/L)	100	100
K (mmol/L)	43.5	43.5
Gluconate (mmol/L)	100	100
Phosphate (mmol/L)	25	25
Trehalose (mmol/L)	120	120
Hydroxyethyl starch (g/L)	30	30
Ulinastatin ($\times 10^3$ U/L)	100	—
Pefabloc (mg/L)	—	1000

Islet Evaluation

The crude number of islets in each diameter class was determined by counting islets after dithizone staining (3 mg/ml, final concentration) (Sigma Chemical Co., St. Louis, MO) using an optical graticule. The crude number of islets was then converted to the standard number of islet equivalents (IE; diameter standardizing to 150 μm) (35). Gross morphology was qualitatively assessed by two independent investigators scoring the islets for shape (flat vs. spherical), border (irregular vs. well-rounded), integrity (fragmented vs. solid/compact), uniformity of staining (not uniform vs. perfectly uniform), and diameter (least desirable: all cells <100 μm/most desirable: more than 10% of the cells >200 μm) (16,35). Each parameter was graded from 0 to 2 with 0 equaling the worst and 2 the best score, so that the worst islet preparations were given a cumulative score of 0 and the best a score of 10. Spherical, well-rounded, solid/compact, uniformly stained, and large islets were characterized as the best islets.

Islet viability after purification was assessed using acridine orange (10 μmol/L) and propidium iodide (15 μmol/L) (AO/PI) staining to visualize living and dead

islet cells simultaneously (3,16,35). Fifty islets were inspected and their individual viability was determined visually, followed by calculation of their average viability (16).

In Vitro Assessment of Islet Function

Islet function was assessed by monitoring the insulin secretory response of the purified islets during glucose stimulation according to a procedure described by Shapiro and colleagues (37). Briefly, 1200 IE were incubated with either 2.8 or 25 mM glucose in RPMI-1640 for 2 h at 37°C and 5% CO_2. The supernatants were collected and insulin levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Insulin ELISA kit; ALPCO Diagnostics, Windham, NH). The stimulation index was calculated by determining the ratio of insulin released from islets in high glucose concentration to the insulin released in a low concentration. The data were normalized to total protein from the cell lysate. All assessments were made in triplicate and the data (mean ± SE) were expressed as a percentage of the control values in each experiment to eliminate variables caused by differences among donor pancreata.

Recently, Goto et al. showed that the measurement of the ADP/ATP ratio correlated with transplantation outcome (8). The ADP/ATP ratio was measured to evaluate the energy status of cultured islets, using the Apo Glow™ kit (Cambrex Bio Science Nottingham Ltd., Nottingham, UK). In brief, 80 IEs were washed in PBS and then mixed with 100 µl of nucleotide-releasing reagent for 10 min at room temperature. Thereafter, 20 µl of nucleotide-monitoring reagent was added to the solution. The ATP levels were measured using a luminometer (FB 12 Luminometer, Berthold Detection Systems GmbH, Pforzheim, Germany) and expressed as the number of relative light units (RLU). After 10 min, the ADP in the solution was converted to ATP by adding 20 µl ADP converting reagent and then measured as the number of RLU. Subsequently, the ADP/ATP ratio of the islets was calculated.

In Vivo Assessment of Islet Function

Mice with severe combined immunodeficiency disease (SCID; CLEA Japan, Inc., Meguro, Tokyo) were used for the experiments. The recipients were rendered diabetic by a single injection of streptozotocin (STZ) at a dose of 220 mg/kg. Hyperglycemia was defined as a glucose level of >350 mg/dl detected twice consecutively after STZ injection. The 2000 IE pig islets obtained from each group were transplanted into the renal subcapsular space of the left kidney of diabetic SCID mice. During the 30-day posttransplantation period, the nonfasting blood glucose levels were monitored three times per week. Normoglycemia was defined when two consecutive blood glucose level measurements showed less than 200 mg/dl. No statistical differences in either pretransplantation blood glucose levels or pretransplantation body weight were observed among the four groups of mice. Mouse studies were approved by the Institutional Animal Research Committees of Kyoto University, Nagoya University, and Fujita Health University.

Statistical Analysis

Values for the data represent the mean ± SE. Two or three groups were compared by Student's *t*-test with Bonferroni correction.

