表2 膵島移植の分類

1．ドナーによる分類
　① 脳死ドナー膵島移植
　② 心停止ドナー膵島移植
　③ 生体ドナー膵島移植

2．術式による分類
　1）単独移植と複合移植
　　① 膵島単独移植（islet transplant alone：ITA）
　　② 腎移植後膵島移植（islet transplant after kidney：IAK）
　　③ 膵島腎同時移植（simultaneous islet kidney transplant：SIK）
　2）門脈アプローチ法による分類
　　① 経皮経肝的アプローチ法（percutaneous transhepatic approach）
　　② 経腸間膜静脈的アプローチ法（transmesenteric approach）

圧，肝障害，リンパ球増殖症などが挙げられる．また，免疫抑制薬が過剰になると肺炎，尿路感染症，髄膜炎，皮膚または肺真菌症などの感染症が発症することがある．

10）その他

糖尿病による心血管系合併症

3．世界の膵臓移植の症例数と成績

膵臓移植は 1966 年，米国ミネソタ大学において世界で初めて行われた．国際膵臓移植登録（IPTR）によると，最近では世界で年間 1,800 件前後実施され，2004 年 6 月の時点で累積症例数は 2 万 3,000 例を超している．膵臓移植のカテゴリー別の成績（2000 年 1 月～2004 年 6 月）は，患者 1 年生存率は SPK 95%，PAK 95%，PTA 98%，グラフト 1 年生着（インスリン離脱）率は SPK 85%，PAK 78%，PTA 76% である．5 年生着（インスリン離脱）率はそれぞれ SPK 69%，PAK 58%，PTA 58% である[1]．

4．日本における膵臓移植の症例数と成績

日本では，臓器移植法施行前の 1984 年から 1994 年までに 15 例の膵臓移植が行われたことがあった．1997 年の臓器移植法施行以降は 28 例の SPK と 4 例の PAK が実施された（うち心停止ドナー移植は 2 例）．移植後の患者生存率は 100% で，グラフト血管の血栓形成（2 例）および十二指腸部分の穿孔（1 例）のため移植膵摘出に至った 3 例以外では膵臓グラフトは生着しており，腎臓グラフトは全例が生着している．

2006 年 4 月 1 日より，脳死および心停止ドナーを用いた膵臓移植は保険適応となっている．

膵島移植

1．膵島移植の分類（表2）

1）ドナーによる分類

膵島移植もドナーによって，① 脳死ドナー膵島移植，② 心停止ドナー膵島移植，③ 生体ドナー膵島移植に分類できる．海外では脳死ドナーを用いているが，日本では心停止ドナーの使用に限られている．生体ドナー膵島移植は 2005 年 1 月に京都大学で実施され，世界で初めて成功した[2]．

2）術式による分類

膵島単独移植（ITA），腎移植後膵島移植（IAK），膵島腎同時移植（SIK）がある．

通常 ITA が行われているが，これはエド

モントンプロトコールで腎機能が保たれている患者が対象となっているためである[3]．しかしながら，最近 IAK による移植腎の保護効果が注目されている[4]．SIK は 1990 年代には最も行われていた術式であるが，現在は一部の施設で行われているのみである．

膵島は通常経門脈的に肝臓内に移植される．門脈へは局所麻酔下で経皮経肝的にアプローチする方法（percutaneous transhepatic approach）と，全身麻酔下で小切開にて開腹し経腸間膜静脈的にアプローチする方法（transmesenteric approach）がある[5]．ほとんどの施設では前者が採用されている．

2．膵島移植後合併症

膵島移植は侵襲の少ない手技で行えるため，移植に関連した重篤な合併症はない．

1）門脈閉塞

移植組織量が多いと門脈閉塞の可能性が高くなる．エドモントンプロトコールでは移植組織量を 10ml 以下とし，さらにヘパリンを同時に投与することでそのリスクを減らしている．

2）肝臓の穿刺部位からの出血

percutaneous transhepatic approach において，穿刺部位からの出血の可能性がある（0～11%）[6]．このリスクを減らすために，移植後カテーテル抜去時に GELFOAM® あるいはコイルを穿刺孔に詰めたり，電気メスの凝固を使って出血を防止する策がとられている．当院では，AVITENE® を生理食塩水で溶いてペースト状にしたものを穿刺孔に詰めている（Iwanaga Y, et al: Manuscript in preparation）．

3）その他の穿刺に伴う合併症

percutaneous transhepatic approach では出血以外に，肺の近くを穿刺するため気胸を起こす可能性，あるいは誤って胆嚢を穿刺する可能性がある．

4）免疫抑制薬による副作用

免疫抑制薬の種類によってさまざまな副作用があり，標準法としてシロリムスと少量のタクロリムスを使用しているためタクロリムスの副作用は少ないが，シロリムスによる高脂血症，口腔内潰瘍，下腿浮腫，タンパク尿の発現頻度が高い．

