RESULTS

Expression Levels of Pig c-FLIP$_{S/L}$ Proteins of Parental PEC and PEC Transfectants

Although low level expressions of endogenous protein of c-FLIP$_S$ and pig c-FLIP$_L$ were detected in parental PEC, the amounts of either one were markedly increased in PEC transfectants (Table 1). We identified 3 positive clones of c-FLIP$_S$ and 2 positive clones of c-FLIP$_L$. Of the pig c-FLIP$_S$ transfectants, 1 clone showed a low protein expression (A-4), while the other 2 clones revealed moderate (B-7) or high (B-2) expression of c-FLIP$_S$ protein. Of the pig c-FLIP$_L$ transfectants, 1 clone had moderate protein expression (E-10), whereas the other clone expressed high levels (F-5). The expression levels of pig c-FLIP$_S$ protein in clone B-2 were approximately 3-fold higher than those in parental PEC, as judged by arbitrary units (Table 1). The expression level of pig c-FLIP$_L$ protein in clone F-5 was approximately 4-fold higher than parental PEC, as judged by arbitrary units (Table 1).

Human CD8$^+$ CTL-Mediated Killing Is Significantly Suppressed by Overexpression of Pig c-FLIP$_{S/L}$ Genes

Human CD8$^+$ CTLs generated in vitro expressed strong killing against parental PEC. These CTLs displayed a lysis of over 80% at an effector-to-target ratio of 50:1 (Fig 1A). The overexpression of pig c-FLIP$_S$ in PEC resulted in marked cytoprotection from human CTLs. An approximate 50% inhibition of killing was observed at an effector-to-target ratio of 50:1 among B-2 PEC transfectants which highly expressed pig c-FLIP$_S$ in the PEC cytosol. In contrast, only 27% suppression of this lysis was observed at the same effector-to-target ratio in A-4, which was the lowest expression clone of pig c-FLIP$_S$ gene (Fig 1B). From the results for pig c-FLIP$_L$ PEC transfectants, the overexpression of pig c-FLIP$_L$ molecule was markedly effective to prevent human CTL killing against PEC (Fig 1B). Approximately 61% and 74% suppression of CTL killing was observed at an effector-to-target ratio of 50:1 in E-10 and F-5 PEC c-FLIP$_L$ transfectants, respectively (Fig 1B). Overexpression of pig c-FLIP$_L$ appeared to be effective for cytoprotective effects compared with c-FLIP$_S$ (Fig 1B).

Overexpression of Pig c-FLIP$_{S/L}$ Molecules Is Effective in the Prolongation of Xenograft Survival

To prove that the overexpression of pig c-FLIP$_{S/L}$ molecules in PEC prolonged xenograft survival, we transplanted PEC transfectants under the rat kidney capsule. The results of the immunohistochemical analysis are summarized in Fig 1C.

At day 3 posttransplantation, numerous well-preserved parental PEC and PEC transfectants were observed under the kidney capsule (Fig 1C). At day 5 posttransplantation, parental PEC xenografts were completely rejected. In contrast, c-FLIP$_{S/L}$ PEC transfected xenografts remained intact (Fig 1C). The prolongation of xenografts with pig c-FLIP$_{S/L}$ PEC transfectants was intact at day 7 posttransplantation (data not shown).

DISCUSSION

Inhibition of mechanisms of cellular xenograft rejection mediated by human NK cells, macrophages, and CD8$^+$ CTLs achieved prolonged graft survival in pig-to-human xenotransplantation.[3–5] We sought to explore novel methods to prevent immune attack of human CD8$^+$ CTLs against xenograft cells by means of the intracellular blocking of death receptor-induced apoptotic signals, such as Fas/FasL pathway by the use of pig c-FLIP$_{S/L}$ molecules.

The functions of c-FLIP$_{S/L}$, as potent inhibitors of Fas-induced apoptosis, have been studied in human or murine immune elements and tumor cells. However, more recent studies have indicated that ectopically expressed c-FLIP$_L$ supports caspase-8 activation at the Fas death-inducing signaling complex (DISC).[10,11] Accordingly, we asked the question whether c-FLIP$_L$ is a dual function regulator of caspase-8 activation and of Fas-mediated apoptosis in pig xenograft cells. The overexpression experiments described herein clearly demonstrated that both pig c-FLIP$_S$ and pig c-FLIP$_L$ act as potent inhibitors of death receptor-mediated apoptosis induced by human CD8$^+$ CTLs. Furthermore, we observed in this study that pig c-FLIP$_L$ seemed more efficient for anti-apoptotic activity in xenograft cells in comparison with pig c-FLIP$_S$.

In the future, transgenic pigs with c-FLIP$_{S/L}$ genes will be generated to obtain their pig islets in attempts to prolong xenograft survival by conferring high resistance toward the immune attack with human CTLs.

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XENOTRANSPLANTATION

Prolonged survival of pig islets xenograft by adenovirus-mediated expression of either the membrane-bound human FasL or the human decoy Fas antigen gene

