American Journal of Transplantation 2008; 8: 288–297
Blackwell Munksgaard

doi: 10.1111/j.1600-6143.2007.02077.x

In Vitro and *in Vivo* Prevention of Human CD8$^+$ CTL-Mediated Xenocytotoxicity by Pig c-FLIP Expression in Porcine Endothelial Cells

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Overcoming cell-mediated immunity, especially of human CD8$^+$ CTLs, is important for the success of xenotransplantation. Our group has previously reported that the cytotoxicity of human CD8$^+$ CTLs against pig endothelial cells (PEC) is highly detrimental and mediated in major part by the Fas/FasL apoptotic pathway. Cellular FLICE inhibitory protein (c-FLIP) was originally identified as an inhibitor of death-receptor signaling through binding competition with caspase-8 for recruitment to Fas-associated via death domain (FADD). Two major c-FLIP variants result from alternative mRNA splicing: a short, 26-KDa protein (c-FLIP$_S$) and a long, 55-KDa form (c-FLIP$_L$). The cytoprotective effects of c-FLIP$_{S/L}$ in xenograft cells remain controversial. This study demonstrates that the overexpression of c-FLIP$_{S/L}$ genes markedly suppress human CD8$^+$ CTL-mediated xenocytotoxicity and, in addition, the cytoprotective effects of c-FLIP$_L$ appear to be significantly stronger than those of c-FLIP$_S$. Furthermore, to prove the prolonged effects of xenograft survival, PEC transfectants with c-FLIP$_{S/L}$ genes were transplanted under rat kidney capsules. Prolonged survival was elicited from FLIP$_{S/L}$ transfectants, whereas parental PEC was completely rejected through day 5, posttransplant. Thus, intracellular remodeling with the overexpression of c-FLIP$_{S/L}$ in xenograft cells may avoid innate cellular attacks against xenografts and facilitate long-term xenograft survival.

Key words: Apoptosis, cellular FLICE-like inhibitory protein (c-FLIP), Fas antigen, human CTL, xenotransplantation

Received 18 July 2007, revised 29 October 2007 and accepted for publication 29 October 2007

Introduction

The clinical application of pig-to-human xenotransplantation has the potential to solve the current shortage of human organs and tissues for transplantation (1,2). However, the potential benefits of xenotransplantation are severely restricted by the antibody-mediated immunologic barrier. Human natural anti-Gal antibodies that bind to the α-gal epitopes (Galα1–3Galβ1–4GlcNAc-R) abundantly expressed on pig cells induce complement activation, and acute vascular rejection of xenografts (3–6). To avoid this immunologic problem, α1,3-galactosyltransferase gene knockout (GT-KO) pigs that do not express α-gal epitopes have recently been produced (7,8). Consequently, the use of organs obtained from GT-KO pigs may prolong xenograft survival by overcoming antibody-mediated hyperacute rejection (9,10).

Even when xenografts overcome hyperacute rejection, however, they will be directly rejected by cell-mediated immunity, which includes NK cells, macrophages and CD8$^+$ CTLs (11–18). Our group has previously demonstrated that human CD8$^+$ CTLs have strong cytotoxicity against xenograft cells, presenting another immunological obstacle to long-term xenograft survival (14–19), and the Fas/FasL apoptotic pathway is a major contributor to this CTL killing. Furthermore, we explored methods for preventing the CTL cytotoxic response against xenograft cells by means of the extracellular remodeling of death receptors with the overexpression of either membrane-bound human FasL, which is not cleaved to the soluble form of FasL by metalloproteinase, or by human decoy Fas antigen, which does not contain a 'death domain' in its cytoplasmic tail. In the present study, we hypothesized the following: because the Fas/FasL apoptotic pathway is a major contributor to CTL-mediated xenocytotoxicity (19), intracellular blocking of death receptor-mediated apoptotic signals induced by the Fas/FasL pathway would provide resistance against human CTL-mediated cytotoxicity, thereby extending xenograft survival.

Apoptosis is an essential mechanism for development, tissue homeostasis and immune function (20). Initiation and regulation of apoptosis is tightly controlled by specific protein-protein interactions and by a family of proteolytic enzymes—the caspases (21,22). Above all,

Fas-associated death domain (FADD)-like interleukin-1-β-converting enzyme-inhibitory protein (FLIP) is a potent inhibitor of death receptor-mediated pro-apoptotic signals—blocking upstream signaling pathways before caspase-8 activation and release (23–30). FLIP was originally identified as a viral gene product (v-FLIP), while investigators were searching genomes for proteins that contain a death effector domain (DED) in an effort to identify molecules that interact with caspases (31,32). Following the characterization of v-FLIP, the mammalian cellular homologue was identified and called c-FLIP (23–26,29,30). c-FLIP is structurally related to procaspase-8 and -10, but lacks enzymatic activity (33,34). At least 10 mRNA splice variants of c-FLIP exist, but often only two c-FLIP proteins are detected: a 26-KDa short form (c-FLIP$_S$) and a 55-KDa long form (c-FLIP$_L$) (35,36). The c-FLIP$_S$ resembles v-FLIP consisting of two DEDs and a short C-terminal tail; however, a caspase-like domain is entirely lacking (23,31). In contrast, c-FLIP$_L$ contains two N-terminal DEDs and a C-terminal caspase-like domain, similar to caspase-8 and -10. However, c-FLIP$_L$ is catalytically inactive, because it lacks a specific cysteine residue in the caspase-like domain that is critical for caspase activity. Both c-FLIP$_S$ and c-FLIP$_L$ are capable of binding to the Fas death-inducing signaling complex (DISC) (33,34), but binding of FLIP$_{S/L}$ molecules does not preclude caspase-8 recruitment; rather, DISC-associated caspase-8/c-FLIP$_{S/L}$ complexes are formed (35,37–39). Thereby, the role of c-FLIP$_S$ as an inhibitor of death receptor-mediated apoptosis is well understood (33,34). In contrast, the specific involvement of c-FLIP$_L$ in death receptor modulation remains controversial. Recent studies with purified components reported that c-FLIP$_L$ activates caspase-8 or -10 through heterodimerization (40,41).

The present study addresses the following question regarding human CD8+ CTL-mediated xenocytotoxicity: will overexpression of c-FLIP$_L$ on pig xenograft cells block death receptor-mediated apoptotic signals and offer cytoprotection? This question is of particular interest in view of the unknown effects of c-FLIP$_L$ on pig xenograft cells. In addition, overexpression of c-FLIP$_S$ on pig xenograft cells also was investigated as a novel approach for protecting xenograft cells against human CTL cytotoxicity.

Materials and Methods

Cell culture

A pig endothelial cell (PEC) line, MYP-30 (42), was cultured in DMEM (Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin and 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, CA) in a 5% CO_2 atmosphere at 37°C.

Gene constructs

The full-length DNA fragments encoding either pig c-FLIP$_S$ or c-FLIP$_L$ were subcloned into the EcoR1 site of pCR3.1 expression vector (Invitrogen), which carried a CMV promoter and the SV40 replication origin with a neomycin-resistant gene, respectively (43,44). These plasmids were transformed into Escherichia coli C600 and amplified using standard techniques.

Gene expression

A total of 20 μg of each plasmid (pCR-FLIP$_S$, pCR-FLIP$_L$) were transfected into the PEC line (MYP-30) using lipofectoamine (Invitrogen), according to the manufacturer's instructions. To identify PEC stably transfected with pig c-FLIP$_{S/L}$ genes, the transfected PECs were cultured in the selection medium (DMEM complete medium containing 1 mg/mL of G-418 (Sigma-Aldrich)) for 3 weeks. PEC stably transfected with pCR3.1 expression vector, which lacks cDNA fragments of pig c-FLIP$_{S/L}$ genes, was also established (i.e. mock) as the vehicle control.