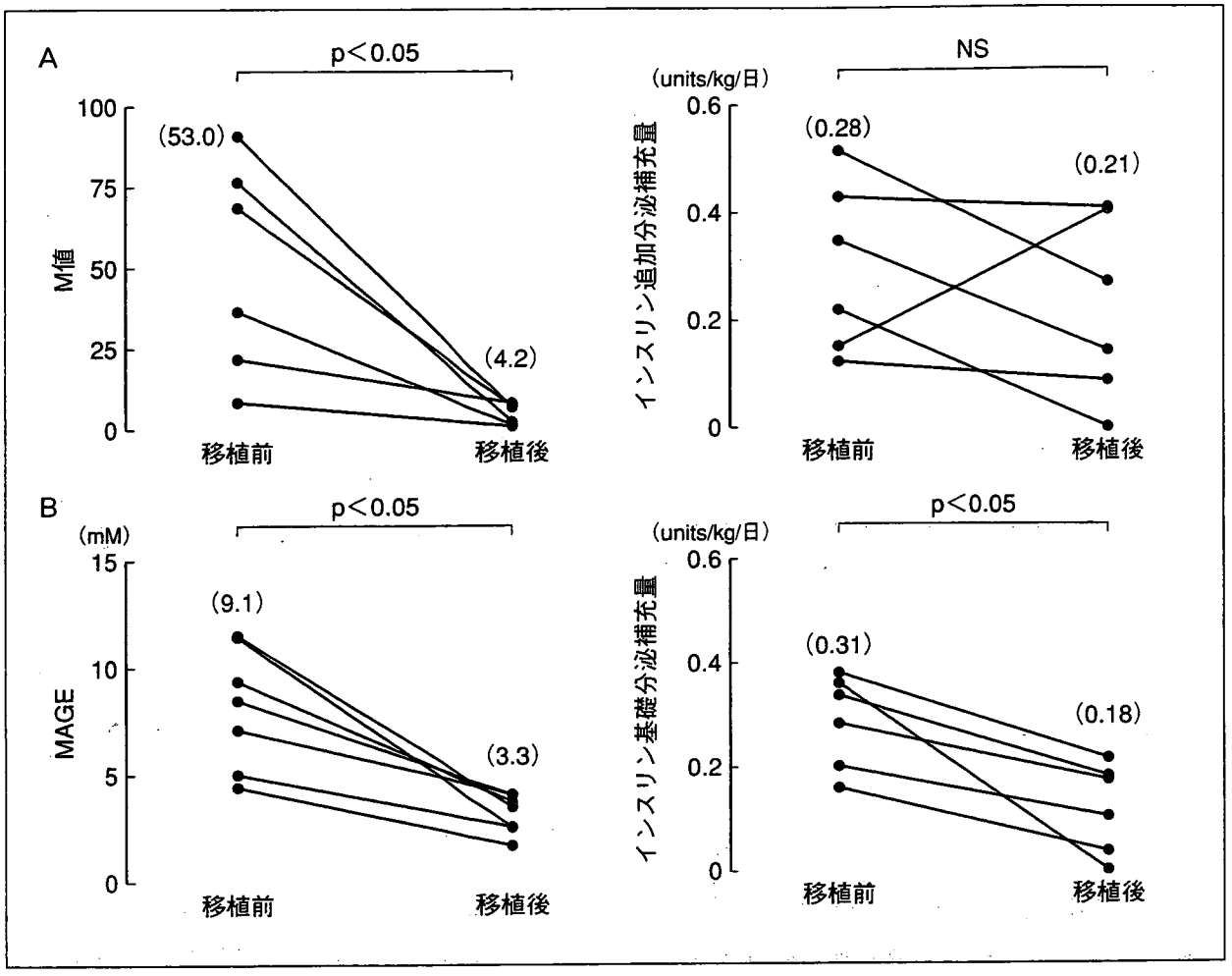
3．世界の膵島移植の症例数と成績

膵島移植は 1970 年代からごく一部の施設で実験的に行われていたが，膵島分離と免疫抑制が困難なため，当初はうまくいかなかった．ところが，2000 年にカナダのアルバータ大学からエドモントンプロトコールが発表され，移植成績が飛躍的に向上し世界中に広まった．このプロトコールが現在の臨床膵島移植の標準となっている．その主な特徴は，① 免疫抑制薬にステロイドを使わずに，シロリムスと少量のタクロリムスを使用すること，② 1 人の患者に複数回の移植を行うこと，③ 腎機能が保たれている患者を対象とすること，である[3]．現在では 60 以上の施設で 600 人以上の患者がすでに移植を受けているが，膵島移植に関連した死亡症例は 1 例もなく，高い安全性が確認されている．移植後 5 年のインスリン離脱率は約 10% と膵臓移植と比べてかなり低い．しかしながら，膵島の生着率（C-ペプチド陽性で判定）は 80% と高く，再びインスリン注射が必要となってもインスリンの基礎分泌があるため，血糖値の安定化は維持できている[7]．

4．日本における膵島移植の症例数と成績

日本では，膵島移植は組織移植の範疇に入るため脳死ドナーを用いることができず，日本組織移植学会が定めたガイドラインに基づいて心停止ドナー膵島移植を行っている．実施に際しては，膵・膵島移植研究会で定められた規則のもとで行っている．2004 年 4 月

図2 膵島移植初回後の血糖値の安定化とインスリン補充量（文献[9]より改変引用）



に京都大学で第1例目が実施され，2006年12月までに全国で15人に28回の移植が行われた．

京都大学では，臨床の膵島移植を開始するに当たって，脳死ドナーに比べて条件が悪い心停止ドナーの膵臓からでも膵島が確実に分離できる方法を開発した（Kyoto Islet Isolation Method）[8]．この方法でヒト膵島分離24例のうち20例が移植基準を満たし，83%という高い膵島分離成功率を記録している．そのうち19例を実際にインスリン依存状態糖尿病患者8人に移植したが，移植後必要インスリン量は減少し，特に複数回の移植を受けた7人中3人がインスリン治療から離脱することができた．また，1回の移植ではインスリン治療から離脱することができなくても，全症例でインスリンの基礎分泌補充量が減少し，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120mg/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）は著明に低下し，重症低血糖が消失した[9]（図2）．

我が国では，エドモントンプロトコールにのっとって腎機能が保たれている患者を対象にITAを実施してきたが，2006年9月に膵・膵島移植研究会でIAKの実施が承認された．その後2人の腎移植後患者に3回の膵島移植が行われている．この場合，基本的に腎移植の免疫抑制法を使うことになっている．SIKの実施については今のところ制度上の問題で不可能である．

膵臓移植と膵島移植

膵臓移植と膵島移植のどちらを選ぶかについては病期によるところが大きく，膵臓移植は糖尿病性腎不全（透析）患者に対して腎臓移植と同時に行われ（SPK），膵島移植は腎機能が保たれている患者が対象（ITA）となるのが一般的である．しかしながら，少数例ではあるが欧米では腎機能が保たれている患者に膵臓移植も行われ（PTA），逆に糖尿病性腎不全（透析）患者に対して SIK も行われている．そのような場合，膵臓移植と膵島移植のメリットとデメリットを十分説明したうえで，移植を受ける患者自身に選択させることになる．

展　望

昨今，外科的治療法はできるだけ侵襲の少ない方法が好まれている．膵臓移植と膵島移植に関しても，もし長期成績に遜色がなければ膵島移植が選ばれることになるであろう．そこで，膵島移植の長期成績を改善する試みが盛んに行われている．具体的には，いかに多くの膵島収量を得て，移植後生着率を上げ，長持ちさせるかである．膵島分離法の改良はすでに行われており[8]，移植後生着率については移植早期の炎症反応を抑えることが鍵であることが分かってきている[10]．また移植膵島を長持ちさせるために，デオキシスパーガリン（DSG），リツキシマブ，LEY29Y などの免疫抑制薬導入が予定されていたり，GLP-1 アナログの使用が試みられている．GLP-1 アナログはもともと2型糖尿病に対する薬剤であるが，以前よりその β 細胞増殖促進作用について研究が行われていた．そこで，膵島移植後にいったんインスリン離脱したがインスリン注射を再開した患者に GLP-1 アナログを投与したところ，再びインスリン離脱することができたとの発表がマイアミ大学からあり，移植膵島の再生による長期成績改善の可能性が示唆されている．

膵臓移植においても，さらなる成績の向上が試みられている．免疫抑制薬に関しては，糖尿病原性が少ない薬剤の導入と多剤併用による副作用の軽減が検討されている．そこには，シロリムスを導入しステロイドを中止する方法など，膵島移植での経験を参考にした移植膵内分泌機能に影響の少ない免疫抑制法の試みなどがある．

移植医療全般に言えることであるが，ドナー不足の問題は深刻である．これを解決するための1つの方法として，心停止ドナーおよび生体ドナーの膵臓が用いられている．生体ドナー膵臓移植は米国で約 140 例行われている．日本でも，2004 年1月の第1例目からこれまでに8例（SPK 5例，PAK 1例，PTA 2例）が行われた．生体ドナー膵島移植については米国ミネソタ大学で 1970 年代に2例行われたがうまくいかず，2005 年1月に京都大学で実施されたのが世界初の成功例となった[2]．生体ドナーの膵臓を用いたことで，死戦期のサイトカインストームがない，冷保存時間がほとんどない，膵体尾部は膵島分離効率が高いといったことから，高品質・高収量の膵島が得られた．レシピエントに関しては，待機手術であるため移植前から免疫抑制薬を服用することで移植時にはその血中濃度を安定させることが可能で，また厳格な血糖コントロールを術前から行っておくことで良い状態で移植膵島を受け入れることができた．また，血縁者からのグラフトなので組織適合が近いということも利点である[11]．生体ドナー移植は今後状況によって考慮されるべき方法である．

膵島移植では，ドナーの供給源としてブタ膵臓を用いた異種移植が検討されている．すでに，細胞性免疫を標的にした免疫抑制法によってブタ-サル間の異種膵島移植における

拒絶反応を制御できることが報告されている[12]．ブタをドナーとした場合，内在性ウイルスのヒトへの感染が問題となるが，さらに研究が進めば近い将来臨床応用される可能性が高いと考えられる．ほかにドナー不足の解決策として，ヒト胚性幹細胞（ES 細胞）あるいは組織幹細胞からインスリン産生細胞を分化誘導させる研究が盛んに行われているが，実用化までにはまだ多くの解決すべき課題がある．

おわりに

膵臓移植と膵島移植の現況と将来展望を概説した．膵島移植は臨床が本格的に始まったばかりではあるが，高い安全性と血糖値の不安定性に対する治療効果が確認されている．膵臓移植は高いインスリン離脱率を維持可能で，特に SPK では QOL の改善のみでなく救命・延命効果も認められ，糖尿病性腎不全に対する根治療法として定着している．現時点では，両者のメリット，デメリットを考慮して選択されるべきである．将来的には，侵襲の少ない膵島移植の技術がトランスレーショナルリサーチのプロトタイプとして，major/minor change を繰り返し発展していくことが予想される．

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Tea Polyphenol Inhibits Allostimulation in Mixed Lymphocyte Culture

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Green tea polyphenols are known to protect allogenic donor tissues from acute rejection by their recipients. This immunosuppressive effect may be generated by a unique chemical property of the major component, epigallocatechin-*o*-gallate (EGCG), which can block specific cell surface molecules of the donor tissues. To test this hypothesis, we examined the effects of EGCG on the murine mixed lymphocyte reactions. EGCG treatment of stimulator cells significantly attenuated the proliferation of responder T cells. The proliferation did not recover upon the secondary stimulations by fresh untreated cells or exogenous IL-2. Flow cytometric analyses showed that EGCG treatment decreased the staining intensities of various cell surface molecules including MHC II, which plays a major role in antigen presentation, and B7.1, B7.2, and their ligand, CD28, which are required for costimulatory signals in T-cell activation. These results suggest that an anergic state of alloreactive T cells may be induced by either weakening of antigen signaling or blockage of costimulatory signals with EGCG. Other possible mechanisms behind the immunosuppressive effect and a potential use of EGCG treatment of donor tissues in transplantation medicine are discussed.