Kawamoto K, Tanemura M, Ito T, Deguchi T, Machida T, Nishida T, Doki Y, Mori M, Sawa Y. Prolonged survival of pig islets xenograft by adenovirus-mediated expression of either the membrane-bound human FasL or the human decoy Fas antigen gene.
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Abstract: Background: Pig islets are considered an attractive alternative treatment for patients with Type 1 diabetes. However, pig islet xenografts, transplanted into non-human primates, are directly rejected by cell-mediated processes. We have previously reported that cell-mediated xenograft-rejections, and especially human $CD8^+$ cytotoxic T lymphocytes (CTL)-mediated cytotoxicity, are highly detrimental to pig xenograft cells. Moreover, we have explored novel strategies for the prevention of CTL killing by overexpression of either human decoy Fas antigen or membrane-bound human FasL in pig endothelial cells. In this study, we assessed the cytoprotective effects of these molecules for pig islets both in vitro and in vivo.
Materials and methods: Pig islets were freshly isolated by modified Ricordi's methods. Subsequently, these islets were transfected with an adenoviral expression vector containing the DNA fragments of either membrane-bound human FasL or human decoy Fas. Transfected islets were transplanted into preimmunized diabetic rats under the kidney capsule. Control pig islets (i.e., MOCK), which were transfected with an adenoviral expression vector containing only the enhanced green fluorescent protein gene, were also transplanted.
Results: Efficiency of adenoviral expressions of these molecules in pig islets was approximately 80% at a multiplicity of infection of 100. In an in vitro assay, approximately 80% suppression of cytotoxicity was observed in membrane-bound human FasL-expressing pig islets and 60% inhibition of CTL killing was displayed in decoy Fas expression pig islets. In an in vivo transplant model, prolonged survival of pig islets xenografts, expressing either membrane-bound human FasL or human decoy Fas genes, was elicited in comparison with that of control islets xenografts.
Conclusion: The extracellular remodeling of either death receptor or death ligand genes by adenoviral expression was effective for the prevention of CTL-mediated xenocytotoxicity in pig islets.

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Key words: cellular rejection – cytotoxic T lymphocyte – human decoy Fas – membrane-bound FasL – pig islet – xenotransplantation

Abbreviations: Ad, adenoviral vector; CMV, cytomegalovirus; DsRed, Discosoma sp. red fluorescent protein; EGFP, enhanced green fluorescent protein; FITC, Fluorescein-isothiocyanate; MOI, multiplicity of infection; PE, phycoerythrin; PEC, pig endothelial cell; TBS, Tris buffered saline; TMRE, tetramethylrhodamine ethyl ester; TRITC, tetramethylrhodamine isothiocyanate; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labeling; vWF, von Willebrand factor

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Introduction

Human pancreatic islet transplantation has been shown to have the potential to treat patients with Type 1 diabetes [1,2]. However, a shortage of human donor pancreas is a serious problem that prevents the development of islet transplantation as an ideal treatment for diabetes mellitus. Therefore, pig islets have been considered an attractive, alternative donor source [3–5]. Recently,

encouraging results have indicated that both adult pig islets and neonatal pig islet-like cell clusters can be engrafted and will elicit sustained, long-term function in immunosuppressed non-human primates [6,7]. However, the severe morbidity associated with strong immunosuppressive therapy, including CD154-specific monoclonal antibody, FTY720, everolimus and leflunomide, may preclude their clinical use [6]. Therefore, a better alternative to immunosuppressive drugs is required for the long-term survival of islet xenografts.

This research team and others have reported that pig xenografts were rejected mainly by cell-mediated immunity, which includes $CD4^+$ T cells [8,9], $CD8^+$ cytotoxic T lymphocytes (CTL) [10,11], macrophages, [12,13] and NK cells [14]. In a pig-to-primate islet transplant model, pig islet xenografts were rejected mainly by T cell-mediated processes [6,7,15]. Our previous studies have demonstrated that human $CD8^+$ CTL are highly detrimental to pig xenograft cells, and this CTL cytotoxicity is mediated mostly by the Fas/FasL apoptotic pathway [10,11]. Moreover, we have explored two novel cytoprotective molecules: membrane-bound human FasL, which is resistant to metalloproteolytic cleavage and induces apoptosis in Fas-expressing effector cells such as CTL, and human decoy Fas antigen, which lacks a death domain in its cytoplasmic region and can compete with the endogenous pig Fas of pig xenograft cells to bind the human FasL of the effector cells. The overexpression of either human decoy Fas antigen or membrane-bound human FasL in pig endothelial cells (PEC) is significantly effective in preventing $CD8^+$ CTL-mediated cytotoxicity in an in vitro assay [11]. These findings led us to hypothesize that the gene delivery of either human decoy Fas antigen or membrane-bound human FasL into pig islets would be advantageous in the prevention of cell-mediated xenograft rejection and would contribute to the prolongation of pig islet xenograft survival. In this study, we used both an in vitro cytotoxicity assay and a pig-to-rat islet transplantation model to verify this hypothesis.

Materials and methods

Pig endothelial cell culture

A PEC line, MYP-30 [16], was cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated FBS (Sigma-Aldrich).

Pig islet isolation, single cell preparation, and viability assay

Adult pig islets were isolated and purified by modified Ricordi's method, as previously described [17,18]. Briefly, pancreatic glands were removed from adult pigs at a slaughterhouse (Large White/Landrace × Duroc, 2-years-old, 200 to 300 kg). The glands were subsequently shipped to our laboratory, using the two-layer method [18]. The pancreas was then infused with 350 ml of a 1.4 mg/ml cold Liberase HI solution (Roche Diagnostics, Basel, Switzerland) and cut into several pieces. Subsequently, these pieces were put into a Ricordi's digestion chamber (Umihira, Kyoto, Japan) and digested according to human islet isolation protocol [17]. The islets were then purified with iodixianol-based continuous gradients and cultured with completed Medium 199 containing 10% heat-inactivated pig serum. The purity of the islets was assessed by the percentage of dithizone-positive cells. For FACS analysis, isolated islets were incubated in 1 ml of TrypLE Express (Invitrogen) for 15 min at 37 °C to prepare single islet cells. Viability of pig islets was assessed by tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, Eugene, OR, USA) [19,20]. Single islet cells were prepared 24-hr post-isolation and then stained with 100 ng/ml of TMRE for 30 min. Subsequently, the fluorescence intensity of TMRE was analyzed at 582 nm with a FACSCalibur flow cytometer (BD Immunocytometry, San Jose, CA, USA).

Generation of human $CD8^+$ CTL

Human $CD8^+$ CTL were generated as previously described [11]. Briefly, 10 to 15×10^6 of fresh peripheral blood mononuclear cells, obtained from healthy volunteers, was co-cultured in a flask containing irradiated PEC and recombinant human IL-2 (50 U/ml, Shionogi, Osaka, Japan) in RPMI-1640 complete medium for 12 to 14 days. Magnetic beads coated with anti-human $CD8^+$ mAb, and employed as effector cells for the in vitro cytotoxicity assay positively isolated $CD8^+$ CTL.