RT-PCR analyses

To identify mRNA expression of either c-FLIP$_S$ or c-FLIP$_L$ in PEC transfectants, RT-PCR analyses were performed. Total RNAs of either parental PEC, mock or PEC transfectants were isolated using a RNeasy minikit (Qiagen, Chatsworth, CA). One microgram of total RNAs was reverse-transcribed with murine leukemia virus reverse transcriptase and the resulting products were PCR-amplified using sets of primers on a Gene Amp thermal cycler (PCR System 9700; Applied Biosystems, Foster City, CA). Reverse transcription of c-FLIP$_S$ and GAPDH was performed using the following PCR cycles: 94°C for 5 min; 35 cycles at 94°C for 30 s; 57°C for 30 s; 72°C for 1 min and a final extension period at 72°C for 7 min. For c-FLIP$_L$ the PCR cycles were as follows: 94°C for 5 min; 35 cycles at 94°C for 30 s; 47°C for 30 s; 72°C for 1 min and a final extension period at 72°C for 7 min. The following forward and reverse primer sets were synthesized: 5′-ATGTC GGCTG AGGTC ATCCA TCA-3′, as the c-FLIP$_S$ forward primer; 5′-TCATG CTGGG ATTCC GCACA CTT-3′, as the c-FLIP$_S$ reverse primer; 5′-ATCAG TGAAA AGTAT GGATT-3′, as the c-FLIP$_L$ forward primer; and 5′-GGCTA AGAGG GGCCT TTGGC TCT-3′, as the c-FLIP$_L$ reverse primer. The amount of mRNA in each transfectant was normalized to the level of GAPDH mRNA, which was determined using 5′-GGACT CATGA CCACA GTCCA T-3′ and 5′-TCAGG TCCAC AACCG ACACG T-3′ as the forward and reverse primers, respectively. PCR products were electrophoresed in 1.5% (W/V) agarose gels; the sizes of the expected c-FLIP$_S$, c-FLIP$_L$ and GAPDH PCR products were 645, 333 and 220 bp, respectively. Subsequently, the gels were recorded with a digital fluorescence-reader (EDAS 290; Kodak, Tokyo, Japan), and fluorescence intensity of each band of PCR products was quantified using NIH's ImageJ (URS list, ImageJ). The relative abundance of specific mRNAs was normalized to that of GAPDH mRNA and was expressed as the c-FLIP$_{S/L}$/GAPDH ratio.

Western blot analysis

For Western blot analysis, parental PEC, mock or PEC transfectants were washed with cold PBS, resuspended in 50 μL of lysis buffer consisting of 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 125 mM NaCl, 10% (W/V) glycerol, 0.1% (W/V) NaN_3, 1% (W/V) NP-40, 5 μg/mL aprotinin and 0.1 mM PMSF, and incubated for 20 min at 4°C. The cell-free supernatants were recovered by centrifugation at 10000 g for 15 min at 4°C. The protein concentration of the extracts was determined by bicinchoninic acid (BCA) protein assay kit (Pierce, IL). For each sample, 10 μg of protein was loaded onto the gel, separated using 8% or 12% SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After transfer, the immunoblots were blocked overnight at 4°C with PBS containing both 3% BSA and 0.1% Tween 20. Next, the blots were probed for 16 h at 4°C with mouse anti-human c-FLIP mAb (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 3% BSA/PBS-Tween 20 at 1:100. The blots were incubated for 1 h at room temperature with the secondary antibody—horseradish peroxidase (HRP)-conjugated anti-mouse IgG (DAKO, Glostnep, Denmark) diluted in 3% BSA/PBS-Tween 20 at 1:2500. Subsequently, the blots were developed with an enhanced chemiluminescence (ECL) Plus Western blotting detection system (GE Healthcare Bio-sciences KK, Piscataway, NJ) according to the manufacturer's instructions. Protein expression levels of pig c-FLIP$_{S/L}$ in PEC transfectants were quantified by Fluor-Chem image analyzer as expressed by arbitrary units. As the loading control for the samples,

protein expression of pig GAPDH in either parental or PEC transfectants was detected by goat anti-pig GAPDH mAb (Santa Cruz Biotechnology). The relative protein expression of pig c-FLIP$_{S/L}$ in PEC transfectants was normalized to that of pig GAPDH and was expressed as the c-FLIP$_{S/L}$/GAPDH ratio.

Preparation of human CD8$^+$ CTL

Human CD8$^+$ CTLs were prepared as previously described (19). Briefly, 10 to 15 $\times$ 10^6 cells of separated PBMCs were cocultured for 14 days with irradiated PEC in the presence of 50 U/mL recombinant human IL-2. Subsequently, human CD8$^+$ CTLs were positively isolated by magnetic beads (Dynal, Oslo, Norway), coated with anti-human CD8 mAb (RPA-T8, BD Biosciences Pharmingen, San Jose, CA), and subjected to an *in vitro* cytotoxicity assay.

In vitro *cytotoxicity assay*

The cytotoxic activity of human CD8$^+$ CTLs incubated under various conditions was assessed using a ^{51}Cr-release assay as previously described (18,19). The target cells parental PEC, mock and PEC transfectants—were plated at 5000 cells/well in 96 well plates. After labeling the target cells with ^{51}Cr, human CTLs were added. The ^{51}Cr released from dead cells was measured in the supernatants. The percentage of specific cell lysis mediated by human CD8$^+$ CTLs was calculated as previously described (19). The effect of c-FLIP$_{S/L}$ expression on CTL-mediated cytotoxicity was determined by comparing PEC transfectants, mock and parental PEC.

Assay for apoptosis

To detect apoptotic cells, a Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using an *In Situ* Cell Death Detection Kit (Roche Diagnostics, Germany). Parental PEC, mock and PEC c-FLIP$_{S/L}$ transfectants were plated at 5000 cells/well in 96 well plates. After overnight incubation, human CD8$^+$ CTLs were added to the wells at an effector to target ratio of 50:1 and the cocultures were incubated for 1 h. Subsequently, the plates were washed with PBS to remove effector cells and the remaining target cells were fixed in freshly prepared 4% formaldehyde in PBS (pH 7.4) for 30 min. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C and then washed by PBS. Subsequently, TUNEL reaction mixture was added to the wells in 50 µL aliquots and plates were incubated in a humidified chamber for 1 h at 37°C in the dark. After a final washing with PBS, cells were observed by Biozero fluorescence microscopy (Keyence, Osaka, Japan) measuring green fluorescence (excitation, 488 nm; emission, 530 nm). Cells that exhibited TUNEL staining were categorized as apoptotic cells. Furthermore, the extent of apoptosis in PEC transfectants, parental cells and mock was determined by annexin V staining using an Annexin-V-Fluos staining kit (Roche Diagnostics). Parental PEC, mock and PEC transfectants were cocultured with human CTLs, the same as for the TUNEL assay. After washing with PBS, cells were incubated with 20 µL annexin V (20 µg/mL in HEPES) for 15 min in the dark at room temperature. Subsequently, treated cells were washed with PBS and analyzed by Biozero fluorescence microscopy (Keyence) measuring green fluorescence in a similar wavelength to those described above. The number of apoptotic cells visible in either the TUNEL assay or in the annexin V staining were counted by VH-analyzer software (Keyence).

Assay of caspase activity in vitro

Caspase-8, -10 and -3 activities were measured using the Caspase Colorimetric Assay Kit purchased from either BD Biosciences Clontech (catalog number K-2027 for caspase-8, K-2029 for caspase-3) or R and D Systems (Abingdon, UK) (catalog number BF-18100 for caspase-10), respectively, according to the manufacturer's instructions, with the following modifications: parental PEC, mock and PEC transfectants were plated at 2 $\times$ 10^6 cells/plate in a 6-cm dish and incubated overnight. The target cells were cocultured with human CD8$^+$ CTLs at an effector to target ratio of 50:1 for 30 min and then washed by PBS to remove effector cells. Subsequently, cells were recovered, resuspended in 50 µL of lysis buffer and then incubated for 10 min on ice. The cell-free supernatants were recovered by centrifugation of the suspension at 10000 g for 5 min at 4°C and then transferred to 96 well plates. Equal volumes (50 µL) of 2$\times$ reaction buffer/DTT and 5 µL of either caspase-8 substrate (IEDT-pNA), caspase-10 substrate (AEVD-pNA) or caspase-3 substrate (DEVD-pNA) were added to each well and incubated for 1 h at 37°C in the dark. The cell lysates also were incubated with reaction buffer in the absence of caspase substrate as a negative control. A pNA standard curve, incubating the concentrations of 0, 2.5, 5, 10 and 20 nmol pNA was constructed and caspase activities were determined from absorbance at 405 nm using the standard curve.