RESULTS

Inhibition of Trypsin Activity

Previous reports show that trypsin inhibition with TLM preservation improves islet yields (17,30). We examined whether MK and PK solutions inhibited trypsin activity. Both solutions inhibited trypsin activity (Fig. 1) compared with ET-Kyoto solution (control) ($p < 0.01$), suggesting that these solutions could be useful in reducing trypsin activity during pancreas preservation.

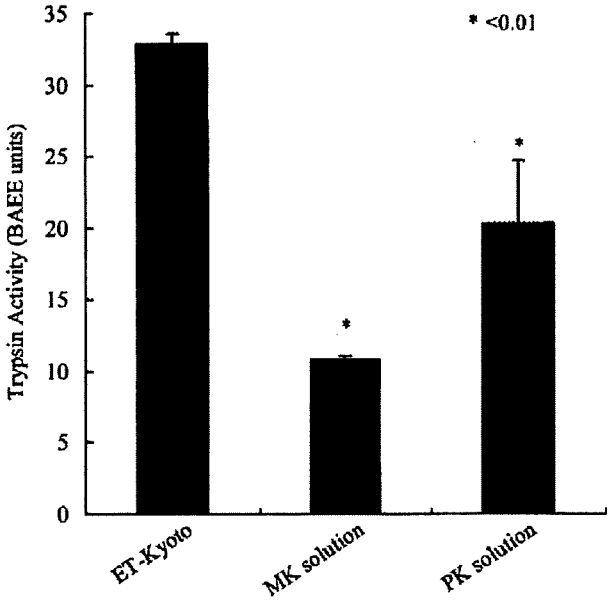

Figure 1. Impact of MK or PK solution on trypsin activity. BAEE was incubated for 5 min at 25°C and then trypsin along with MK solution ($n = 7$), PK solution ($n = 7$), or ET-Kyoto (control; $n = 7$) was added. Trypsin activity was measured by absorption spectrophotometry (λ253 nm) using BAEE reagent. Absorbance was measured every minute for 6 min. A BAEE unit was defined as a change in optical density of 0.001/min. MK and PK solutions inhibited trypsin activity significantly more than ET-Kyoto ($p < 0.01$). Data are expressed as the mean ± SE.

Table 2. Pig Islet Isolation Characteristics

	MK	PK
Pancreas size (g)	105.0 ± 8.9	87.3 ± 3.4
Operation time (min)	8.0 ± 1.0	4.3 ± 0.9
Warm ischemic time (min)	27.2 ± 1.7	25.7 ± 2.8
Cold ischemic time (min)	123.6 ± 1.6	122.3 ± 1.2
Phase I period (min)	10.2 ± 1.8	7.7 ± 1.2
Phase II period (min)	35.0 ± 3.0	29.7 ± 3.2

Data are expressed as mean ± SE.

Table 3. Pig Islet Characteristics

	MK	PK
Viability (%)	97.5 ± 0.5	96.5 ± 1.6
Score	9.2 ± 0.3	8.7 ± 0.2
Purity (%)	66.0 ± 6.8	70.0 ± 5.8
Recovery rate (%)	68.1 ± 8.4	70.5 ± 13.6
Stimulation index	2.50 ± 0.21*	1.37 ± 0.18

Data are expressed as mean ± SE.
*Stimulation index was higher for the MK/PFC group than for the PK/PFC group ($p < 0.05$).

Porcine Islet Isolation Characteristics

The characteristics of porcine islet isolation protocols are shown in Table 2. There were no significant differences in pancreas size, operation time, WIT, or CIT between the two groups. Phase I and phase II periods were also similar for the two groups.

Islet yield before purification was significantly higher in the MK/PFC group ($n = 5$) than the PK/PFC group ($n = 3$) (MK/PFC; 9676 ± 635 IE/g, PK/PFC; 6999 ± 844 IE/g, $p < 0.05$) (Fig. 2A). The islet yield after purification for the MK/PFC group was higher than the PK/PFC group (MK/PFC; 6608 ± 927 IE/g, PK/PFC; 4964 ± 1153 IE/g) but not significantly so (Fig. 2B). Other porcine islet characteristics are shown in Table 3. The stimulation index was higher for the MK/PFC group than for the PK/PFC group ($p < 0.05$). There were no other significant differences in characteristics between the two groups.