Key words: Costimulatory signals; Allorecognition; Polyphenol; EGCG

INTRODUCTION

Studies on transplantation immunology have shown that some plant-derived chemicals can prevent transplantation-associated problems such as graft rejection and graft versus host disease (GVHD) (5,19,21). Among these chemicals, green tea polyphenols, better known as anti-cell proliferation agents, were recently found to prevent allogenic rejection of nerve transplants in rats, where immersing peripheral nerve bundles in a polyphenol solution before transplantation completely avoided rejection by the host (7). This process should involve immunological interactions between the polyphenol-treated donor tissue and the host immune cells but not a direct proliferation arrest of the latter, which is not treated with polyphenols.

The exact mechanism of this immunosuppressive effect and a specific active component of the polyphenols have not yet been defined (21). Because the polyphenols are known to bind to cell surface macromolecules (12) and impair receptor–ligand interactions (2), we hypothesized that they would attach to such cell surface molecules that are involved in antigen recognition and interfere with the recognition of a donor tissue as foreign by its recipient. In the present study, we tested this hypothesis by examining the influence of (−)-epigallocatechin-*o*-gallate (EGCG), a major green tea polyphenol component, on various parameters of allostimulation using a murine mixed lymphocyte culture (MLR) system.

MATERIALS AND METHODS

Mice

Female BALB/c and C57Bl/6 mice were purchased from Japan SLC (Shizuoka, Japan) and housed under specific pathogen-free conditions. Mice were used at the age of 9–11 weeks old. All experiments were approved by the local review board of Kyoto University and were conducted in accordance with the national and international guidelines for laboratory animal care.

EGCG Treatment Protocol

Spleens were aseptically removed from BALB/c (H-2K^d) and C57Bl/6 (H-2K^b) female mice, and cell suspen-

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sions were prepared by teasing and flushing the spleens with forceps and filtering through a 50-μm filter in Hank's balanced salt solution (HBSS) containing 1% FCS. Cells were centrifuged at 300 × *g* for 5 min, resuspended in 10 ml RPMI-1640 (Sigma-Aldrich, Tokyo, Japan) containing 0, 200, or 400 μM EGCG/10^7 cells, and incubated at 4°C for 1 h. After incubation, cells were immediately washed twice with RPMI-1640 (10% FCS) before being used in mixed lymphocyte reactions or cell surface marker analyses.

Mixed Lymphocyte Reaction (MLR)

Splenocytes derived from BALB/c mice were used as "stimulator" cells and those from C57Bl/6 as "responder" cells. In both one-way and two-way MLRs, the stimulator cells were untreated or treated with EGCG before coculture. In a one-way MLR, cells from BALB/c mice were treated with 20 ppm mitomycin C (MMC) (MP Biomedicals, Aurora, OH) in RPMI-1640 supplemented with 10% FCS at 37°C for 30 min to arrest their proliferation before EGCG treatment. In a two-way MLR, both stimulator and responder populations were left capable of responding. Each cell population was resuspended in complete medium (RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 ppm streptomycin) to a final concentration of 5.0×10^6 cells/ml. The stimulator and responder cells were mixed at a 1:1 ratio (100 μl each) in 96-well microplates and incubated triplicate at 37°C with 5% CO_2 for 72 h. Cell proliferation was assessed by counting an aliquot of harvested cells from each MLR well with a trypan-blue exclusion method.

Measurement of IL-2

After the period of MLR incubation, supernatants from the cultures were assayed for IL-2 production using cytokine quantification ELISA kits according to manufacturer's instruction (eBioscience, San Diego, CA). The detection limit of IL-2 was 10 pg/ml.

Secondary Stimulation of MLR Cultures

One-way MLR wells were separately prepared, and after 48 h of incubation the cells were recultured with MMC-treated fresh BALB/c splenocytes (5.0×10^6 cells/ml) in complete medium. Some MLR wells were cultured with exogenous recombinant murine IL-2 (1.7 IU/ml) (eBioscience). These MLR cultures were incubated for an additional 48 h, and cell proliferation was assessed as described earlier.

Flow Cytometric Analyses of Cell Surface Molecules

Flow cytometric analyses of cell surface molecules of the control and EGCG-treated splenocytes were performed using a series of fluorescence-labeled monoclonal anti-mouse CD/MHC antibodies and corresponding isotype-matched control antibodies. These antibodies are to detect CD28, CD49d, B7.1 (CD80), B7.2 (CD86), MHC I, MHC II (eBioscience), and TCRαβ (Pharmingen, San Diego, CA). The splenocytes were treated with red blood cell lysis buffer (eBioscience) for 5 min, and mononuclear cells were treated with EGCG-containing media and washed with RPMI (10% FCS) and a staining buffer (eBioscience) at 4°C. Each splenocyte sample was reconstituted in 50 μl of the staining buffer and subjected to antibody labeling for 15 min. After washing the samples twice with the staining buffer, a minimum of 10,000 events per sample was collected and analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Detection of Cell Proliferation by Flow Cytometry

To examine if the proliferation of responder T cells was attenuated by coculturing with EGCG-treated stimulator cells, cell samples from MLR cultures after 72 h were stained with FITC-labeled anti-mouse CD71 monoclonal antibody (BD Pharmingen) and with PE-labeled anti-mouse Thy 1.2 monoclonal antibody (Caltag Laboratories, Burlingame, CA) and analyzed on FACScan.

Detection of Apoptosis by Flow Cytometry

To examine whether apoptosis is induced in the responder and EGCG-treated stimulator cells in MLR culture, apoptosis was examined by flow cytometry. Cells collected from MLR plates after 24 h of incubation were washed once with PBS. The cells were stained with PE-labeled anti-mouse H-$2K^b$ monoclonal antibody (Caltag Laboratories, CA), which stains the responder cells (C57Bl/6). Then apoptotic cells were stained with FITC-labeled Annexin V according to the manufacturer's instructions (R&D Systems, MN) and analyzed on FACScan.

Statistical Analysis

All results are expressed as mean ± SD. Differences between the experimental groups of triplicate samples were analyzed by ANOVA and Fisher's PLSD using StatView (SAS Institute Inc., Cary, NC). Values of $p < 0.05$ were considered significant.