Construction of plasmids and adenoviral vector

The DNA fragments encoding either human decoy Fas antigen (FD2) or membrane-bound human FasL (D4) were generated as previously described, respectively [21,22]. Adenoviral vector (Ad) containing both the enhanced green fluorescent protein (EGFP) and FD2 cDNAs (Ad-EGFP-FD2) was constructed by the full-length insertion method, using an Adenovirus Expression Vector Kit

(Takara Bio, Otsu, Japan) [23,24]. Briefly, cDNA encoding human decoy Fas antigen was cloned into an expression plasmid containing cytomegalovirus immediate early promoter ($P_{CMV\ IE}$), internal ribosome entry site 2 (IRES2) gene, EGFP, and SV40 poly A (Clontech, Pal Alto, CA, USA). This expression unit was then inserted into the SwaI cloning site of the cosmid cassettes, pAxcwit. These cosmids contained the adenovirus genomic sequence, except for E1 and E3. *Escherichia coli* DH5α was transformed with the packaging mixture of ligated DNAs to generate the desired shuttle vector. Adenoviral vector encoding the *Discosoma* sp. red fluorescent protein (DsRed) and D4 (Ad-DsRed-D4), and adenoviral vector encoding EGFP only (Ad-EGFP), were also constructed in a manner similar to that described above. Adenoviruses were amplified by the infection of 293 cells, purified on Cs discontinuous gradient, and stored at −80 °C until use. The titer of recombinant adenoviruses (multiplicity of infection; MOI) was measured, using the 50% tissue culture infectious dose ($TCID_{50}$) method [23].

Gene expression in pig islets

Fresh pig islets were incubated for 1 hr in complete Medium 199 and then rinsed with serum-free RPMI1640. Subsequently, the islets were resuspended in 200 μl of serum-free RPMI1640 and then exposed to either Ad-EGFP, Ad-EGFP-FD2 or Ad-DsRed-D4 for 1 hr at 37 °C at various MOI. The transfected islets were then washed and resuspended in complete Medium 199. To confirm the expression of these molecules in pig islets, fluorescence images were obtained after 48 hr post-transfection by confocal fluorescence microscopy (Biozero; Keyence, Osaka, Japan). To further determine the expression of either FD2 or D4 on the surface of pig islets, single cell preparations were stained with either PE-conjugated anti-human Fas (DX2) or FITC-conjugated anti-human FasL (4H9), respectively. Subsequently, stained cells were analyzed by FACSCalibur (BD Immunocytometry).

^{51}Cr release assay

For an in vitro cytotoxicity assay, a ^{51}Cr release assay was performed, as previously described [11]. Parental or transfected islets were incubated with $Na_2{}^{51}CrO_4$ for 24 hr (1μCi^{51}Cr/100 islets). Single cell preparations of the labeled pig islets were prepared and then washed three times to remove the free ^{51}Cr. Subsequently, these labeled islets were re-suspended in RPMI complete medium and human $CD8^+$ CTL were added as effector cells at various effector-to-target ratios. The plates were then incubated for 4 hr at 37 °C. The ^{51}Cr released from the dead cells was measured in the supernatants. To assess the mechanism of the CTL-mediated xenocytotoxicity toward pig islets, a blocking assay using Concanamycin A, which is a potent inhibitor for the perforin/granzyme pathway, and anti-human FasL mAb (4H9), which is a blocking antibody for Fas/FasL pathway, was performed as previously described [11]. The amelioration of CTL killing against Ad-EGFP-FD2- or Ad-DsRed-D4-transfected islets was assessed by comparison with either Ad-EGFP-transfected or parental pig islets.

Islet transplant studies

It is well established that Fas/FasL interaction is fully cross-reactive between humans and rodents [25]. Hence, to determine the in vivo prevention of CTL cytotoxicity by the overexpression of these molecules in pig islets, pig-to-rat islet transplants were performed [26]. Male Lewis rats (8 to 10 weeks old; Charles River Laboratories, Wilmington, MA, USA) were used as xenograft recipients. To immunize the recipient rats, pig kidney membranes were prepared as previously described [27]. Subsequently, the recipient rats were immunized intraperitoneally three times with 250 mg of pig kidney membrane at 1-week intervals prior to the islets transplantation. Recipient rats were also rendered diabetic by a single injection of 60 mg/kg streptozotocin (Sigma-Aldrich) at 2 weeks before transplantation. Their diabetic condition was confirmed by blood glucose levels of greater than 300 mg/dl on 2 separate days. The recipient rats were then distributed randomly between experimental groups that received either Ad-EGFP-, Ad-EGFP-FD2-, or Ad-DsRed-D4-transfected pig islets at an MOI of 30. The pellets of transfected pig islets containing 3000 IEQ were transplanted under the kidney capsules of recipient rats. The blood glucose level was monitored at several time points during the first 24 hr. The transplanted rats were then euthanized either at day 1, 3 or 5 post-transplant, and grafted kidneys were retrieved, formalin-fixed and embedded in a paraffin block to assess the immunohistological findings.

Cell transplant studies

To further investigate the mechanism of the rejection of xenografted cells in vivo, cell transplant studies were also performed [28,29]. Stable PEC transfectants of either human decoy Fas or membrane-bound human FasL were established by

lipofection. A 2×10^6 cell-pellet of either parental PEC, human decoy Fas-PEC, or membrane-bound human FasL-PEC was transplanted under the kidney capsules of preimmunized rats. The transplanted rats were then euthanized either at day 1, 3 or 5 post-transplant.