Transplant studies and immunohistochemical analysis

Lewis rats, 8–10 weeks old, were purchased from Oriental Yeast (Tokyo, Japan) and were randomized to receive either parental PEC, mock or PEC transfectants (n = 5 rats per group). Recipient rats were preimmunized three times intraperitoneally with 250 mg of pig kidney membranes with a 1-week interval between injections. PECs were recovered with 2 mM EDTA/PBS, washed twice with PBS and stored on ice until transplantation. In each case, a pellet of 2.5 $\times$ 10^6 cells was mixed with 30 µL of syngenic blood to form a clot. The clots, which included either parental PEC, mock or PEC transfectants, were transplanted into the rats under the kidney capsule. Transplanted rats were monitored until euthanization on day 2, day 3, day 5 and day 7 posttransplantation. Each grafted kidney was analyzed by immunohistochemistry. Kidney specimens were cut into small blocks, fixed in formalin and then embedded in paraffin. Tissue sections 2 µm thick were deparaffinized with xylene, rehydrated in graded concentrations of alcohol and washed in water. Endogenous peroxidase activity was quenched by exposing the sections to 3% H_2O_2/methanol for 15 min. After blocking with 10% BSA-TBS-Tween 20 for 30 min at room temperature, the sections were incubated with a rabbit anti-human Von Willebrand Factor (vWF) polyclonal antibody (1:200, DAKO) in TBS-Tween 20 for 16 h at 4°C to detect endothelial cells. The sections were then rinsed and incubated with link antibody for 30 min at room temperature, followed by incubation with HRP-conjugated streptavidin for 30 min at room temperature. Immunostaining was visualized with 0.02% diaminobenzidine (DAB, Sigma-Aldrich) as the chromogen. The specificity for the primary vWF Ab was verified by control sections, in which the primary Ab was omitted. To further determine the specificity of vWF Ab, the sections were incubated with the manufacturer's recommended control Ab (1:200, DAKO, code x0936) as the isotype control for this Ab. To identify the phenotype of the infiltrated rat lymphocytes in PEC xenografts, cells were stained with the following mAbs: biotinylated anti-rat CD8 mAb for staining CD8$^+$ T cells; biotinylated anti-rat CD4 mAb for staining CD4$^+$ T cells; biotinylated anti-rat CD68 mAb for staining CD68$^+$ macrophages; biotinylated anti-rat CD161 mAb for staining CD161$^+$ NK cells. All Abs were purchased from Serotec Ltd. (Oxford, UK). After incubation with HRP-conjugated streptavidin for 30 min at room temperature, immunostaining was developed with DAB as described above.

Double labeling

Kidney sections from rats euthanized at day 3 posttransplant, were doubly labeled for vWF and TUNEL, using the *In Situ* Apoptosis Detection Kit (Chemicon, Temecula, CA). The labeling procedure was a modification of the manufacturer's instructions. Following incubation with a rabbit anti-vWF polyclonal antibody as described above, free-floating sections were rinsed with PBS and incubated with tetra-methyl-rhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG (1:100; DAKO) for 30 min at room temperature in the dark and rinsed again. Subsequently, the sections were incubated with 20 µg/mL proteinase K in PBS for 15 min at room temperature followed by incubation with TUNEL reaction mixture (38.5 µL TUNEL label solution conjugated with fluorescein and 16.5 µL TdT enzyme) for 1 h at

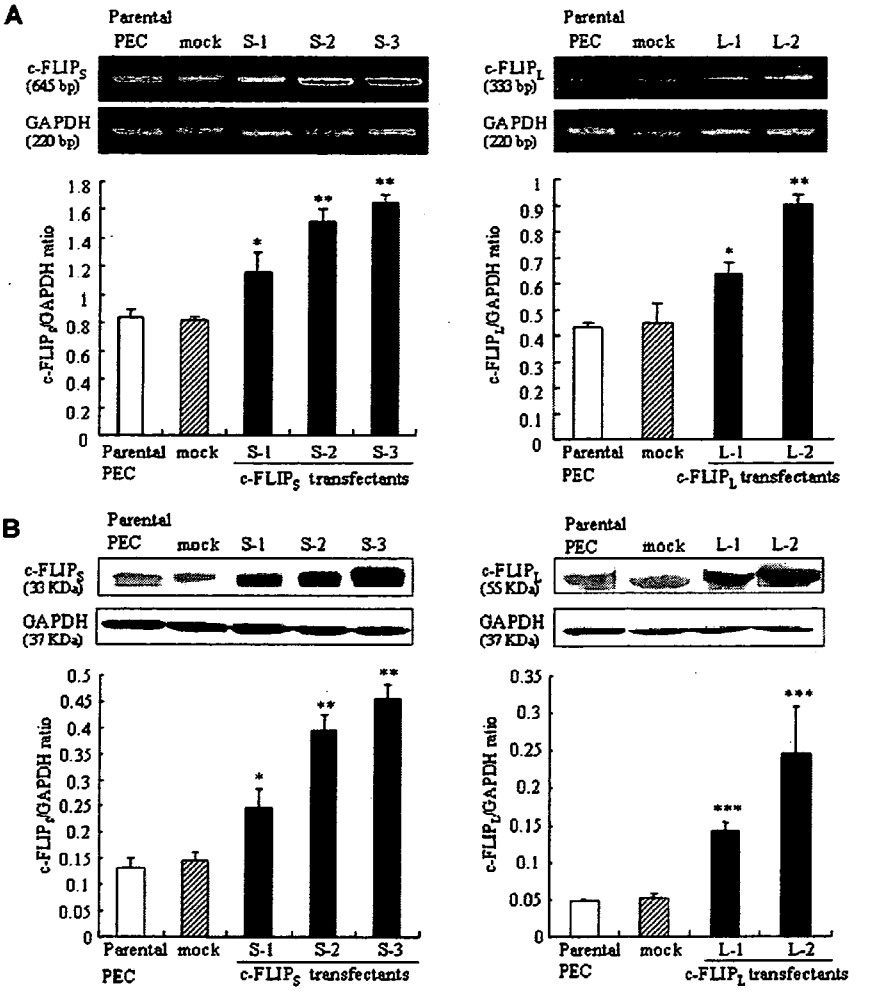

Figure 1: Changes in the expression levels of c-FLIP$_{S/L}$ mRNAs or proteins in PEC transfectants. (A) The expression levels of either c-FLIP$_S$ or c-FLIP$_L$ mRNAs were examined by RT-PCR. As an intrinsic control, GAPDH mRNA expression was measured in the same samples. Representative photographs of the electrophoresis are shown in A. The intensity of each bands of PCR products was quantified, subsequently, the relative abundance of specific mRNA was normalized to the GAPDH mRNA level as expressed by c-FLIP$_{S/L}$/GAPDH ratio. Each value is expressed as the mean ± SD from three independent experiments. An asterisk indicates a significant difference versus parental PEC and mock samples (*$p < 0.05$, **$p < 0.01$). (B) The expression levels of either c-FLIP$_S$ or c-FLIP$_L$ proteins were examined by Western blotting. Protein samples extracted from either parental PEC, mock or PEC transfectants were separated by electrophoresis in 8% or 12% SDS-polyacrylamide gel and transferred onto PVDF membrane. Representative photographs are shown in B, together with pig GAPDH levels as an internal control. Image scanner profiles were employed to evaluate protein expression levels of either c-FLIP$_{S/L}$ or pig GAPDH in parental PEC, mock and PEC transfectants, expressed by c-FLIP$_{S/L}$/GAPDH ratio. Each value is expressed as the mean ± SD from three independent experiments. An asterisk indicates a significant difference versus parental PEC and mock samples (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

37°C. A tissue section incubated with an equal volume of TUNEL labeling solution instead of TUNEL reaction mixture served as a negative control. Fluorescence signals were analyzed by Biozero fluorescence microscopy (Keyence).

Statistical analysis

The statistical significance was analyzed using a Student's *t*-test. Values were presented as the means ± SD. Differences were considered to be significant at $p < 0.05$.

Results

Generation of stable PEC transfectants overexpressed either pig c-FLIP$_S$ or pig c-FLIP$_L$ gene

The mRNAs of c-FLIP$_{S/L}$ were faintly expressed in both parental PEC and mock (Figure 1A). Three positive clones of c-FLIP$_S$ were isolated as follows: S-1 had low mRNA expression; S-2, moderate and S-3, high. Two c-FLIP$_L$ clones were identified: L-1 expressed moderate levels of mRNA

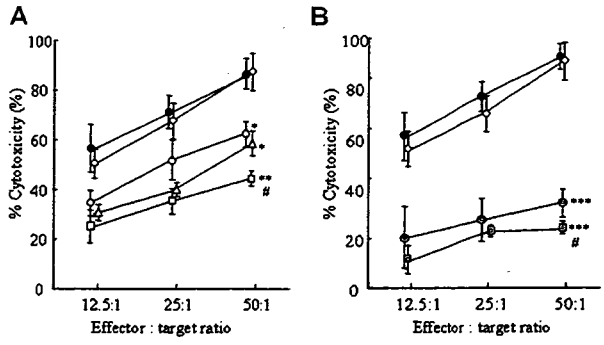

Figure 2: *In vitro* cytotoxicity assay of PEC transfectants with pig c-FLIP$_{S/L}$ genes. (A) Amelioration of human CD8$^+$ CTL-mediated cytotoxicity by c-FLIP$_S$ transfectants, control parental PEC and mock transfectants were assessed by ^{51}Cr release assay. Parental PEC (●), mock (◇), S-1 (○), S-2 (△), S-3 (□). Each value is expressed as the mean ± SD from five independent experiments. *The difference was statistically significant ($p < 0.05$ S-1, S-2 vs. parental PEC, mock). **The difference was statistically significant ($p < 0.01$ S-3 vs. parental PEC, mock). (B) Suppression of human CD8$^+$ CTL-mediated killing by c-FLIP$_L$ transfectants, mock transfectants and control parental SEC was also investigated by ^{51}Cr release assay. Parental PEC (●), mock (◇), L-1 (◐), L-2 (◧). Data are shown as the mean ± SD from five independent experiments. ***The difference was statistically significant ($p < 0.001$ L-1, L-2 vs. parental PEC, mock). #The difference was statistically significant ($p < 0.05$ S-3 vs. L-2).

and L-2 high levels. Western blot analysis showed low levels of endogenous c-FLIP$_{S/L}$ expression in both parental PEC and mock, and significantly elevated protein expression of either pig c-FLIP$_S$ or pig c-FLIP$_L$ in the transfectants (Figure 1B). No significant differences of both c-FLIP$_{S/L}$ mRNA and protein expression were observed between parental PEC and mock transfectants.