RESULTS

Mixed Lymphocyte Reaction

We first examined the effects of EGCG treatment on allorecognition of responder T cells using one-way and two-way MLR cultures. In one-way MLR, responder T cells (C57Bl/6, H2K^b) recognized untreated stimulator cells (BALB/c, H-$2K^d$) as foreign and showed substan-

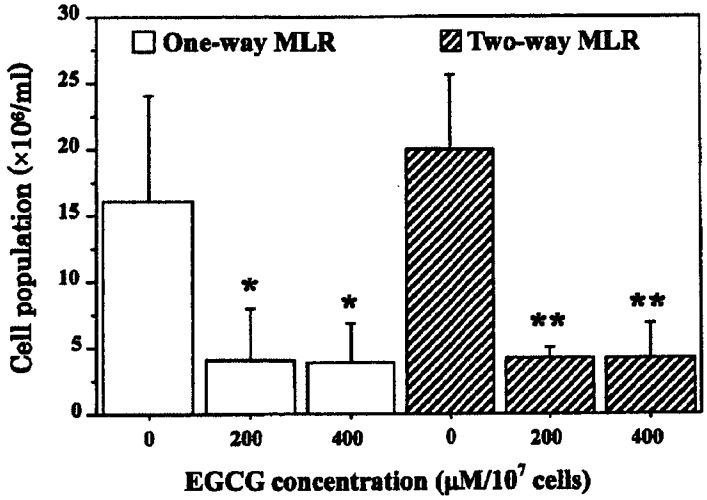

Figure 1. Effects of EGCG treatment on induction of one-way and two-way MLR. C57Bl/6 splenocytes were cocultured with EGCG-treated stimulator BALB/c splenocytes either treated (one-way MLR) or untreated with MMC (two-way MLR). Differences were significant between treated and untreated groups (*$p < 0.0001$; **$p < 0.018$).

tial proliferation (Fig. 1). The proliferation was attenuated down to 20–30% when stimulator cells were treated with 200 or 400 μM EGCG. Microscopic images of MLR cultures for these cells showed the suppressed levels of foci formation (Fig. 2). In two-way MLR, where both stimulator and responder cell populations are capable of proliferation, EGCG treatment similarly resulted in the attenuated proliferation (Fig. 1). We next examined the effects of EGCG treatment on IL-2 production in MLR cultures and found that EGCG treatment strongly reduced the production of IL-2 in one-way MLR cultures as detected by ELISA after 72 h of incubation (Fig. 3). IL-2 production was also reduced in two-way MLR cultures after EGCG treatment of BALB/c splenocytes (Fig. 3).

Detection of Cell Proliferation by Cytometry

CD71, transferrin receptor, is expressed on the cell surface of actively dividing cells and has been used as an activation marker of T-cell proliferation. To examine if the proliferation of responder T cells was attenuated by coculturing with EGCG-treated stimulator cells, cells

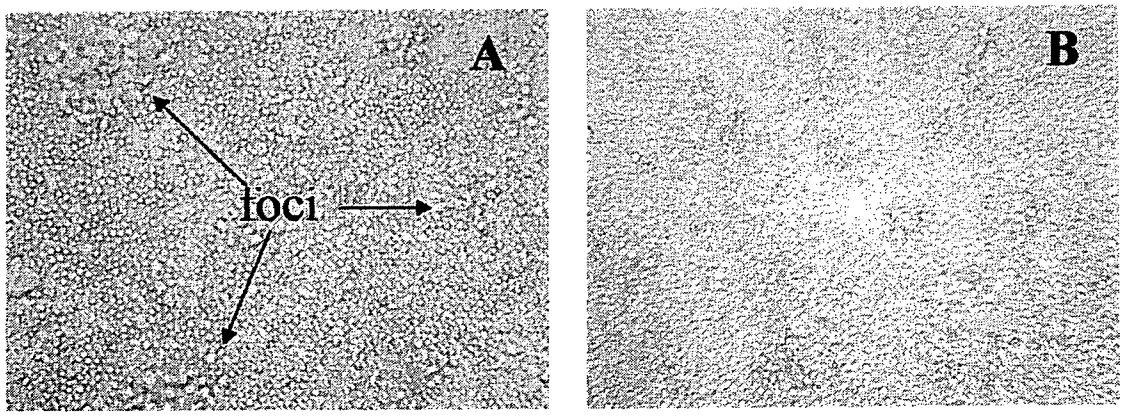

Figure 2. Foci formation of responder T cells in one-way MLR cultures. C57Bl/6 responder cells were cocultured with BALB/c simulator cells that had been either untreated (A) or treated with 400 μM EGCG (B). Microscopic images of cultured cells in a flat-bottomed 96-well plates were photographed after 72 h of incubation.

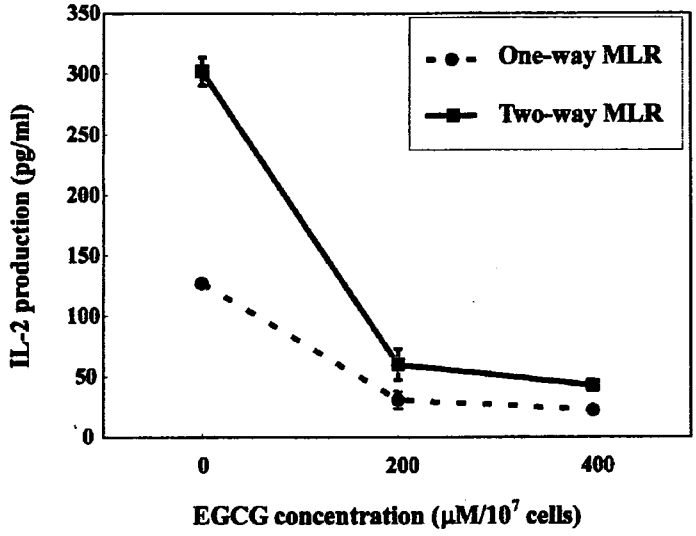

Figure 3. Inhibition of IL-2 production by EGCG treatment of stimulator cells in both one-way and two-way MLR cultures. Differences were significant between treated and untreated groups ($p < 0.0001$) for both MLRs.

from MLR culture were stained with FITC-labeled anti-mouse CD71 and PE-labeled anti-mouse Thy 1.2 antibodies, and analyzed by flow cytometry. The expression level of CD71 of EGCG-treated groups was significantly reduced down to the level of the control or the unstimulated responder cells (C57Bl/6) (Fig. 4).

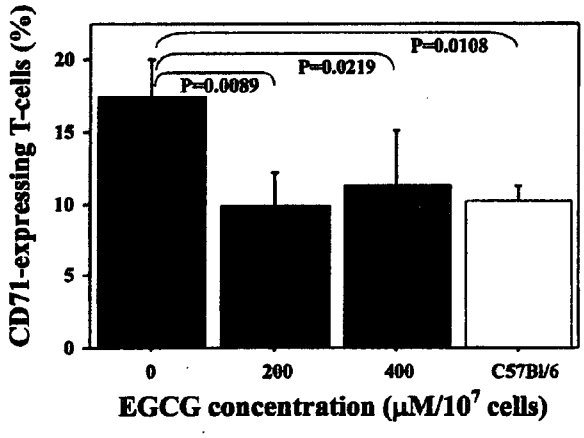

Figure 4. CD71 expression in T-cell population stimulated with EGCG-treated stimulator cells in MLR culture. Cells harvested from the MLR cultures (black columns) and from C57Bl/6 alone (white column) as a background reference were stained with FITC-anti-CD71 and PE-anti-Thy 1.2 antibodies and analyzed by FACScan.

Flow Cytometry of Cell Surface Molecules

To investigate whether the cell surface molecules of splenocytes are masked by EGCG, we performed the flow cytometric analysis of various cell surface molecules of splenocytes after EGCG treatment. Detection of most of the tested cell surface molecules was reduced by EGCG treatments, and the levels of reduction appeared to vary among different surface molecules (Table

Table 1. Detection of Cell Surface Epitopes

	EGCG Concentration (/10^7 Cells)		
Epitope	0 μM	200 μM	400 μM
TCRαβ	31.0	29.9 (3.5)	29.7 (4.2)
MHC I	96.3	92.5 (3.9)*	90.5 (6.0)*
MHC II	43.8	32.5 (25.8)*	33.2 (24.2)*
CD28	17.6	8.7 (50.6)*	9.4 (46.6)*
CD49d	42.9	19.7 (54.1)*	20.5 (52.2)*
B7.1 (CD80)	21.2	3.1 (85.4)*	6.0 (71.7)*
B7.2 (CD86)	11.6	4.2 (63.8)*	9.7 (16.4)†

Percent of epitope-positive cells measured by flow cytometry. Values in parentheses for EGCG-treated samples (200 and 400 μM/10^7 cells) represent the average percent reduction in the detection relative to the untreated control samples (0 μM/10^7 cells). Each value is an average of triplicate samples.
*$p < 0.0001$ compared with untreated controls.
†$p < 0.03$ compared with untreated controls.