Immunohistochemistry

To detect the grafted pig islets, immunostaining for pig insulin (DakoCytomation, Glostrup, Denmark) was performed. In the cell transplant studies, PEC was identified using a rabbit anti-human von Willebrand factor (vWF) polyclonal antibody (1 : 200, DakoCytomation) [29]. To identify the phenotype of the infiltrating cells, immunostaining for rat CD4, CD8, CD68 for macrophage, and CD161 for NK cells (Serotec, Oxford, UK) were also performed [26]. Sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked by incubation in methanol containing 3% H_2O_2 for 15 min. After blocking with 10% BSA-Tris buffered saline containing 0.1% Tween (TBS-T) for 30 min, the sections were incubated with the primary antibodies (described above) for 16 hr. The sections were then incubated in biotinylated link antibody for 30 min, followed by incubation with HRP-conjugated streptavidin (DakoCytomation, LSAB2 kit) for 30 min. The immune complexes were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) in the presence of hydrogen peroxide. The sections were counterstained with hematoxylin solution, washed in running water, dehydrated, and mounted. All procedures were performed at room temperature, except for the primary antibody, which was done at 4 °C. The sections were rinsed with TBS-T three times between steps. The specificity for the primary antibody was verified by control sections, in which the primary antibody was omitted.

Double staining

Kidney sections from rats euthanized at day 3 post-transplant were doubly labeled for CD8, CD68, or von Willebrand factor (vWF) and terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labeling (TUNEL) [29,30]. Sections were stained with CD8, CD68, or vWF (DakoCytomation), as mentioned above, and then incubated with TRITC-conjugated goat anti-mouse IgG (DakoCytomation) instead of link antibody. Subsequently, the sections were incubated with 20 μg/ml of proteinase K for 15 min at room temperature. After rinsing with PBS, the sections were incubated with TUNEL reaction mixture (in situ cell death detection kit; Chemicon) composed of 45 μl TUNEL label solution conjugated with fluorescein and 5 μl of TUNEL enzyme for 60 min at 37 °C. The negative control section for TUNEL staining was incubated with 50 μl TUNEL label solution replacing the TUNEL reaction mixture. The fluorescence signals were observed, using confocal microscopy (Biozero). Apoptosis was detected as green nuclear staining, whereas infiltrating cells were detected by CD8 or CD68 as red membrane staining. Double-positive cells (green circled by red) in randomly selected high-power fields (HP) were counted, using a VH analyzer (Keyence).

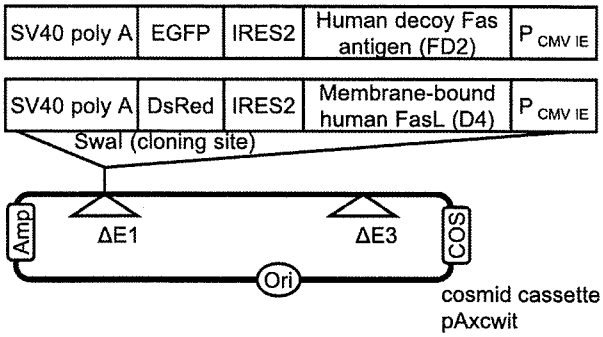

Fig. 1. Gene structures of EGFP-FD2 (human decoy Fas antigen) and DsRed-D4 (membrane-bound human FasL). These cosmids contain the adenovirus genomic sequence except for E1 and E3 (open triangles). Replication-defective adenovirus recombinants expressing either EGFP, EGFP-FD2, or DsRed-D4 were constructed using Adenovirus Expression Vector Kit. Amp, Ampicillin-resistance gene; COS, cos site.

Statistical analysis

Comparisons between each group were analyzed with a two-sided *t*-test, using Statcel2 software (oms-publishing, Tokorozawa, Saitama, Japan).

Results

Pig islets are susceptible to human $CD8^+$ CTL-mediated xenocytotoxicity through the Fas/FasL apoptotic pathway

The schemes of the cosmid cassettes and expression units of either EGFP-FD2 or DsRed-D4 are shown in Fig. 1. The endogenous expression of pig Fas in freshly isolated parental pig islets was determined by FACS analysis. As shown in Fig. 2A, the single cell preparation of parental pig islets clearly expressed pig Fas antigen on its cell surface. This finding may indicate that isolated pig islets may be sensitive to Fas-mediated cell death. An in vitro cytotoxicity assay demonstrated that fresh lymphocytes displayed no cytotoxic activity toward pig islets (data not shown).

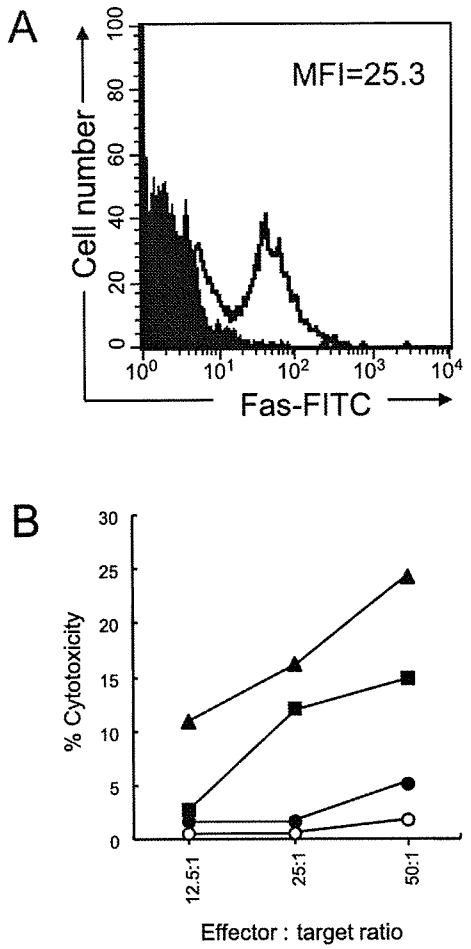

Fig. 2. (A) Flow cytometry analysis of freshly isolated pig islets. The data are from one of three representative islets. (open histogram) Single islet cells stained with anti-Fas mAb (clone 13). (closed histogram) Unstained pig islets. Mean fluorescence intensity (MFI) is expressed in the upper right of the histogram. (B) Cytotoxicity of cultured and purified human $CD8^+$ cytotoxic T lymphocytes (CTL) against non-transfected pig islets. Control killing of $CD8^+$ CTL (closed triangles). CTL killing blocked with 10 nM Concanamycin A (closed squares). CTL killing blocked with 10 μg/ml anti-human FasL mAb (closed circles). CTL killing blocked with both Concanamycin A and anti-FasL mAb (open circles). The data shown are representative of five different experiments.