Assessment of Islet Function In Vitro and In Vivo

Recently, Goto et al. showed that the measurement of the ADP/ATP ratio correlated with transplantation outcome (8). To assess the islet graft function of each group in vitro, the ADP/ATP ratio was measured. There was no significant difference in ADP/ATP ratio between the groups (data not shown).

To assess the islet graft function of each group in vivo, 2000 IE of each group were then transplanted below the kidney capsule of STZ-induced diabetic SCID mice. There was no significant difference between the groups with respect to the attainability of posttransplantation normoglycemia (data not shown). Morphologic

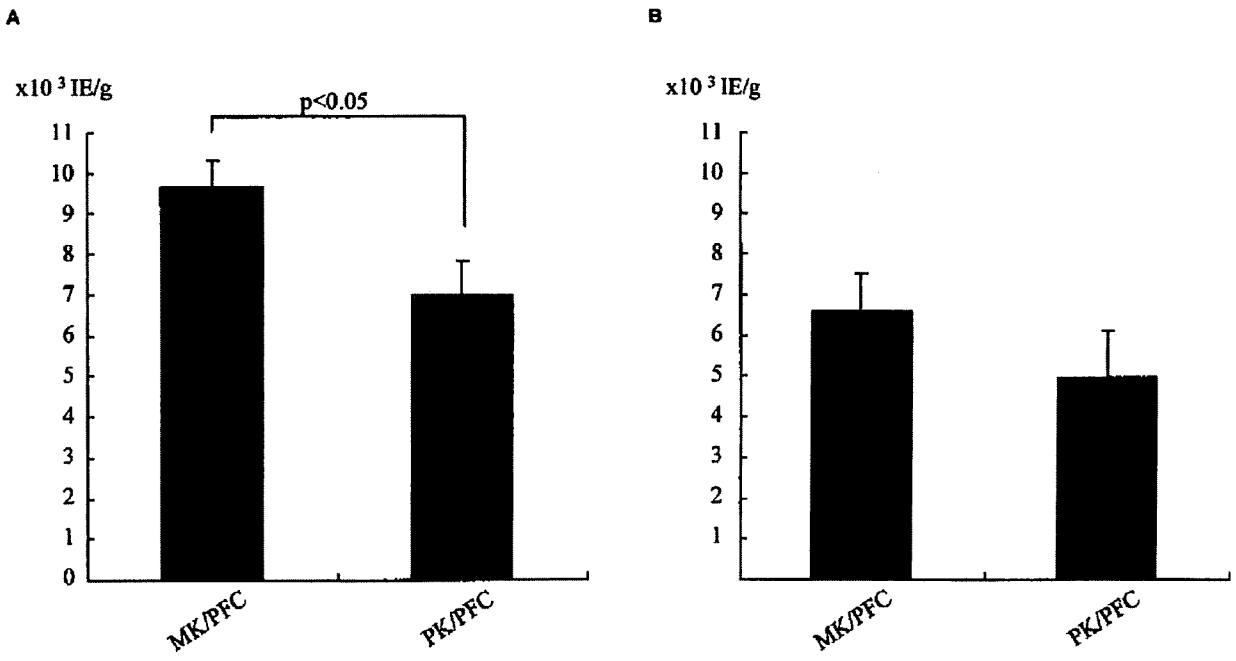

Figure 2. Islet yield before purification (A) and after purification (B). The MK/PFC group had significantly better islet yield before purification than the PK/PFC group ($p < 0.05$). Data are expressed as the mean ± SE.

studies showed the presence of islets under the kidney capsule of all SCID mice 30 days after transplantation. The islet grafts of each group in the normoglycemic mice showed intense insulin staining (data not shown).

Taken together, these data suggest that MK/PFC preservation is superior to PK/PFC preservation.

DISCUSSION

Islet allotransplantation can achieve insulin independence in patients with type 1 diabetes (37). Since the Edmonton protocol was announced, islet transplantations from brain-dead donors (9,10) as well as from non-heart-beating donors (13,14,21) and living donors (15) have been performed. These advances were based on advanced pancreas transport systems (10,16,17,30), revised immunosuppressant protocols (24,25), improved islet isolation methods (17), and enhanced islet engraftment (26). Although experiments of β-cell regeneration from stem cells have proceeded (20,22,29), there is still no reliable method for producing β-cells. Until a new method to generate β-cells is developed, improving the efficacy of islet transplantation seems the most realistic and prudent method to cure diabetes.