1). While a moderate reduction was observed for MHC II, detection of its ligand, TCRαβ, remained unchanged (Fig. 5, Table 1). Detection of CD28, B7.1, and B7.2, which are involved in the generation of costimulatory signals for T cells, was significantly reduced by EGCG treatment (Fig. 5, Table 1). Greater reduction was also shown in CD49d, an adhesion molecule (Table 1).

Unresponsiveness of Responder T Cells Prestimulated With EGCG-Treated Stimulator Cells

To analyze whether reduced T-cell responses after EGCG treatment are reversed by secondary stimulation with fresh stimulator cells or exogenous IL-2, after one-way MLR cultures we restimulated MLR cells with

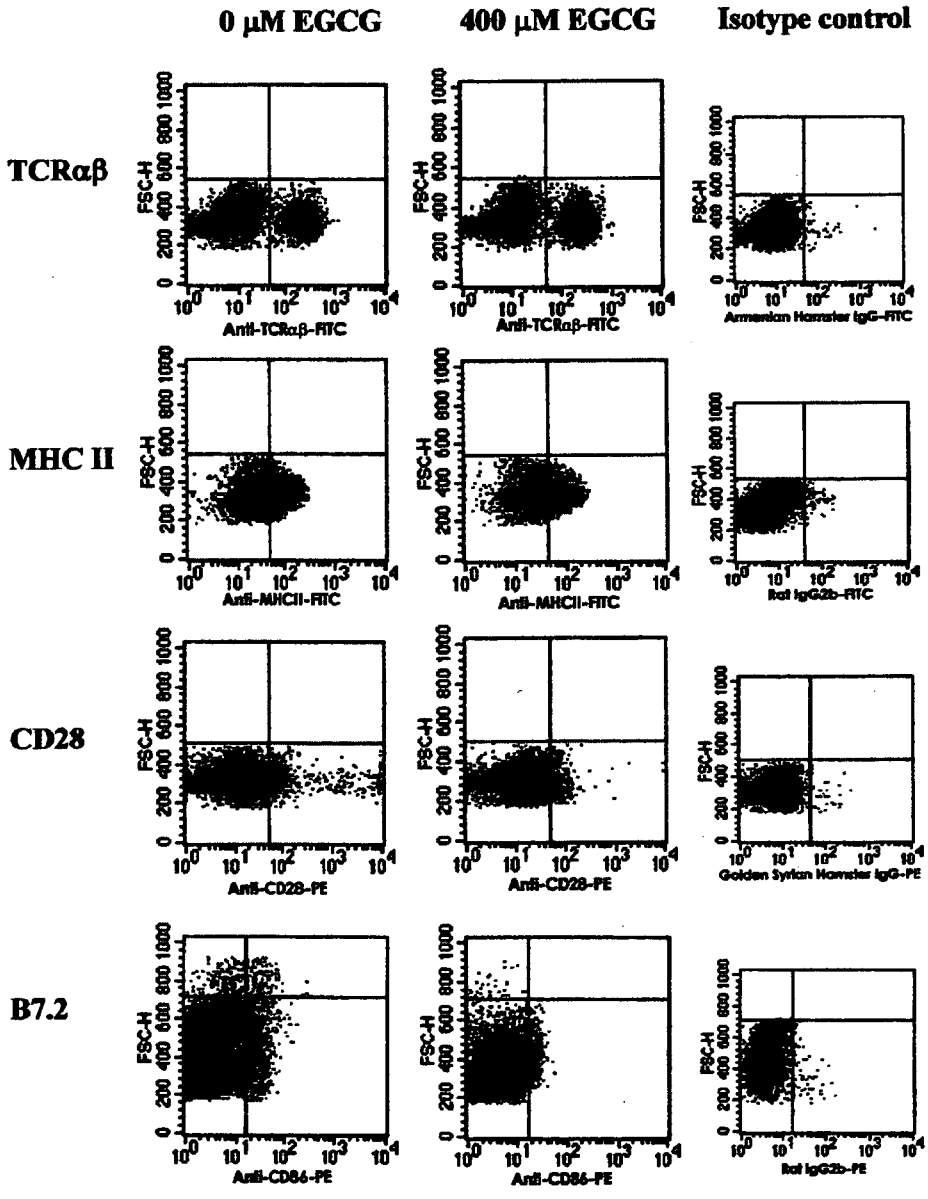

Figure 5. Flow cytometric analysis of cell surface molecules after EGCG treatment. C57Bl/6 splenocytes were either untreated or treated with EGCG (200 and 400 μM/10^7 cells), and then stained by epitope-specific monoclonal antibodies. A minimum of 10,000 counts per samples was analyzed by FACScan, and the representative results are shown.

EGCG-untreated fresh stimulator cells or IL-2 (1.7 U/ml) and analyzed the T-cell proliferation in secondary cultures. There was significant suppression of T-cell proliferation down to or below that of the unstimulated control cultures (Fig. 6).

Detection of Apoptosis by Cytometry

To examine the proportions of apoptotic cells in responder cells (C57Bl/6) and EGCG-treated stimulator cells (BALB/c), cells from MLR cultures after 24 h were analyzed by Annexin V assay. For the responder cells that were cocultured with the stimulator cells treated with EGCG, there was no substantial change in the proportion of apoptotic cells due to the EGCG treatment (0 μM: 81.8 ± 2.1%; 200 μM: 77.9 ± 1.1%; 400 μM: 78.7 ± 1.7%, based on one-way ANOVA of triplicate samples, $F = 4.43$, $p > 0.05$) (Fig. 7). In contrast, for the stimulator cells, the proportion of apoptotic cells significantly decreased with the EGCG treatment (Fig. 7).

DISCUSSION

Our present study demonstrated that EGCG can prevent allostimulation in a murine in vitro allograft context. EGCG treatment of murine splenocytes decreased the cell signaling for optimal allostimulation and attenuated the activation and proliferation of T cells in the MLR cultures.

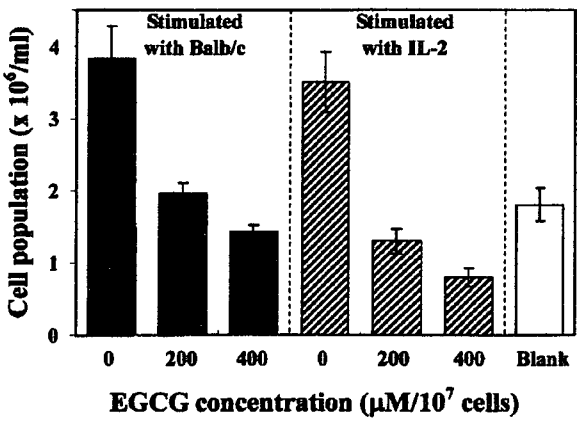

Figure 6. Unresponsiveness of responder T cells that have been prestimulated with EGCG-treated stimulator cells. After coculturing C57Bl/6 splenocytes with EGCG-treated BALB/c splenocytes for 48 h, MLR wells were restimulated with either fresh untreated BALB/c splenocytes (black columns) or exogenous IL-2 (hatched columns), and incubated for an additional 48 h. C57Bl/6 without any stimulation is shown as a background (blank column). Differences were significant between untreated and treated groups (200 or 400 μM/10^7 cells) for cell-stimulated cultures ($p < 0.0001$) and IL-2-stimulated cultures ($p < 0.0001$).

Tissue or organ transplantation is increasingly applied for the treatment of many medical problems, but its major drawbacks are transplant rejection and GVHD. One of the ordinary therapeutic methods to prevent these problems is the application of pharmacological agents that suppress T-cell activation (18). Although these chemical agents mitigate the problems by inhibiting T-cell signaling and activation, they often cause significant side effects on various tissues such as renal, hepatic, gastrointestinal, and mucosal systems (3). To avoid these side effects, more amenable methods have been developed (10,11,14). One of the promising methods is immunocamouflage where the donor tissues bearing cell surface antigenic determinants are coated with nonimmunogenic agents such as polyethylene glycol (PEG) (14). PEG can block immunogenic recognition by the host immune system and decrease graft rejection and GVHD (4,13). In the present study, we hypothesized that a tea polyphenol, EGCG, also blocks antigenic determinants and attenuates the allostimulation and subsequent activation of host T cells.