However, human $CD8^+$ CTL, generated in an in vitro culture, were highly detrimental to parental pig islets. These human CTL displayed approximately 25% cytotoxicity at an effector-to-target ratio of 50 : 1. Blocking experiments showed that about 80% inhibition of $CD8^+$ CTL cytotoxicity was observed with anti-FasL mAb (4H9) treatment at the concentration of 10 μg/ml, whereas only 40% inhibition of CTL killing was shown with Concanamycin A blocking at 10 mM. Furthermore, more than 90% inhibition of CTL cytotoxicity was detected with the double blocking, using both Concanamycin A and anti-FasL mAb treatment (Fig. 2B).

Adenoviral vectors efficiently express human decoy Fas antigen and membrane-bound human FasL on the surface of pig islets

Confocal microscopy was used to detect the fluorescence of reporter proteins. Representative microphotographs of pig islets transfected with either Ad-EGFP-FD2 or Ad-DsRed-D4 at an MOI of 10, 30 or 100 for 48 hr post-transfection are shown in Fig. 3A. The fluorescence of transfected pig islets was markedly increased in an MOI-dependent manner. The protein expression of either FD2 or D4 was also detected by FACS analysis. The efficiency of transfection of human decoy Fas and membrane-bound human FasL was 81.4% and 79.3%, respectively. Therefore, the fluorescence intensity of EGFP or DsRed detected from transfected pig islets correlated well with the protein expression on their cell surfaces (Fig. 3B). To determine whether membrane-bound FasL that were overexpressed on the cell surfaces of pig islets may injure adjacent pig islets by binding with endogenous pig Fas antigen, TMRE staining was employed to assess the viability of transfected pig islets (Fig. 3C). The majority (86.2%) of single cell preparations from non-transfected pig islets belonged to a viable population, as judged by TMRE fluorescence levels (M1). The viability of pig islets transfected with either Ad-EGFP only or Ad-EGFP-FD2 only was not affected by high concentrations of both adenovirus and protein expression; on the other hand, the viability of Ad-DsRed-D4-transfected islets (81.2%) was actually down-regulated (Fig. 3C). A large quantity of membrane-bound FasL in pig islets may be harmful toward other pig islets.

Expression of either human decoy Fas antigen or membrane-bound human FasL in pig islets was effective for the inhibition of $CD8^+$ CTL-mediated xenocytotoxicity in vitro

As shown in Fig. 4, approximately 35% cytotoxicity was observed for parental islets at an effector-to-target ratio of 50 : 1. The cytotoxicity of human $CD8^+$ CTL against Ad-EGFP-transfected islets was approximately 50% and was higher than that against parental islets. In contrast with these controls, the overexpression of human decoy Fas in pig islets resulted in marked cytoprotection against human $CD8^+$ CTL. Approximately 60% suppression of $CD8^+$ CTL killing was observed at an effector-to-target ratio of 50 : 1 in Ad-EGFP-FD2-transfected pig islets. From the results of the membrane-bound human FasL transfected islets, the overexpression of this molecule was also quite effective in inhibiting $CD8^+$ CTL killing of pig islets. Approximately 80% suppression of $CD8^+$ CTL