Overexpression of either pig c-FLIP$_S$ or pig c-FLIP$_L$ were responsible for the inhibition of human CD8$^+$ CTL-mediated xenocytotoxicity

Human CD8$^+$ CTLs generated *in vitro* resulted in >80% lysis of both parental PEC and mock (Figures 2A and B). Overexpression of pig c-FLIP$_S$ in PEC resulted in approximately 50% inhibition of CTL-mediated lysis in S-3 PEC transfectants, which highly expressed pig c-FLIP$_S$ in the PEC cytosol. In contrast, only 30% suppression of lysis was found in S-1—the clone that expressed the lowest levels of the pig c-FLIP$_S$ gene (Figure 2A). Overexpression of pig c-FLIP$_L$ in PEC effectively prevented human CTL cytotoxicity (Figure 2B). Approximately 63% and 75% suppression of CTL cytotoxicity was observed in L-1 and L-2 PEC transfectants, respectively (Figure 2B). Overexpression of pig c-FLIP$_L$ appeared to be significantly more effective in cytoprotective effects than that of pig c-FLIP$_S$ ($p < 0.05$ S-3 vs. L-2).

PEC transfectants with either pig c-FLIP$_S$ or pig c-FLIP$_L$ gene were highly resistant to apoptosis induced by human CD8$^+$ CTLs

Representative pictures of either TUNEL assay or annexin V staining were shown in Figure 3, and the number of cells visible in these assays are summarized in Table 1. Many parental PEC and mock showed positive staining in both TUNEL and annexin V staining (Figure 3, Table 1). Apoptosis of S-1 c-FLIP$_S$ PEC transfectants did not significantly differ from parental PEC and mock cells; however, the number of apoptotic cells was markedly reduced in the S-2 and S-3 PEC c-FLIP$_S$ transfectants (Figure 3, Table 1). Similarly, the number of apoptotic PEC c-FLIP$_L$ transfectants was much lower than that observed in both parental PEC and mock (Figure 3, Table 1). Remarkably, in L-2 PEC transfectants, which expressed the highest levels of pig c-FLIP$_L$ genes, a very small number of cells exhibited positive staining for both TUNEL and annexin V staining (Figure 3, Table 1).

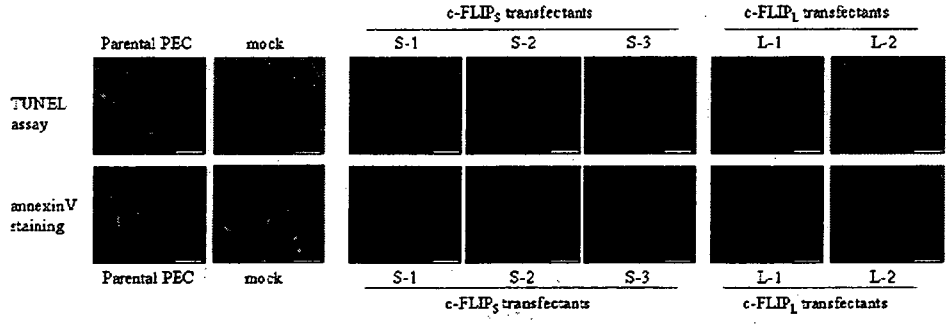

Figure 3: Detection of cell apoptosis by either TUNEL assay or annexin V staining. Parental PEC, mock and PEC transfectants were cocultured with human CD8$^+$ CTLs to induce apoptotic signals. Subsequently, cells were washed by PBS, then the reaction mixtures of either TUNEL or annexin V staining were added. Treated cells were analyzed by Biozero fluorescence microscopy. Pictures are representative of these assays obtained from five experiments per transfectant. White bars shown in the lower right of each pictures indicate 100 μM.

Table 1: The number of apoptotic cells in parental PEC, mock and PEC transfectants xenografts

Cells	TUNEL assay Positive cells (cells/HP[1])	p-Value	Annexin V staining Positive cells (cells/HP)	p-Value
Perental PEC (n = 5)	46.7 ± 14.2	–	201.5 ± 40.3	–
Mock (n = 5)	42.0 ± 16.4	NS[2]	229.6 ± 55.0	NS
S-1PEC transfectant (n = 5)	30.0 ± 5.0	NS	153.0 ± 22.0	NS
S-2 PEC transfectant (n = 5)	5.7 ± 3.1	$p < 0.05$	81.3 ± 60.8	$p < 0.05$
S-3 PEC transfectant (n = 5)	2.7 ± 0.9	$p < 0.01$	49.7 ± 25.8	$p < 0.01$
L-1 PEC transfectant (n = 5)	5.0 ± 4.2	$p < 0.05$	68.7 ± 18.4	$p < 0.01$
L-2 PEC transfectant (n = 5)	3.0 ± 0.8	$p < 0.01$	31.0 ± 20.0	$p < 0.01$

The number of apoptotic cells were counted by VH-analyzer software. Data are expressed as the mean ± SD from five independent experiments.
[1]HP = high-power fields (magnification, ×300).
[2]NS = not significant.

Pig c-FLIP$_{S/L}$ down-regulated both caspase-8, -10 and -3 activities

Caspase-8, -10 and -3 activities were measured to determine if overexpression of pig c-FLIP$_{S/L}$ in PEC down-regulates the activities of these enzymes. Coincubation of either parental PEC or mock with human CD8+ CTLs strongly activated caspase-8, -10 and -3 (Figures 4A–C). In contrast, pig c-FLIP$_{S/L}$ PEC transfectants had significantly reduced the activities of caspase-8, -10 and -3. The percent reduction in caspase-8 activity was 25–43% in the pig c-FLIP$_S$ transfectants and 51–58% in the pig c-FLIP$_L$ transfectants, respectively. Caspase-10 activity was reduced 37–47% in the pig c-FLIP$_S$ transfectants and 57–61% in the pig c-FLIP$_L$ transfectants, respectively. Furthermore, caspase-3 activity was also inhibited 43–66% in the pig c-FLIP$_S$ transfectants and 60–66% in pig c-FLIP$_L$ transfectants.

Overexpression of pig c-FLIP$_{S/L}$ molecules can prolong xenograft survival

Immunohistochemistry of transplanted pig c-FLIP$_{S/L}$ transfectants suggested that the overexpression of these molecules in PEC was effective in prolonging xenograft survival. At day 2 posttransplant, the majority of both parental PEC, mock and PEC transfectants were intact, as shown in Figure 5A, and there was slight to moderate infiltration of CD4+ and CD8+ T cells in these xenografts (data not shown). At day 3 posttransplant, no major differences in surviving xenograft cells were noted between parental PEC, mock and PEC transfectants. However, large numbers of CD8+ T cells and CD68+ macrophages had infiltrated to both the parental and transfected PEC xenografts (Figure 5B). On the other hand, CD4+ T cells and CD 161+ NK cells were the minor infiltrating cells in these PEC xenografts (Figure 5B). No significant differences were observed between parental PEC and PEC transfectants in regard to the immunopathological findings, including either the phenotype or the number of infiltrated lymphocytes (Figure 5B). At day 5 posttransplant, both the parental PEC and mock xenografts were completely rejected. Five and 7 days posttransplant, the c-FLIP$_{S/L}$ PEC-transfected xenografts still remained intact (Figure 5A). At day 10 posttransplant, most of the c-FLIP$_{S/L}$ PEC-transfected xenografts were finally rejected.

Double-staining xenografts with vWF Ab and TUNEL reagent demonstrated that, in the parental PEC and mock xenografts, apoptosis, induced by the infiltrating lymphocytes, had started at day 3 posttransplant—despite the intact appearance of surviving xenografts. In contrast, there was no evidence of apoptosis in PEC-transfected xenografts at day 3 posttransplant (Figure 5C).

Discussion

The mechanism of cellular xenograft rejection, including human NK cells, macrophages and CD8+ CTLs, seemed to play a crucial role in achieving prolonged graft survival in pig-to-human xenotransplantation (18,19,45). The objectives of this study were to explore a novel strategy by means of the intracellular blocking of death receptor-induced apoptotic signals to prevent a human CD8+ CTL response against xenograft cells. Specifically, blocking the Fas/FasL pathway by the use of pig c-FLIP$_{S/L}$ molecules was investigated in the present study.