Donor pancreata are usually preserved with University of Wisconsin (UW) solution. Recent reports have shown that the two-layer method (TLM), which employs oxygenated perfluorochemical (PFC) and UW solution, is superior to simple cold storage in UW to preserve not only the whole pancreas but also individual islets for transplantation (16,17). However, UW solution has several disadvantages: it is chemically unstable, it must be cold stored until use, and its short shelf life makes it expensive. It is also highly viscous, which may complicate the initial organ flush (39). Recently, our university developed a new preservation solution, ET-Kyoto solution, and its effectiveness in cold lung storage has been demonstrated in clinical lung transplantation (5,32). It also is effective for skin flap storage and its clinical application is beginning in this field (40). Although high potassium in UW solution causes insulin release from pancreatic β-cells (7), ET-Kyoto solution has a high sodium/low potassium composition. Moreover, UW solution inhibits the activity of Liberase, an enzyme blend for pancreatic digestion (6,36), but ET-Kyoto solution with ulinastatin inhibits Liberase less (30).

Trypsin from pancreatic acinar cells destroys islets. Previous studies have shown that trypsin inhibition by Pefabloc during human pancreas digestion improves islet yield and reduces the fraction of embedded islets (11,17), suggesting that trypsin may degrade the ductules and thus reduce the delivery of collagenase solution to the immediate neighborhood of the islets. Previously, we demonstrated that modifying TLM preservation, by including ulinastatin, eliminated trypsin activity during pancreas preservation, and ET-Kyoto/PFC preservation without ulinastatin resulted in lower islet yields (30). In this study, we showed that MK solution was synthetically superior to PK solutions. It may be due to differences in inhibitory effects of cytokines. Ulinastatin has been shown to inhibit not only trypsin activity but also the release of neutrophil elastase. It also downregulates transcription of tumor necrosis factor mRNA, the activation of endothelial cells, and the expression of ICAM-1 induced by endotoxin in vitro (1,2,18). It has been shown that administration of ulinastatin decreased the ischemia-reperfusion injury (38) and attenuated the elevation of inflammatory cytokines and C-reactive protein, a marker of inflammation (41) in transplanted small intestine.

In conclusion, we show that ET-Kyoto with ulinastatin is a better combination for pancreas preservation than ET-Kyoto with Pefabloc. Based on these data, we now use the ET-Kyoto solution with ulinastatin for clinical islet transplantation from NHBD pancreata. MK/PFC preservation makes it feasible to use NHBDs for efficient islet transplantation into type 1 diabetes patients.

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トピックス

IV. 最近の話題
3. 膵島移植と再生医療

佐々真理子　岩永　康裕　山田祐一郎

要　旨

血糖が不安定なインスリン依存状態の患者に，内因性のインスリンを分泌する膵β細胞を補充する方法の一つとして，ドナーの膵臓から分離した膵島を用いる膵島移植がある．わが国でも2004年に開始され，生体内にある一定量の膵β細胞が存在することは，無自覚低血糖や重症低血糖の軽減などに効果が得られることが報告されている．今後，iPS細胞などから分化させた膵β細胞やブタの膵島を用いた異種移植ができれば，糖尿病患者にとって福音になるであろう．

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Key words：膵島移植，インスリン依存状態，iPS細胞

はじめに

インスリン依存状態の糖尿病では，内因性インスリン分泌が枯渇している．したがって，刻々と変動する血糖に対しては，インスリン頻回注射又はCSII(continuous subcutaneous insulin injection)療法に血糖自己測定を併用した，強化インスリン療法を用いて治療している．

しかし，外からのインスリン注射をいかに最適化しても，高血糖と低血糖を繰り返す症例がある．低血糖発作は時に生命に危険を及ぼすものとなり，インスリン依存状態の糖尿病患者における血糖管理のlimiting factorとなっている．また長期にわたる血糖コントロール不良は，糖尿病合併症の発症・進展を引き起こす．このように血糖が不安定なインスリン依存状態の患者に，内因性のインスリンを分泌する膵β細胞を補充する方法の一つとして，膵島移植がある．