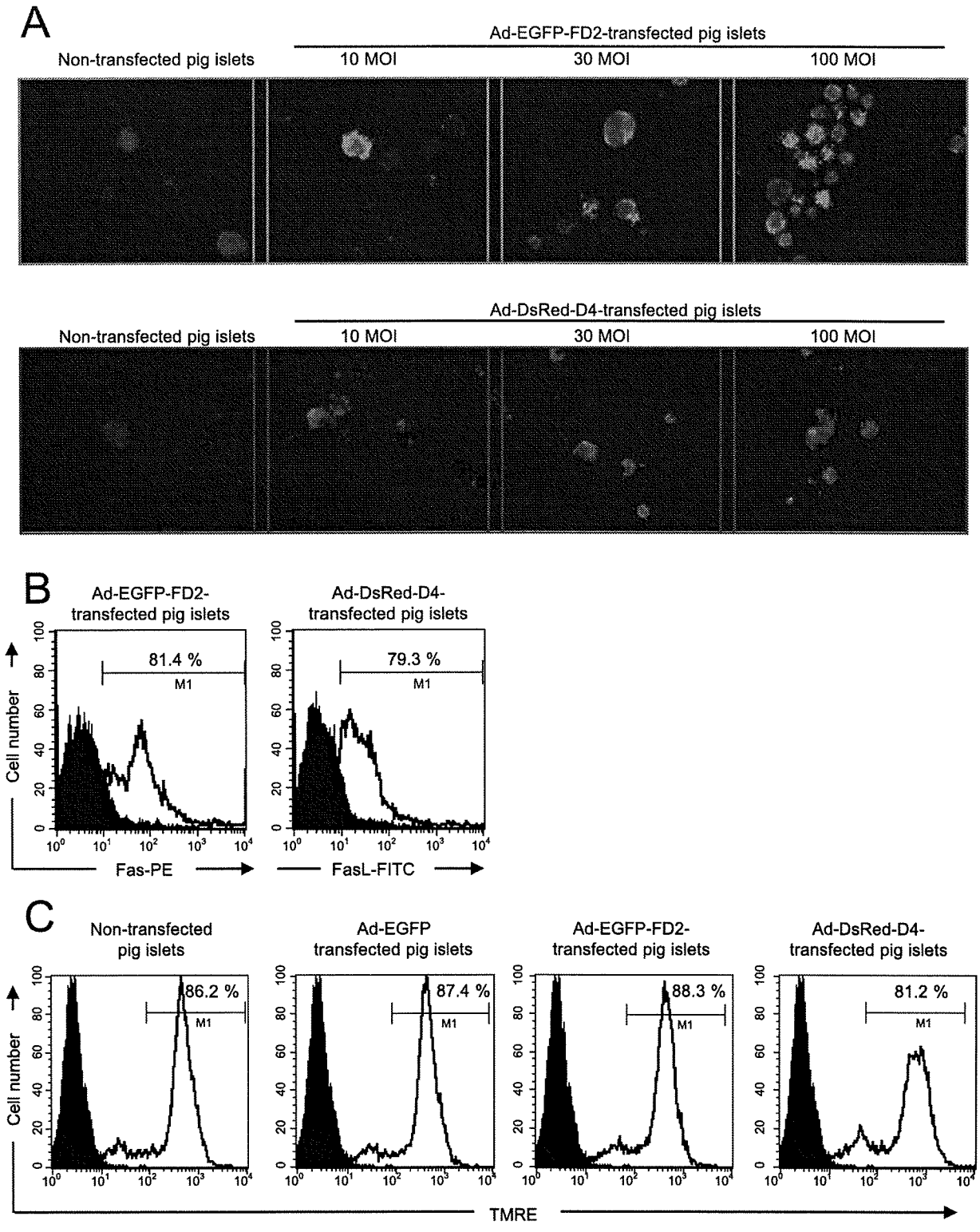

Fig. 3. Adenovirus-mediated overexpression of human decoy Fas antigen with a standard GFP-filter (upper panels) and membrane-bound human FasL with a TRITC-filter (lower panels). (B) Overexpression of human decoy Fas or membrane-bound human FasL in transfected pig islets. (closed histogram) Unstained islets. (open histogram) Stained islets with anti-human Fas mAb (DX2) or anti-human FasL mAb (4H9). The percentage of transfected islets displaying fluorescence in channels greater than 10 in each of the preparations are indicated in the upper right of each histograms (M1). (C) Viability of isolated pig islets. (closed histogram) Unstained pig islets. (open histogram) TMRE-labeled pig islets. The percentage of high TMRE cells displaying fluorescence in channels greater than 100 in each of the preparations is indicated in the upper right of each histogram (M1).

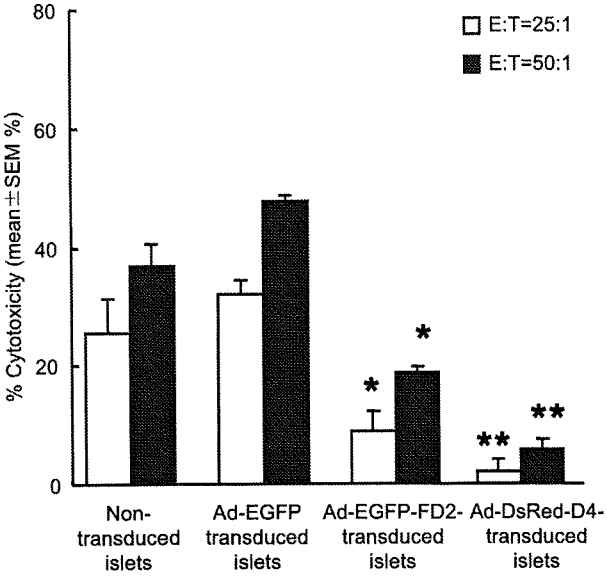

Fig. 4. Cytotoxicity assay of human decoy Fas antigen or membrane-bound human FasL transfected pig islets. Amelioration of $CD8^+$ cytotoxic T lymphocytes-mediated cytotoxicity by decoy Fas or membrane-bound FasL and controls (Non-transfected and EGFP-transfected islets). White bars indicate the cytotoxicity at E : T = 25 : 1. Black bars indicate the killing at E : T = 50 : 1. Data represent the mean ± SEM of five independent experiments with different volunteers. A statistical difference between cytotoxicity against EGFP-transduced islets and that against the experimental groups is indicated by asterisks ($^\star P < 0.01$, $^{\star\star}P < 0.001$).

cytotoxicity was observed at an effector-to-target ratio of 50 : 1 in Ad-DsRed-D4-transfected islets.

Overexpression of human decoy Fas and membrane-bound human FasL in pig islets was effective for the prolongation of pig islet xenograft survival

To determine the in vivo effectiveness of these molecules, a pig islet transplant study was performed. As described in relation to the viability assay assessed by TMRE, a high concentration of adenovirus (i.e., 100 MOI of adenovirus) may be toxic for pig islets. We transplanted pig islets, which were transfected with 30 MOI of adenovirus, under the rat kidney capsules. In the Ad-EGFP-transfected islets graft, the islet graft was intact at 1 day after transplantation, assessed by insulin staining (data not shown). However, numerous infiltrating mononuclear cells were observed in the islets graft. The pig islets xenograft transfected with Ad-EGFP was rejected at day 3 post-transplant (Fig. 5A). Compared with this control group, both Ad-EGFP-FD2- and Ad-DsRed-D4-transfected pig islets xenografts clearly survived at day 3 post-transplant (upper panels). Furthermore, at day 5 post-transplant, insulin-positive cells were still observed in both FD2- and D4- transfected pig islets (lower panels). These findings indicate that both human decoy Fas and membrane-bound human FasL elicited prolonged xenograft survival. To elucidate the phenotype of infiltrating cells, immunostaining of rat $CD4^+$, $CD8^+$, $CD68^+$, and $CD161^+$ cells were performed (Fig. 5B). The majority of infiltrating cells were $CD8^+$ CTL and $CD68^+$ macrophages, and, by contrast, the infiltration of $CD4^+$ T cells and $CD161^+$ B cells was weak.