Triggering of pig Fas antigen, endogenously expressed on the pig cell surface by human FasL expressed on human CTLs, led to oligomerization of pig Fas antigen. Subsequently, the intracellular adaptor molecule, FADD and procaspase-8, were recruited to oligomerized pig Fas antigen, forming a DISC in pig cells. In the DISC, procaspase-8 was autoproteolytically cleaved, forming the active enzyme. When stably overexpressed, c-FLIP$_{S/L}$ competitively bound procaspase-8, interfering with the generation of the large active subunit of caspase-8. Therefore, because of the intra- and inter-species binding compatibilities of intracellular pig molecules, pig c-FLIP$_{S/L}$ genes were employed in this study.

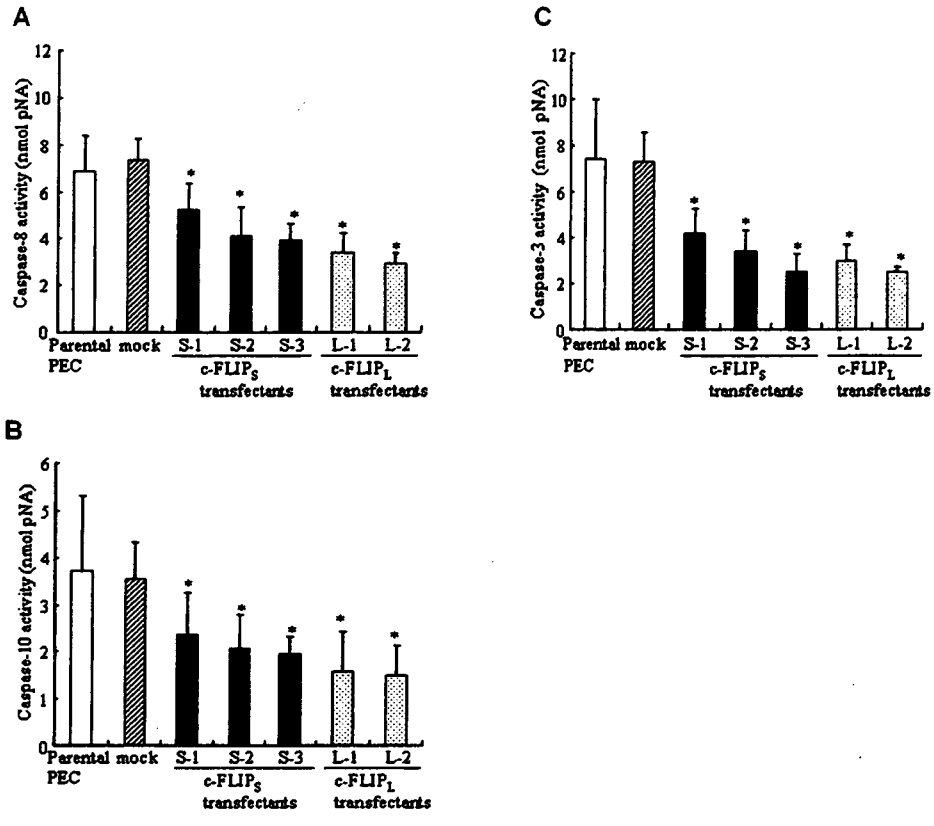

Figure 4: Inhibitory effects on caspase-8, -10 and -3 enzyme activities in PEC transfectants. Parental PEC, mock and PEC transfectants were cocultured with human $CD8^+$ CTLs. Subsequently, cells were recovered and then incubated with caspase-8, -10 and caspase-3 substrates, respectively. Caspase enzyme activities were measured as indicated by the absorbance at 405 nm. (A) Inhibitory effects of c-$FLIP_{S/L}$ overexpression on caspase-8 activation. (B) Inhibitory effects of c-$FLIP_{S/L}$ overexpression on caspase-10 activation. (C) Suppressive effects of c-$FLIP_{S/L}$ overexpression on caspase-3 activation. Parental PEC (□), mock transfectant (▨), c-$FLIP_S$ PEC transfectants (■), c-$FLIP_L$ PEC transfectants (▩). Each value is expressed as the mean ± SD. from five independent experiments. *The difference was statistically significant ($p < 0.05$ PEC transfectants vs. parental PEC, mock).

Two alternative splicing isoforms, pig c-$FLIP_S$ (642 bp and 214-aa) and pig c-$FLIP_L$ (1446 bp and 482-aa) were identified from the cDNA library prepared from follicular granulosa cells obtained from pig ovaries (43,44). Unexpectedly, the amino acid sequence homologies between pig and human c-$FLIP_S$ were as follows: overall, 79%; DED1, 88% and DED2, 84% (43). Similarly, the amino acid sequence homologies between pig and human c-$FLIP_L$ were as follows: overall, 76%; DED1, 88%; DED2, 84% and the caspase-like domain, 74% (43). Accordingly, these findings may indicate that the major functions of c-$FLIP_{S/L}$ molecules are highly conserved between mammalian homologues and that the DEDs of human c-$FLIP_{S/L}$ can interact with the DEDs of the pig endogenous adaptor protein (FADD), resulting in competitive binding with endogenous pig initiator caspase, pig procaspase-8. Future studies are required to further confirm the binding compatibilities of these molecules between pig and other mammalian species.

The functions of c-$FLIP_{S/L}$, as potent inhibitors of Fas-induced apoptosis, have been well studied in immune cells and in human and murine tumor cell lines (34,46). However, more recent studies indicate that ectopically expressed c-$FLIP_L$ can support caspase-8 activation at the Fas DISC (38,39). Thus, the possibility is raised that c-$FLIP_L$ regulates both caspase-8 activation and Fas-mediated apoptosis in pig xenograft cells. This possibility may be better explored by overexpression studies.

The overexpression experiments, described in the present study, reinforce the conclusion that both pig c-$FLIP_S$ and c-$FLIP_L$ are significant inhibitors of death receptor-mediated apoptosis induced by human $CD8^+$ CTLs. Furthermore, pig c-$FLIP_L$ seemed to exhibit greater anti-apoptotic activity in xenograft cells compared pig c-$FLIP_S$. Based on these observations, the following reasons are suggested for the sufficient anti-apoptotic activity of pig c-$FLIP_L$ in pig xenograft cells. First, the anti-apoptotic activity of pig c-$FLIP_L$ is

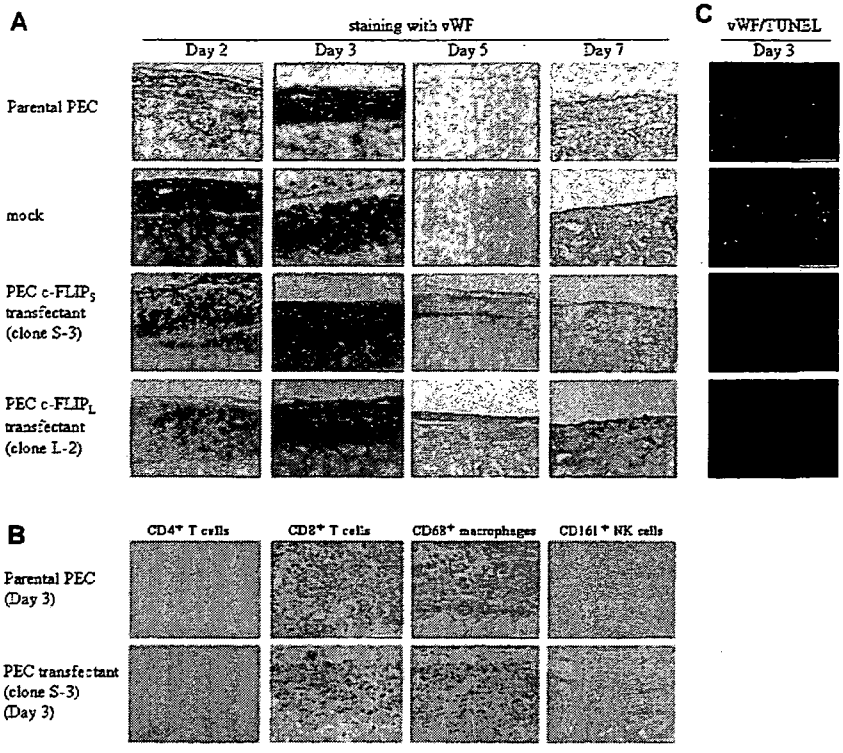

Figure 5: Histological appearance of rat kidney tissue of parental PEC, mock or PEC transfectants transplanted rats. (A) Immunostaining with anti-vWF Ab for transfected PEC of kidney specimens obtained at day 2, day 3, day 5 and day 7 posttransplant. To assess the efficacy of overexpression of c-FLIP$_{S/L}$ *in vivo*, we transplanted Lewis rats with either parental PEC, mock or PEC transfectants under kidney capsule. Pictures are representative of immunostaining of kidney sections obtained from 10 animals per transfectant group. At least five sections per individual kidney were studied. Non-specific bindings of the primary vWF Ab were not detected by control sections. (B) Immunostaining with appropriate mAbs for different lymphocytes subpopulations at day 3 posttransplant. CD8$^+$ T cells and CD68$^+$ macrophages were the main infiltrating cells in both the parental and transfected PEC xenografts. The phenotype of the infiltrate lymphocytes was similar in comparison with parental PEC and PEC transfectants xenografts. (C) Double labeling with both anti-vWF Ab and TUNEL staining for transplanted PEC of kidney specimens obtained at day 3 posttransplant. TUNEL-positive cells were observed in the xenografts of both parental PEC and mock, however, no TUNEL-positive cells were found in the xenografts consisting of pig c-FLIP$_{S/L}$ PEC transfectants. The bars in the lower right of each picture indicate 100 μM.