Our results indicate that responder T-cell proliferation in the MLR culture was attenuated when the stimulator cells were treated with EGCG (Fig. 1). The EGCG treatment of the stimulator cells representing the transplant led to the attenuation of T-cell proliferation of the responder cells (Fig. 4) representing the host, which is usually targeted by immunosuppressive drugs. Because the stimulator cells in one-way MLR culture had been arrested of proliferation by mitomycin C, the decrease of T-cell proliferation was largely due to the influence of EGCG on the stimulator cells and/or their stimulatory activities. The choice of C57Bl/6 as the responder rather than the stimulator in the one-way MLR in these experiments was to avoid overestimating the attenuating potential of EGCG treatment because C57Bl/6 T cells are classified as Th-1 type and are generally considered to have stronger and more sustained response toward cellular antigens than BALB/c T cells, which belong to Th-2 type.

It must be noted that the decrease of T-cell proliferation was not due to the direct toxicity of EGCG on the stimulator cells. In fact, EGCG-treated stimulator cells in MLR cultures had reduced levels of apoptosis than untreated controls (Fig. 7). For the same reason, the production of IL-2 in the one-way MLR cultures decreased supposedly due to the reduced stimulation of T cells (Fig. 3) by the EGCG-treated stimulator cells but not loss of viability of the latter. IL-2 production was also reduced in two-way MLR cultures (Fig. 3). In two-way MLR cultures, we cannot simply determine which cell type (C57B/6 or BALB/c) is actually contributing to IL-2 production, and this reduction seemed assigned to the inactivation of both responder and stimulator T cells,

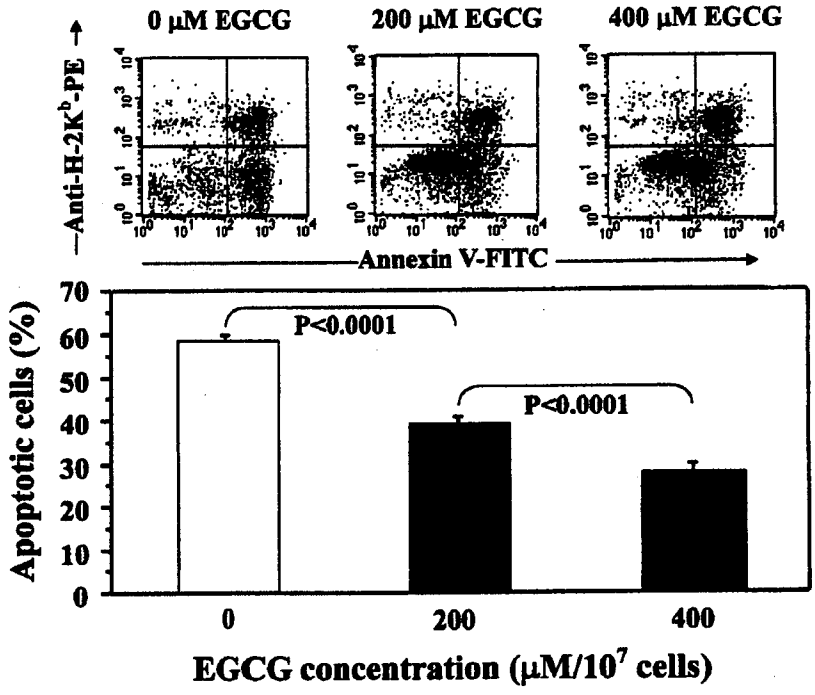

Figure 7. Analysis of apoptosis in responder and EGCG-treated stimulator cells in the MLR culture. C57Bl/6 splenocytes were cocultured with EGCG-treated BALB/c splenocytes for 24 h, and cultured cells were stained with PE-conjugated anti-mouse H-2K^b and FITC-conjugated Annexin V. The upper figures show three representative scatter grams (lower left quadrant: nonapoptotic stimulator; lower right quandrant: apoptotic stimulator; upper left quadrant: nonapoptotic responder; upper right quadrant: apoptotic responder cell populations). The lower graph shows the proportions of stimulator cell populations treated with 0, 200, or 400 μM EGCG before MLR culture with responder cells.

because activation of stimulator (BALB/c) T cells was also attenuated by the treatment with EGCG (our unpublished results).

The T cells stimulated with EGCG-treated stimulators were not only attenuated of proliferation but rendered somehow unresponsive toward allostimulation (i.e., they were in an anergic state). In fact, the proliferation of the responder cells did not recover upon secondary stimulation with fresh stimulator cells or even exogenous IL-2 (Fig. 6). The lack of responsiveness to the secondary stimulation with cells is not necessarily due to the mortality of responder cells, as indicated by the result of apoptosis detection (Fig. 7). Although T-cell apoptosis is known to be required in the induction of peripheral transplantation tolerance (20), the level of responder cell apoptosis occurring in our experimental system suggests that the induction of anergy due to EGCG treatment of stimulator cells is involved in the unresponsiveness of responder T cells (Fig. 7) Furthermore, the lack of responsiveness to the secondary stimulation with exogenous IL-2, in turn, suggests that the stimulation of CD8$^+$ T cells may be prevented by the stimulation with EGCG-treated stimulators. These results provide further evidence to support that EGCG treatment prevents acute graft rejection (7) and present a question as to what mechanisms are involved in the influence of EGCG on the stimulator cells and their activities.

In light of the present hypothesis, the simplest interpretation is that stimulatory activities were abrogated by blocking cell surface molecules with EGCG, resulting in weak or incomplete stimulation of alloreactive T cells. Indeed, staining intensity of cell surface molecules involved in allorecognition, costimulation, and cell-to-cell adhesion were reduced by the EGCG treatment (Table 1). Weakening or blockage of interactions of any of these molecules and their ligands could lead to incomplete activation of T cells (4).

Particular attention is paid to the different effects exhibited among these cell surface molecules. Normally,

T cell is stimulated at an allorecognition site that constitutes a main recognition domain of T-cell receptor (TCR) that binds to a complex formed by a foreign peptide and a major histocompatibility complex class I or II (MHC I or II) molecule. As the results of surface marker analysis indicate, on one hand, EGCG treatment moderately reduced the detection of MHC I and II molecules but not that of TCRαβ (Fig. 5, Table 1). On the other hand, EGCG treatment strongly reduced the detection of costimulatory molecules B7.1 (CD80) and B7.2 (CD86) as well as their ligand, CD28 (Fig. 5, Table 1), indicating potential abrogation of pivotal costimulatory signals (6,8,15–17) that may lead to the induction of anergic state in T cells. The detection of adhesion molecule, CD49d, was also strongly reduced, suggesting weakening of cell-to-cell adhesion prerequisite to allostimulation (Table 1).

Besides the anergy induction, other modes of action of EGCG can be suggested with respect to its possible influence on antigen-presenting cells. The EGCG molecules, with their amphipathic property depending on treatment conditions, are considered to penetrate the cell membrane and interfere with specific biochemical pathways underlying cell activation processes. For example, EGCG is known to downregulate the activity of NF-κB, which is involved in the activation of macrophages and dendritic cells (1,9). In addition, there is a large number of cytokines that are involved in the activation of antigen-presenting cells, and EGCG may influence the activity or production of these cytokines. EGCG may not only influence these indirect immunosuppressions but also the direct suppression by means of regulatory T cells.

The EGCG treatment presented in this study can provide a novel and useful method to prevent allorejection of foreign tissues, because donor tissues are simply to be immersed in culture media containing EGCG before being transplanted to a recipient body system. In terms of its potential clinical application, this plant-derived agent is expected to require less preclinical toxicological tests and lower manufacturing costs than synthetic chemicals. Regarding dose–effect comparison, EGCG dosage used in the present study was somewhat higher than that of methylated PEG, but the overall level of the attenuation effect was in a similar range to that reported for PEG-camouflaged cells (4). Although further investigation is necessary with respect to the mechanisms behind the immunosuppressive actions, the treatment of donor tissues with EGCG may have a potential value for the prevention of allorejection and can be applicable in other transplantation situations.