Evaluation of graft survival and apoptosis in grafted kidney of parental or transfected PEC

To assess the cytoprotective mechanism of these molecules, cell transplant studies were performed using stably transfected PECs. At day 3 post-transplant, no significant differences in surviving xenografts were observed between parental PEC and PEC transfectants (Fig. 6A). However, at day 5 post-transplant, parental PEC xenografts were completely rejected, whereas both PEC transfectant xenografts remained intact. Large numbers of $CD8^+$ CTL and $CD68^+$ macrophages had already infiltrated into both parental and transfected PEC xenografts at day 3 post-transplant, and no significant differences were observed in either parental PEC or PEC transfectants xenografts (data not shown). Double-labeling analyses with apoptotic cells (i.e., TUNEL) and either vWF, CD8 or CD68 were performed, using the specimens at day 3 post-transplant (Fig. 6B). In parental PEC xenografts, apoptotic cells (green nuclear staining) were clearly detected at day 3 post-transplant. On the contrary, no apoptotic cells were observed in decoy Fas-PEC xenografts at day 3 post-transplant. In membrane-bound FasL-PEC grafts, many of the infiltrated cells were detected as apoptotic cells. Furthermore, the number of double-positive cells with either $CD8^+$ T cells or $CD68^+$ macrophages and TUNEL increased in the membrane-bound FasL-PEC grafted group (right panels), compared with the parental group or human decoy Fas-PEC grafted group. Double-positive cells (both TUNEL and CD8) from the section of FasL-PEC transplanted kidney (3.33 ± 0.58) were significantly increased, compared with either the parental PEC-transplanted group (0.67 ± 1.15) or the decoy Fas-PEC-transplanted group (0.67 ± 1.15) ($^\star P < 0.05$). Similarly, double-positive cells (both TUNEL and CD68) from the section of FasL-PEC transplanted kidney (8.33 ± 1.53) were statistically upregulated compared with either the parental PEC-transplanted cohort (1.33 ± 1.15) or the decoy Fas-PEC-transplanted cohort (1.33 ± 1.15) ($^\star P < 0.01$).

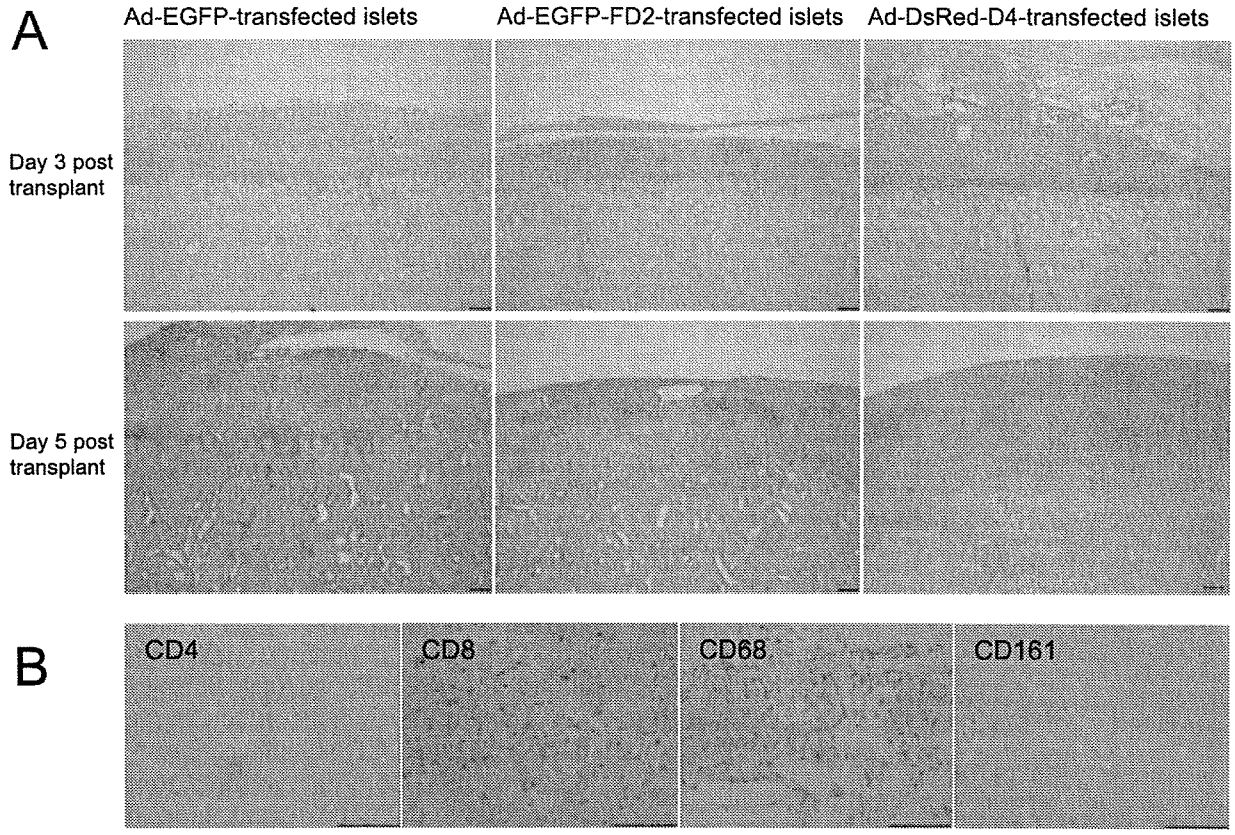

Fig. 5. Histological analysis of grafts from rats transplanted with either Ad-EGFP-, Ad-EGFP-FD2-, or Ad-DsRed-D4-transfected islets. (A) Insulin staining of representative sections from graft-bearing kidney transplanted with either Ad-EGFP, Ad-EGFP-FD2-, or Ad-DsRed-D4-transfected islets harvested at 3 days (upper panels) or 5 days (lower panels) post-transplantation. Black bars indicate 100 μm. (B) Immunohistochemical assessment of non-transfected islets-transplanted grafts (day 3 after transplant). Immunohistochemistry for rat CD4, CD8, CD68 or CD161 of a representative section was shown. Black bars indicate 100 μm.