precisely controlled by its heterodimerization with caspase-8 through the caspase-like domain that is present in c-FLIP$_L$, but not in c-FLIP$_S$. Accordingly, it has been speculated that the affinity of the caspase-8/c-FLIP$_L$ heterodimer for FADD is higher than those of either the caspase-8 homodimer or the caspase-8/c-FLIP$_S$ heterodimer. In addition, considering the molecular mechanisms of c-FLIP$_{S/L}$ degradation, the c-FLIP$_L$ protein may have a significantly longer half-life than the c-FLIP$_S$ protein in pig xenograft cells. To prove these hypotheses, further studies will be required, and regulation of c-FLIP$_{S/L}$ expression and degradation may be important for understanding regulation of apoptosis in pig xenograft cells.

The pig pancreas is considered to be the most suitable source of islets for xenotransplantation into the patients with type 1 diabetes. Overexpression of c-FLIP$_{S/L}$ may have practical applications to xenografts, including pig pancreatic islet cells. Several investigators have reported that islet cell death, especially of insulin-producing β-cells, is triggered by apoptosis, immediately after the islet isolation (47,48). Saldeen previously reported that the combination of cytokines, including IL-1β, INF-γ and TNF-α, induces apoptosis in isolated rat islets (49). Therefore, these findings indicate that the islets may be exposed to these cytokines released from activated macrophages in the islets during and after isolation, and subsequently undergo apoptosis. Taken together, the overexpression of c-FLIP$_{S/L}$ in islets may be beneficial to not only prevention of human CD8$^+$ CTL-mediated xenocytotoxicity but also to the cytoprotective effects against apoptosis induced by the islet isolation procedure and by the exposure of cytokines. Moreover, up-regulation of c-FLIP$_L$ switches Fas signaling toward proliferation and increases insulin secretion and PDX-1 expression in human β-cells (50,51). Accordingly, c-FLIP$_L$ overexpression in pig islets (i.e. transgenic pig with c-FLIP$_L$ gene) may reduce xenograft rejection and islets apoptosis, enhance β-cell secretory function and

stimulate β-cell proliferation after transplantation of pig islet cells.

Finally, as described above, c-FLIP$_L$ mRNA and protein were abundantly expressed in granulosa cells of healthy follicles (44). In contrast, expression of c-FLIP$_S$ mRNA was low and did not differ between follicular stages (44). Therefore, c-FLIP$_L$ may play a crucial role in follicular selection and the overexpression of c-FLIP$_L$ may effectively improve fertility in gene-manipulated pigs, such as the α1, 3-galactosyltransferase gene-knockout pig.

Acknowledgments

We thank Dr. James L. McDonald for editing this manuscript, Prof. Noboru Manabe for kindly providing plasmids encoding pig c-FLIP$_{S/L}$ and Dr. Shuji Miyagawa for generously providing the pig endothelial cell line, MYP-30. This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 19591481), to M. T. from Japan Society for the Promotion of Science.

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Improvement of pancreatic islet cell isolation for transplantation

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Pancreatic islet transplantation is a promising treatment for diabetes but still faces several challenges. Poor islet isolation efficiency and poor long-term insulin independence are currently two major issues, although donor shortage and the need for immunosuppressants also need to be addressed. We established the Kyoto islet isolation method (KIIM), which has enabled us to isolate and transplant islets even from non–heart-beating donors. KIIM involves 1) cooling the donor pancreas in situ, 2) preserving the ducts with modified Kyoto solution, 3) using a modified two-layer pancreas preservation method, and 4) adjusting the density of the density gradient centrifugation and using an iodixanol-based solution for purification. KIIM has enabled us to transplant 17 islet preparations out of 21 isolations (an 81% success rate). All transplanted islets functioned, and all transplanted patients had improved glycemic control without hypoglycemic unawareness. Recently, we used KIIM for islet isolation from a brain-dead donor at Baylor, which resulted in a very high islet yield (789,984 IE) with high viability (100% by fluorescein diacetate/propidium iodide staining and a stimulation index of 4.7). This preliminary evidence suggests that KIIM may also be promising for islet isolation from brain-dead donors. In addition, to assess engrafted islet mass, we developed a secretory unit of islet transplant objects (SUITO) index: fasting C-peptide (ng/dL) / [fasting blood glucose (mg/dL) − 63] × 1500. This simple index has enabled us to monitor the engrafted islet mass. This index should be useful when deciding whether to perform additional islet transplantations to maintain insulin independence. Poor islet isolation efficacy and poor long-term results could be resolved with ongoing research.

Type 1 diabetes still represents a therapeutic challenge and remains a substantial burden for patients and their supporters. The Diabetes Control and Complications Trial showed that intensive insulin therapy improved glycated hemoglobin A_{1c} and protected against diabetic triopathy (1), but the penalty was a thrice-increased risk of serious hypoglycemic events, including recurrent seizures and coma (2). Whole-pancreas transplantation can make those patients insulin independent, but the morbidity of that procedure is too high to advocate it for most patients (3).

An attractive alternative is islet transplantation. The islet transplantation procedure doesn't involve major surgery, general anesthesia, or the complications related to exocrine enzymes. Since the first human islet allograft transplant was done in 1974 (4), this treatment has continuously improved, and a dramatic improvement was achieved with the Edmonton protocol in 2000 (5). Shapiro et al demonstrated that seven out of seven preuremic type 1 diabetic patients who received islet transplants became insulin independent, with a dramatic decrease in the frequency of hypoglycemic unawareness at 1 year posttransplantation.

Key elements of the Edmonton protocol are 1) avoidance of corticosteroids with combined sirolimus, tacrolimus, and anti-interleukin-2-receptor antibody therapy to protect against rejection and autoimmunity and 2) the use of two or more fresh islet preparations (within 3 to 4 hours after isolation) processed by the Edmonton islet isolation protocol. The Edmonton islet isolation protocol includes 1) procurement of the donor pancreas from a brain-dead donor and organ preservation in cold University of Wisconsin solution with a minimal storage period, 2) collagenase infusion via the main pancreatic duct using a pressure-controlled method, 3) pancreas digestion using the Ricordi system, 4) islet purification by continuous density gradient using Ficoll with a chilled COBE 2991 cell processor, and 5) removal of all xenoproteins from the islet isolation process (5). Isolated islets were transplanted immediately by simple gravity infusion.

There has been an exponential increase in clinical islet transplantation activity, with 471 patients transplanted at 43 international institutions (6). The University of Alberta, the University of Minnesota, and the University of Miami demonstrated that 82% of 118 recipients of completed transplants were insulin independent within the first year after transplantation (6). The University of Alberta further demonstrated progressive loss of insulin independence over time, leaving approximately 10%