1. 膵島移植とは

膵島移植とは，提供された膵臓から特殊な技術を用いて膵島を分離し（図1），インスリン依存状態の糖尿病患者の門脈へ，点滴の要領で移植を行う細胞移植療法である．移植された膵島は，門脈の末梢又は類洞に生着する．生着した膵島は，血糖値に応じてインスリンを分泌し，血糖値のコントロールを行う．

膵島移植の最大の特徴は，移植にあたって全身麻酔や手術を必要とせず，移植に要する時間も10～20分と短く，侵襲が非常に小さいことである．また，臓器移植ではリンパ節なども一緒に移植されるが，膵島移植では単離の際に，膵

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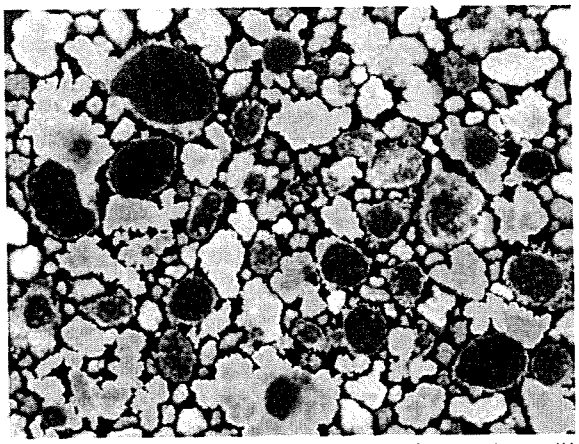
やまだ　ゆういちろう：秋田大学医学部内科学講座内分泌・代謝・老年医学分野



2005年1月生体膵島移植症例．ジチゾンで染色した単離膵島．赤く染色されているのが膵β細胞である．

図1．分離された膵島

島以外はできる限り除去されるため，感染源となりにくい．さらに，移植膵島が機能しない場合は自然に吸収され，再移植も容易である．しかし，膵島分離技術が難しいことや，拒絶反応を知るすべがないことから，最近まで普及しなかった．

2．エドモントンプロトコールと世界の膵島移植の現況

2000年にAlberta大学のShapiroらは，膵島移植の方法を確立し，7人の1型糖尿病患者に膵島移植を行い，すべての患者でインスリンから離脱することができたことを報告した[1]．この方法は，エドモントンプロトコールと呼ばれ，現在の臨床膵島移植の標準となっている．その内容とは，①膵島分離の方法を最適化し，②膵島分離後直ちに移植する，③免疫抑制薬として，耐糖能を悪化させる可能性のあるsteroidを使わず，sirolimusと少量のtacrolimusを使用する，④インスリン離脱するまで膵島移植を続ける（通常2～4回），というものである．

この方法は世界中に普及し，現在までに60以上の施設で約700人の患者が膵島移植を施行されている．最近のエドモントンの報告では，2～3人のドナーから1人の患者に移植することによって，一旦インスリン注射が不要となっても，それを持続できる症例は年々減少し，移植5年後のインスリン離脱率は7.5%であった．しかしながら膵島の生着率（C-peptideの陽性で判定）は移植5年後でも80%以上と良好である[2]（図2）．膵島が生着している患者では，血糖値の安定化が得られ，重症低血糖が消失し，HbA_{1c}も6.5～7%前後で推移している[2,3]．なお，Clinical Islet Transplantation Consortiumにより，臨床膵島移植のphase III trialが計画されており，一般医療に向けての準備が進められている．

3．本邦での膵島移植

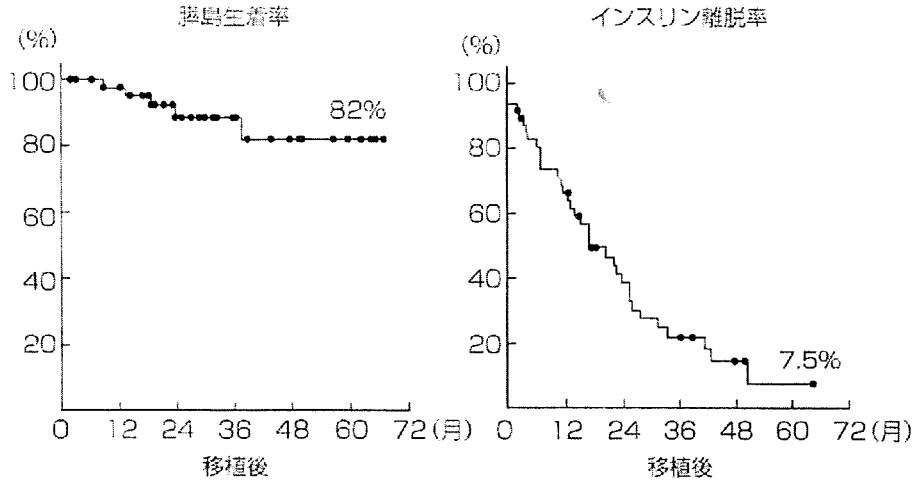
海外では，脳死ドナーから膵臓の提供を受けることが一般的であるが，本邦では臓器移植法により，脳死ドナーから提供を受けることはできないため，阻血のために膵島機能がより低下する可能性のある心停止ドナーからの提供が主体となっている．