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Successful Engraftment of Cryopreserved Human Pancreatic Islets into Diabetic Nude Mice.

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Abstract

For the purpose of realization of clinical cryopreserved islet transplantation, frozen-thawed human islets were examined *in vitro* by static incubation and *in vitro* by transplantation models using diabetic nude mice. Stimulation index of static incubation was 5.44±2.38 (n=5) in fresh islets and 2.85±1.48 (n=6) in frozen-thawed islets. By injection of 1,000 IEq fresh human islet transplantation into the space beneath th left kidney capsule, diabetic nude mice showed normoglycemia (blood glucose level; <200mg/dl). On the other hand, 1,000 IEq frozen-thawed human islet transplantation failed to realize normoglycemia of diabetic nude mice. 2,000IEq frozen-thawed human islet transplantation, however, normalized blood glucose levels as well as 1,000 IEq fresh human islet transplantation. In a histological study, round and well-shaped-shape islets were detected beneath the kidney capsule, demonstrating good function of the frozen-thawed human islets.

Cryopreservation is the most promising technique for long-term storage of human islets and has many advantages. From the results of the present study, it was demonstrated that frozen-thawed human islets had an ability of insulin secretion and potential diabetes cure when enough islets were transplanted. In addition to fresh islet transplantation, frozen-thawed islet transplantation has great potential for clinical application.

Key words : cryopreservation, human islet, nude mice, islet transplantation

Introduction

Pancreatic islet transplantation, which is considered to be the most physiologically safe procedure for the treatment of type I diabetes mellitus, has been started in Japan in 2004[1]. According to the medical law of our country, the pancreata were able to be collected only from the non-heart beating donors for islet transplantation. When the yield and purity of islet isolation do not fulfill the criteria for fresh islet transplantation, the isolated islets must be cryopreserved for future use of clinical islet transplantation. According to the Edmonton protocol, sufficient of fresh islets (>12,000IEq/kg) should be needed to transplant in order to realize insulin independency[2]. Although cryopreserved islets have also been used for clinical islet transplantation[3,4], the effect of cryopreserved islets as compared to fresh islets has not been examined, especially in clinical studies.

Cryopreservation of human pancreatic islets offers many advantages for clinical islet transplantation. Cryostorage allows the accumulation of a large amount of donor tissue so that a sufficient number of islets with a desired HLA tissue type can be provided for transplantation. Frozen islets can be shipped to other institutions worldwide. In addition, an accurate pre-transplant evaluation in terms of safety and efficacy is possible during the cryopreservation period. However, the major problem of cryopreservation of islets is a decreased number and function of frozen-thawed islets as compared to the fresh islets.

In the present study, we compared the effect of cryopreserved human islets and fresh human islets on complete reversal of diabetes using *in vivo* nude mice model. These data may be helpful to decide the number of cryopreserved islets to transplant for the diabetic patient in clinical application.

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Materials and Methods

Human Islet Isolation

All human pancreata were harvested with appropriate consent from brain dead cadaveric organ donors obtained through the two organ procurement agencies of Southern California, the Regional Organ Procurement Agency and the Southern California Organ

Procurement Center. The pancreas was transported to the laboratory in University of Wisconsin (UW) solution at 4°C. Islet Isolation was performed using two-step digestion method as previously reported[5]. In brief, the pancreata was first expanded by injecting 150-200 mL warm collagenase (Collagenase P, Boehringer Mannheim Co., Indianapolis, IN, USA) solution (approximately three times the volume of the pancreas weight in grams). Expanded pancreas was cut into approximately 2- to 3-cm^3 pieces. The sectioned pancreas was first digested in the plastic chamber in a 37°C water bath via a swing-arm shaker for 12-15 minutes(Step1). Subsequently, Cold LAP solution[5] was added to perform cold digestion phase(Step2). Islet purification was performed by discontinuous gradient centrifugation on three layers of Euro-Ficoll solutions (density, 1.100, 1.087, and 1.056) using a COBE2991 cell processor (COBE Laboratories, Inc., Lakewood, CO, USA).

Islet Culture

Isolated islets were cultured in plastic Petri dishes (Falcon # 1005, Becton Dickinson Co., Franklin Lakes, NJ, USA) at 37°C with RPMI 1640 medium containing 20% fetal bovine serum, 10mM nicotonamide, 25mM HEPES, 24mM $NaHCO_3$, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. After overnight culture in a CO_2 incubator, the islets were transplanted into diabetic nude mice or used for the cryopreservation procedure.

Cryopreservation of the islets

The islets were cryopreserved in a fully automated cryounit as previously reported using a cooling rate of 1°C/mim and 5% DMSO CPA.[6] Islets were counted before cryopreservation. After cryostorage for five to 21 days, the frozen islets were thawed in a 37°C water bath in a rapid thawing manner[6]. Withim 1 hour frozen-thawed islets were transplanted into diabetic nude mice .

Static incubation for insulin assecsment

To assess the function of the fresh or the frozen-thawed islets, static incubation was performed. Briefly, 50 islets were placed into plastic Petri dish (Falcon # 1005) with RPMI 1640 containing 3.3 mmol/L D-glucose. After 60 minutes, the islets were transferred into a new plastic Petri dish with RPMI 1640 containing 20 mmol/L D-glucose and 0.1% BSA (glucose stimulation). After 60 minutes the islets were transferred again to the basal medium again for an additional 60-minute culture. Each medium was centrifuged and immediately frozen for later assay of insulin concentration by ELISA. The stimulation index was calculated by comparing the insulin content in the glucose stimulation medium with the second basal medium.

Islet transplantation into diabetic nude mice

Diabetes was induced in nude mice by administration of streptozotocin (180mg/kg IV). The islets were injected into the space beneath the left kidney capsule. 1,000 IEq fresh human islets were transplantaed into five diabetic nude mice (Fresh1000 group), and 1,000 and 2,000 IEq frozen-thawed islets were transplanted into six mice (CP1000 group, CP2000 group). After transplantation, blood glucose levels were measured for 21 days. Left nephrectomy was performed after 21 days to examine histological study and confirm that normoglycemia was made by transplanted islets.

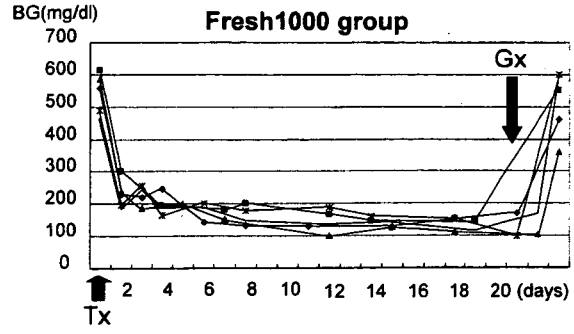

Figure 1. Blood glucose levels in Fresh1000 group. All five diabetic nude mice showed normoglycemia (< 200mg/dl) immediately after transplantation (Tx) of 1,000 IEq fresh human islets. After left nephrectomy (Gx), prompt increase of blood glucose levels were shown.

Results

Static incubation

As shown table 1, the fresh islets showed the good function against in vitro glucose challenge. Stimulation index was 5.44 ± 2.38 (n=5). While, stimulation index was deteriorated to 2.85 ± 1.48 (n=6) when using frozen-thawed islets.

Results of transplantation

In Fresh1000 group, all five mice showed prompt decrease of blood glucose levels after transplantation. Blood glucose levels decrease below 200mg/dl (normoglycemia) within five days (Figure 1). Blood glucose levels were maintained below 200mg/dl for 21 days after transplantation. After nephrectomy, immediate increase of blood glucose levels was shown in all five mice (Figure 1). While, in CP1000 group, two out of three mice (66.7%) showed hyperglycemia even after transplantation. Although the blood glucose levels of the other one mouse decreased to 200mg/dl at 5days after transplantation, they were maintained at the levels of 200-300mg/dl for 21 days (Figure 2a). In CP2000 group, however, all three mice showed normoglycemia after transplantation (Figure 2b). In CP2000group, 7 to 10 days were needed to realize normoglycemia. As well as Fresh1000 group, all mice showed hyperglycemia immediately after left nephrectomy. In the histological study, round and good-shape islets were detected beneath the kidney capsule, demonstrating the good function of the frozen -thawed islets.