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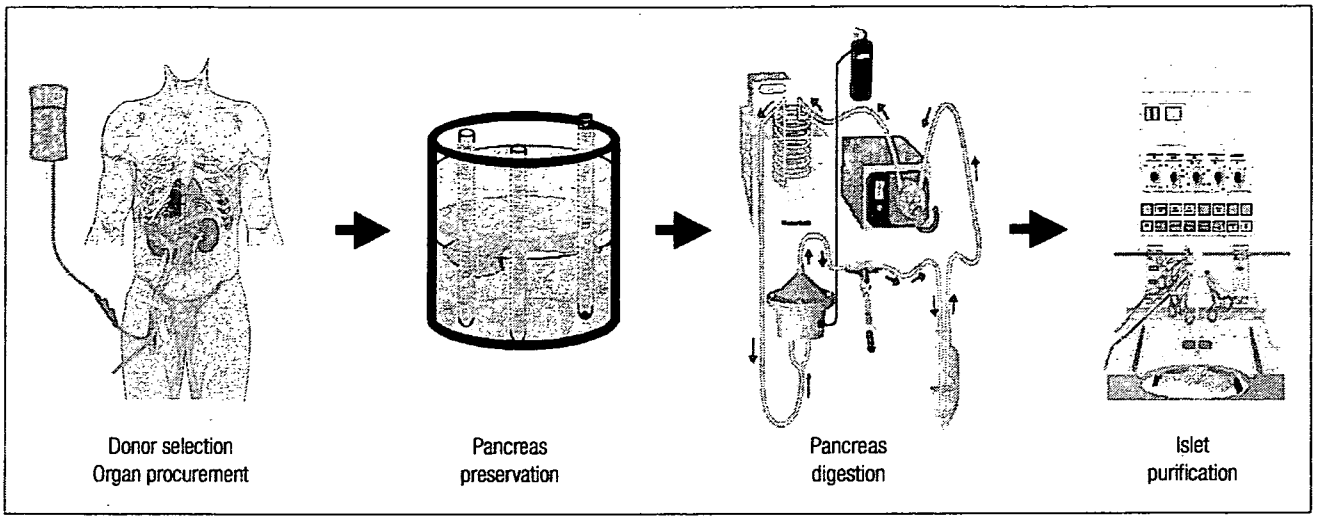

Figure. Donor selection and pancreas procurement is the first step for islet isolation. The procured pancreas is typically preserved by the two-layer method. When a pancreatic graft arrives at the islet isolation facility, the pancreas is distended with collagenase and digested in a Ricordi chamber. The final step is purification of isolated islets from exocrine tissues. A COBE 2991 cell processor is used for purification.

of patients still insulin independent at 5 years (7). However, more than 80% of patients continued to demonstrate persistent islet function at 5 years with effective prevention of recurrent hypoglycemia or severe lability combined with correction of hemoglobin A_{1c} (7). In addition, patients' quality of life was significantly improved by islet transplantation (8). Therefore, it should be reasonable to consider islet transplantation an option for the treatment of unstable type 1 diabetes.

The most difficult part of islet transplantation is islet isolation from a donated pancreas. Indeed, even in the leading centers, a transplantable yield of isolated islets is obtained in <50% of processed pancreata (9–11). In Japan, there is an extremely low number of brain-dead donors, and it has been almost impossible to utilize such donors for islet isolation. Therefore, alternative resources for islet isolation were sought, including islet isolations from non–heart-beating donors (NHBDs). The quality of the donor pancreas has a substantial impact on islet isolation. Therefore, using NHBDs for islet isolation is very challenging (12, 13).

In order to utilize pancreata from NHBDs for islet isolation, numerous protocols have been tested. Ultimately, an islet isolation method was established that enabled islet transplantation using pancreata from NHBDs (14–16). Very recently, this isolation method was applied to a regular brain-dead donor with promising preliminary data. In this article, we describe our endeavor to develop a new islet isolation method and our current effort to adapt this method to brain-dead donors.

THE PROCESS OF PANCREATIC ISLET ISOLATION

Pancreatic islet cell isolation starts at the point of donor selection and pancreas preservation *(Figure)*. When a pancreatic graft arrives at the islet isolation facility, the pancreas is distended with collagenase and digested in a Ricordi chamber. The final step is purification of isolated islets from exocrine tissues. After purification, islets are washed and put into a transplantation bag or culture medium before transplantation.

Donor selection

The quality of the donor pancreas is an important factor in successful islet isolation (17–20). However, pancreata are offered as whole organs first and only next for islet transplantation since whole-pancreas transplantation is considered an established treatment and islet transplantation is considered experimental.

Pancreata from obese donors give higher islet yield than those from lean donors (11, 17). Recently, researchers at the University of Minnesota demonstrated that the average islet yield from a pancreas donor with a body mass index (BMI) >30 was 319,129 IE and from a donor with a BMI <30 was 215,753 ($P = 0.0002$) (11). In addition, the donor with a BMI >30 had a higher islet isolation success rate—defined as isolations yielding >300,000 islet equivalents per pancreas, with purities of >50% (37.3% vs 15.9%; $P = 0.009$). This study showed that successful islet isolation can be difficult with low-BMI donors.

Previously, older donors were considered more suitable for islet isolation than younger donors (17). However, investigators have recently confirmed the advantages of islets from young donors, both in vitro in terms of insulin secretory function (21) and in vivo after transplanting islets in diabetic mice (22). In addition, we discovered that high islet yields could be obtained from younger donors with a modified islet isolation and purification method (18). The main obstacle to gaining high postpurification islet yields from young donors lies in the higher percentage of mantled islets embedded in acinar tissue. To recover mantled islets, we individualized the density of the high-density purification solution for each islet preparation (18). We believe that if we can recover mantled islets from young donors, the islet preparations should be of high quality, both in function and in islet number.

The stability of the donor during brain-dead status is another important factor. Based on the Edmonton protocol, many islet centers, including ours, have several exclusion criteria related to stability. The first concerns circulation and blood pressure:

prolonged hypotensive episodes are exclusion criteria when they have caused significant biochemical abnormalities (e.g., elevation of serum creatinine levels by >50% of the initial value or elevation of transaminases to levels >2 times the normal values). Cardiac arrest is an exclusion criterion when constantly stable circulation cannot be achieved in the 2 days following the event or when significant biochemical abnormalities have occurred, as described above. A third exclusion criterion relates to vasopressors, specifically norepinephrine, if required for maintenance of stable circulation. However, we have recently developed an islet isolation method for marginal donors, especially NHBDs. With this method, we have successfully isolated islets from marginal donors, and we have changed the criteria from absolute contraindications to relative contraindications. This change will significantly increase the islet isolation number, and we estimate that about 50% of donor pancreata will be used for islet isolation (Matsumoto et al, manuscript in preparation).

We have found that histology-proven chronic pancreatitis has led to the worst islet isolations (18). The duration of pancreatic digestion was significantly longer, and the undigested tissue volume was significantly larger with chronic pancreatitis. In addition, the purity of isolated islets was significantly lower with chronic pancreatitis. This finding suggests that fibrotic pancreata are resistant to collagenase digestion, resulting in poor islet isolation.

Pancreas preservation

Traditionally, the pancreas is preserved in University of Wisconsin solution. However, even for a short duration, oxygenated perfluorocarbon (PFC) provides the best method for pancreas storage (23–25). With the oxygenated PFC, pancreas grafts are directly oxygenated and continuously generate ATP (26), and the viability of endothelial cells is maintained (27, 28). Because of these effects, oxygenated PFC seems to be the most suitable substance for preservation before pancreas transplantation and islet isolation.

When PFC is added to the University of Wisconsin solution or another solution—the "two-layer method"—it is necessary to oxygenate the PFC just before pancreas preservation, ensure adequate oxygenation of PFC just before storage, and ensure sufficient attachment of PFC to the pancreata. With inadequate oxygenation to the pancreas, ATP production was low, and the benefit of the two-layer method was lost (29).

Complete immersion of the pancreas into oxygenated PFC—the "one-layer method"—seems to be better than the two-layer method (30). Even for the one-layer method, the top layer is necessary to keep oxygen from escaping from the surface of the PFC.

Pancreatic ductal preservation seems important because collagenase is delivered through the pancreatic duct (31). Sawada et al demonstrated that a small amount of University of Wisconsin solution perfused into the pancreatic duct significantly improved the results of islet isolation in a rodent model (31), and a European group introduced the method in humans (32). We have demonstrated that ductal injection of a large amount of modified Kyoto solution into the main pancreatic duct significantly reduced apoptotic cell death of both exocrine tissue and islet cells (Noguchi et al, *Cell Transplantation*, in press). We avoided University of Wisconsin solution for ductal injection since it inhibits collagenase activity, which is essential for pancreas digestion and islet isolation (33). We preferred a large ductal injection because the solution protects not only pancreatic ducts but also exocrine tissues.

Collagenase selection and delivery

Selection of collagenase is important for successful islet isolation, and currently Liberase is used exclusively (34). Liberase is considered to be the best collagenase, but lot-to-lot variation has been a concern. Kin et al demonstrated that optimization of thermolysin dosage based on caseinase unit per gram of pancreas contributed to the islet isolation outcome, but the collagenase dosage provided by the manufacturer (Wünsch unit per gram of pancreas) was not a major determinant of islet isolation outcome (9). In addition, they pointed out that the lot-to-lot inconsistency of the enzyme's performance was explained not by the activity values provided by the manufacturer but rather by the proportion of class I collagenase and class II collagenase, as determined by an in-house assay (9). Specifically, the odds of successful isolation were 8.67 times higher when a vial with a class II:class I ratio of <0.204 was used than when a vial with a ratio of ≥0.204 was used.