1）心停止ドナーからの膵島移植

（1）レシピエントの適応

本邦では，膵・膵島移植研究会・膵島移植班[4]によって膵島移植の適応基準ならびに禁忌が定められている（表1）．膵島移植の適応は，内因性インスリン分泌が枯渇（高感度測定法によっても血中C-peptideが0.1 ng/ml以下で，グルカゴン負荷試験でも反応が認められない）し，糖尿病専門医の治療努力によっても血糖コントロールが困難な，75歳以下の患者であり，本人，家族，主治医の同意が得られていることである．無自覚低血糖や，第三者の介助が必要な重症低血糖の有無，低血糖の頻度などが，血糖コントロールの困難性の判定に重要である．また重度の心疾患，肝疾患，アルコール中毒，感染症，5年以内の悪性腫瘍の既往，未処置の網膜症，肥満などの禁忌事項のない患者となっている．

なお，腎不全のレシピエント候補は当初エド



(文献2より)

図2. 膵島移植の成績(エドモントン)

表1. 心停止ドナー膵島移植レシピエント条件

[適応]
1. 内因性インスリンが著しく低下し，インスリン治療を必要とする
2. 糖尿病専門医の治療努力によっても，血糖コントロールが困難
3. 原則として75歳以下
4. 膵臓移植，膵島移植につき説明し，膵島移植に関して，本人，家族，主治医の同意が得られている
5. 発症後5年以上経過していること

[禁忌]
1. 重度の心疾患，肝疾患(心移植または肝移植と同時に行う場合には考慮する)
2. アルコール中毒
3. 感染症
4. 悪性腫瘍(5年以内に既往がないこと)
5. 重症肥満(BMI 25以上)
6. 未処置の網膜症(ただし失明例は除く)
7. その他移植に適さないもの

表2. 新鮮膵島移植基準

分離膵島が以下の条件を満たすときに，新鮮膵島を移植する．
1. 膵島量5,000 IE/kg(患者体重)以上
2. 純度30%以上
3. 組織量10 ml以下
4. Viability 70%以上
5. Endotoxin 5 EU/kg(患者体重)以下
6. グラム染色陰性

(文献4より)

モントンプロトコールに則って選択しないことになっていたが，欧米で，腎移植後の膵島移植によって移植腎機能が保護されることが示され，腎移植後の膵島移植症例が増加していることを受けて，本邦でも2006年9月より腎移植後膵島移植が開始された．レシピエントの条件は，腎移植後6カ月以上経過しており，血清Cre 1.8 mg/dl以下で，直近6カ月の血清Creの上昇が0.2 mg/dl以下で，持続上昇を認めないことである．またステロイドは内服量が10 mg/日以下であることが望ましい．

(2) 申請と登録

膵島移植の申請[1]の手順は，主治医が当該患者に膵島移植の説明をして上記のような評価を行い，膵・膵島移植研究会・膵島移植班事務局に膵島移植適応判定申請書を請求してこれを作成し，「膵島移植適応判定に関する承諾書」を添えて，膵島移植班事務局に送付する．膵島移植班事務局は，膵島移植適応検討委員会に適応判定を依頼する．この際に，患者は膵島移植を受ける移植希望施設を選択する．適応検討委員会の審査の結果「適応あり」とされた場合，患者の膵島移植希望の意志を確認し，膵島移植班事務局にてレシピエント登録を行う．

現在の膵島分離・移植施設は，7施設(東北大学医学部附属病院，福島県立医科大学医学部附