Discussion

Although clinical islet transplantations have been successful in a limited patients[7] before 2000, the Edmonton protocol, which was introduced by the Alberta University group, markedly improved the results of clinical islet transplantation[2,8]. In the Edmonton protocol, only fresh islets were used for transplantation to achieve insulin independency of diabetic patients. Cryopreserved islets were not used in this protocol in spite of previous clinical experience in islet allograft[9,10].

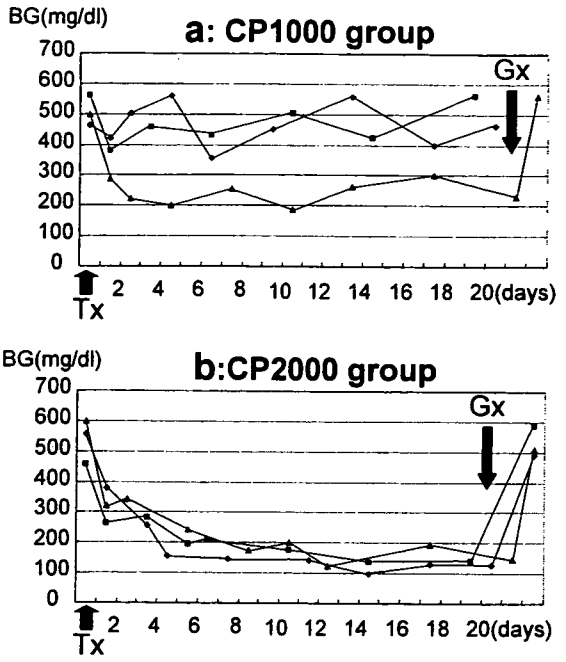

Figure 2. Blood glucose levels in CP1000 and CP2000 groups. In CP1000 group, two mice (66.7%) failed to normalize the blood glucose levels of diabetic nude mice and the other one mouse showed incomplete effect on decrease of blood glucose level (a). In contrast, In CP2000 group, all mice showed normoglycemia and prompt increase of blood glucose levels were shown after left nephrectomy (Gx).

Cryopreservation is still belived to be an ideal method for long-term storage of human pancreatic islets and numerons investigations concerned with islet cryopreservation have been previously performed[11~15]. Cryopreservation of islets has many advantages in clinical islet transplantation. Test for bacteria and fungus contaminations during the procedure of islet isolation is impossible except for a gram staining in fresh islet transplantation. Also, evaluation of the endocrine function including a static incubation is impossible in fresh islet transplantation. Such data can be obtained during cryostorage of the islets before transplantation. Cryostorage allows the accumulation of large amounts of donor tissue so that sufficient numbers of islets with a desired HLA tissue type can be provided for transplantation. Furthermore, frozen islets can be shipped to other institutions. Immunogenicity of the islets may deteriorate during cryostorage according to the reduction of MHC antigen[16].

The major disadvantage of cryopreservation is, however, decreased number and function of the islets after thawing. These may be caused by a direct cryo-injury, the toxicity of DMSO as widely used for cryoprotectant, and thawing procedure. Instead of DMSO, various materials including trehalose[17], ethylene glycol[18] and polyethylene glycol[19] have been used as cryoprotectant for cryostorage of islets in order to decrease the toxicity for the islets in the previous studies. We also tried hydroxyethyl starch (HES) as an extracellular cryoprotectant with DMSO to simplify the freeze-thawing procedure[20].

Table 1 Data of static incubation of fresh and frozen-thawed human islets.

Human islets	Stimulation Index	Mean±SD
Fresh	8.6, 6.8, 5.5, 3.5, 2.8	5.44±2.38
Frozen-thawed	1.8, 1.9, 2.1, 2.2, 3.5, 5.6	2.85±1.48

In our country, pancreata from non heart beating donors were usually used for the islet isolation and, thus, more than 50% of isolations were used for cryopreservation for islet banking to use the future clinical islet transplantation. To realize a clinical transplantation of frozen-thawed islets, evaluation of frozen-thawed islets including contamination check and function should be done. In particular, a number of frozen-thawed islets, which is effective after transplantation, is, still, unknown.

In the present study, a stimulation index from static incubation of frozen-thawed human islets decreased to an approximately half level of fresh human islets. These data demonstrated that frozen-thawed human islets were able to secrete insulin against glucose challenge, but their endocrine function was decreased as compared to fresh human islets. From the data using nude mice in the present study, it was clearly demonstrated that a two-fold number of frozen-thawed islets (2,000 IEq) was needed to realize euglycemic state in diabetic nude mice as compared to fresh islets (1,000 IEq). Furthermore, frozen-thawed islets were able to make euglycemic state with prolonged period (7-8 days). It may be caused by direct injury of islets during frozen-thawed procedure, and inhibition of capillary formation to the islets because of the injury of capillary endothelial cells for the islets.

In conclusion, cryopreservation is the most promising technique for long-term storage of human islets and has many advantages. From the results of the present study, it was demonstrated that frozen-thawed human islets had the ability of insulin secretion and diabetes cure when enough islets (more than two-fold of fresh islets) were transplanted and, as well as fresh islet transplantation, frozen-thawed islet transplantation could be realized in clinical application in our country.

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膵臓移植の最近の動向*

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* Current status of pancreas transplantation

キーワード：膵臓移植，生体膵臓移植，膵島移植，免疫抑制法

要旨：膵臓移植は1967年に米国で臨床例が開始され，現在では23,000例以上が施行されている．わが国では臓器移植法の施行後，40例の脳死・心停止膵臓移植，12例の生体膵臓移植が施行されている．膵臓移植の成績は，最近は他の臓器移植と同等に良好である．わが国では膵臓移植を移植施設＋National Teamで行い，40例に死亡例はなく良好な成績を得ている．脳死ドナー不足を背景に，当院では生体膵腎同時移植（SPK）を7例に施行した．ドナーは合併症もなく退院し，レシピエントは全例インスリン離脱が可能であった．膵島移植は成績が向上し，わが国でも臨床で開始された．全例で低血糖発作の消失・減少，血糖の安定化が得られ膵島移植の有効性が臨床的に示されたが，長期成績の改善が必須である．

はじめに

膵臓移植は1型糖尿病や高度慢性膵炎・膵全摘後などのインスリン依存型糖尿病に対する究極的治療法である．1967年，米国においてKellyら[1]により最初の臨床例が施行されたが，当時は拒絶反応に加えて移植手技に起因する合併症のため移植膵が廃絶する例が多く[1]，移植後血栓症予防，膵液ドレナージ法の実験的・臨床的研究が多数されている．臨床例が急増し，成績が飛躍的に向上したのは1980年代に入ってからであり，他の臓器移植と同様cyclosporine A（CsA）による免疫抑制法が登場したことによる．現在では膵臓移植は世界で23,000例以上が施行されており[2]，糖尿病根治療法として定着している．

わが国では1984年に筑波大学で脳死ドナーからの膵腎同時移植（simultaneous pancreas-kidney transplantation：SPK）が行われたが[3]，その後は心停止ドナーからのSPKが主流となり，1993年までに東京女子医科大学を中心に14例が行われた[4]．以後，法整備のため一時中断したが，2000年4月に大阪大学において臓器移植法施行後初めての脳死・膵腎同時移植が行われた[5]．以後，現在までに40例の脳死・心停止膵臓移植が行われている．また，12例の生体膵臓移植も施行されている．

本稿では膵臓移植の方法，世界およびわが国の現況と成績に加え，当院での生体膵臓移植の成績，さらには膵臓移植のオプションである膵島移植について最近の動向を中心に述べる．

膵臓移植の方法

膵臓移植の手技は長く実験的・臨床的に研究されてきた．全膵を用いるか部分膵（膵体尾部）を用いるかの検討のほか，膵液ドレナージ法として腸管ドレナージ，膀胱ドレナージがあり，さらに