Collagenase delivery with pressure monitoring is the current standard (35). It is widely believed that during the infusion of collagenase into the pancreas, the goal is excellent distension with minimum leakage. An important modification that we have made is the use of only one cannula, inserted from the duodenal orifice of the main pancreatic duct (one-cannula method) (25). For collagenase delivery, usually a pancreas is cut and cannulas are inserted into two or three pancreatic ducts; this has been done since if the pancreatic duct is not adequately preserved, one cannula cannot deliver collagenase through the pancreas. However, this technique inevitably causes collagenase leakage. The one-cannula method, which requires the pancreas to be preserved intact and not cut, has resulted in minimal collagenase leakage with excellent distension.

Pancreas digestion

The Ricordi method is a standard for pancreas digestion in clinical islet transplantation (36). The key component of this method is a special Ricordi chamber for pancreas digestion and the effective collection of digested pancreatic tissue (36). The Ricordi chamber is designed for effective pancreas digestion with meticulous temperature control and is useful for effective dilution and collection of digested pancreatic tissue with a large volume of solution. Besides the Ricordi method, other static digestion methods have been effective for pancreas digestion (37, 38); however, those methods may not be effective for dilution. Since islets are sensitive to overdigestion, effective dilution may be important. Therefore, dilution, temperature control, and neutralization of digestive enzymes are all important, and the Ricordi method is the best to provide these conditions.

Trypsin inhibitors may help to avoid overdigestion (38). Previously, we have shown that the use of the trypsin inhibitor

Pefabloc during islet isolation using the simple open-pan islet isolation method improved islet yield in nonhuman primate and human models (38). The University of Alberta also demonstrated that human islet isolation was improved with Pefabloc when pancreata were preserved for extended time periods (39). However, trypsin inhibition had no effect on improved islet isolation when pancreata were procured from brain-dead heart-beating donors using the Ricordi islet isolation method (40, 41). In addition, when collagenase activity is not strong enough, trypsin may actually help to digest a pancreas. Therefore, trypsin inhibition during islet isolation might not be important when an optimal pancreas is processed with the Ricordi islet isolation method.

Islet purification

Purification of islets from exocrine tissue is a critical step for maintaining high islet yields. The common method of islet purification is density gradient centrifugation. Ficoll is widely used for density gradients (42) with a COBE 2991 cell processor (43). However, an iodixanol-based solution has contributed to increased islet yield, especially for porcine islet isolation (44–47). Iodixanol has low viscosity and, therefore, it needs less force during centrifugation. Iodixanol-based purifications were clinically applied by others as well as by our own group with promising results (15, 16, 48, 49). We diluted iodixanol with ET-Kyoto solution. However, other solutions, such as culture media or other preservation solutions, could be examined.

THE KYOTO ISLET ISOLATION METHOD FOR NHBDS

In Japan, it is difficult to use brain-dead heart-beating donors for islet isolation and transplantation. Therefore, we pursued islet transplantation from NHBDs (14, 15) or living donors (50–52). To initiate islet transplantation using NHBDs, we established several criteria that resulted in the Kyoto islet isolation method (KIIM) (14–16).

First, we inserted a double-balloon catheter before cessation of heart beating to chill the pancreas immediately after cardiac arrest (53). This technique enabled us to minimize warm ischemic time. It was demonstrated that islet yield and function deteriorated after 30 minutes of warm ischemia in rat and dog models (54). As a matter of fact, without this technique, warm ischemic time is >30 minutes and results in unsuccessful islet isolation. Second, we introduced ductal injection immediately after procurement, as described in the pancreas preservation section. Third, we modified the two-layer (modified Kyoto solution and oxygenated PFC) method of pancreas preservation and recently switched to a one-layer method. Fourth, we used ulinastatin for trypsin inhibition during islet isolation. Ulinastatin is not only a trypsin inhibitor but also an antiinflammatory drug (55). Therefore, this drug might be useful for an ideal donor with the Ricordi method. Finally, we performed density measurements on exocrine tissue since acinar tissue density could decrease during warm ischemia. Adjusting the density of the gradient solution enabled us to recover embedded islets. In addition, we used iodixanol instead of Ficoll for islet purification because iodixanol has low endotoxin activity and low viscosity, which should be less harmful for islets.

We have isolated 21 human pancreata using KIIM from NHBDs. Double-balloon catheters inserted before cardiac arrest were combined with kidney retrieval in 18 cases (18). The average transplanted islet yield was 382,945 ± 44,146 IE (4,589 ± 504 IE/g), and the purity was 46.8 ± 3.3%. The viability of transplanted islets, as assessed by acridine orange/propidium iodide, was 96.2 ± 0.7%, and all of the samples were above 85%. The average insulin stimulation index was 4.2 ± 1.8. Islet preparations from 17 cases (16 cases with a double balloon and one case without a balloon) met transplantation criteria. These islet preparations were transplanted into eight type 1 diabetic patients. In all cases after islet transplantation, hemoglobin A_{1c} levels were improved and there was no hypoglycemic unawareness. Thus, 17 out of 21 islet preparations (81%) achieved success. Compared with results of leading institutes, KIIM provided a very high success rate of transplantation, even using NHBDs (9–11).

SECRETORY UNIT OF ISLET TRANSPLANT OBJECTS INDEX

To assess engrafted islet mass, we developed the secretory unit of islet transplant objects (SUITO) index (56, 57). The formula of the SUITO index is as follows: fasting C-peptide (ng/dL) / [fasting blood glucose (mg/dL) – 63] × 1500. A SUITO index of 100 reflects 100% pancreatic beta-cell function in a healthy person. If the fasting C-peptide level is 0.8 ng/dL and blood glucose is 103, the SUITO index will be 0.8 / (103–63) × 1500 = 30.

Previously, we have shown that a SUITO index of >25 is necessary for insulin-independent status (56). The SUITO index of islet-transplanted patients with cultured islets from NHBDs was significantly lower than that of patients with fresh islets from NHBDs (56). In addition, living-donor islet-transplanted patients showed the highest SUITO index with insulin-independent status (56).

Recently at Baylor, we performed islet transplantation from a brain-dead donor without culturing the islets. After a single islet infusion, the average SUITO index from day 3 to day 30 was 29.7 ± 10.4. The patient's glycemic control improved substantially without hypoglycemic unawareness. The insulin dosage has been substantially reduced, and the patient is expected to be insulin independent. After single islet infusion with NHBDs, the average SUITO index from day 3 to day 30 was approximately 12. Therefore, islets from brain-dead donors seem to be of higher quality than those from NHBDs.

APPLICATION OF KIIM FOR ISLET ISOLATION FROM A BRAIN-DEAD DONOR

Recently, we applied KIIM for islet isolation from a brain-dead donor (except for the double-balloon technique, which is necessary only for NHBDs). In this case, the islet team joined the Baylor University Medical Center donor team for the pancreas procurement. The donor was a 52-year-old woman with a BMI of 39.1 and a pancreas weight of 98 g. After the pancreas was retrieved, the attached spleen, duodenum, and fat tissue were immediately removed. A single cannula was inserted from the duodenal orifice of the main pancreatic duct. Approximately 100

mL of modified Kyoto solution was infused. An accessory pancreatic duct was identified and was ligated with a hemoclip. The pancreas graft was preserved by the two-layer method (modified Kyoto and oxygenated PFC) and transported to the islet isolation laboratory at the Baylor Institute for Immunology Research. The cold storage period was approximately 3 hours. Upon arrival at the islet isolation laboratory, the pancreas was immediately immersed in the decontamination solution since it had already been trimmed. Chilled collagenase solution (Serva collagenase with neutral protease) was infused using a single cannula with pressure control. The pancreas distended excellently with minimum collagenase leakage. The distended pancreas was cut into nine pieces and put into the Ricordi chamber for digestion. Islets were purified using a density-adjusted Kyoto-iodixanol continuous density gradient with a COBE 2991 cell processor.

Islet yield after digestion and before purification was 803,467 IE. After purification, the islet yield was 789,984 IE with approximately 40% purity. A viability assay with fluorescein diacetate/propidium iodide showed that 100% of the islets were viable after purification, and the stimulation index with static glucose challenge was 4.7. Thus, the first trial of KIIM for islet isolation from a brain-dead donor is promising.

CONCLUSIONS

At this time, islet transplantation is the most promising method to cure diabetes with minimum risks—although the success rate for islet isolation is still relatively poor. However, continuous improvements in islet isolation are occurring. Single-donor islet transplantation for insulin independence should be established with an advanced islet isolation technique. The SUITO index is a powerful tool to estimate engrafted islet mass (56). We should supply islets to maintain a SUITO index of >30; this practice should result in patients who remain insulin independent for the long term. Current major concerns of poor islet isolation efficacy and long-term results could be resolved with ongoing research.

Acknowledgments

This study is partially supported by All Saints Health Foundation and Otsuka Pharmaceutical Company (Tokushima, Japan). We thank Dr. Carson Harrod for his careful review of this manuscript.

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