Discussion

Human islet allotransplantation has recently received attention because of advances in the isolation of human islets, the survival of pancreatic islet allografts, and the immunosuppression regimens employed in preventing allograft rejection [1]. However, insulin independence is gradually lost over time in most cases [2,31]. Moreover, broad implementation of this therapy is limited by the large amount of human islets required per recipient. Pig islets appear to be a potential and unlimited source for pancreatic islet transplantation, and may resolve the worldwide shortage of pancreas donors [3–5]. Fortunately, two groups reported that insulin independence was achieved for more than 6 months in pig-to-primate islet xenotransplantation employing wild-type pigs as pancreatic donors [6,7]. However, this xenotransplantation eventually resulted in cell-mediated rejection [8–14]. Our previous studies demonstrated that cellular rejection by $CD8^+$ CTL is of particular importance for the long-term survival of pig xenografts [10,11]. Several immunosuppressive drugs reportedly show promise for the inhibition of cell-mediated immunity [32]; however, severe side effects resulting from immunosuppressive drugs may preclude their clinical use [6]. Therefore, novel strategies involving the remodeling of either death receptors or death ligands of pig islets are required to prevent cell-mediated xenocytotoxicity. In the present study, we assessed the effectiveness of human decoy Fas and membrane-bound human FasL for the inhibition of $CD8^+$ CTL-mediated xenocytotoxicity by adenoviral expression in pig islets. Taken together, our findings indicate that the overexpression of human decoy Fas in PEC can protect the xenograft cells from cellular rejection, including $CD8^+$ CTL and macrophages, without causing apoptosis for both PEC and infiltrated cells. On the other hand, expression of membrane-bound human FasL in PEC displayed cytoprotective effects for xenograft cells by killing the effector cells, including $CD8^+$ CTL and macrophages, through the Fas/FasL apoptotic pathway.

In both the pig islets and cell transplant models, we found severe infiltration of both $CD8^+$ CTL and macrophages into pig islet xenografts

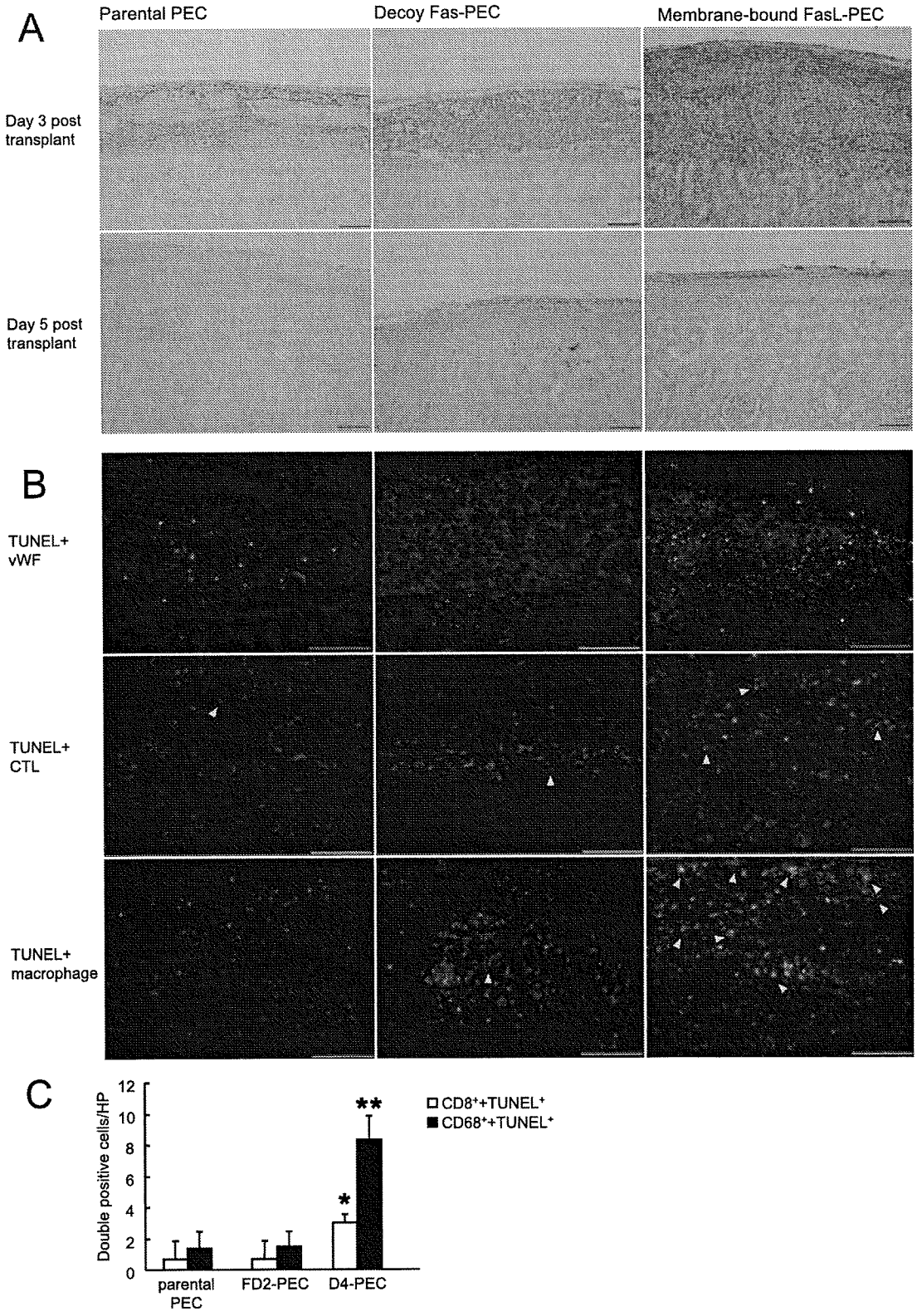

Fig. 6. Histologic analysis of grafts from rats transplanted with parental PEC, FD2-, or D4-transfected PEC. (A) Immunohistochemistry for vWF of a representative section of a parental PEC graft, decoy Fas transfected PEC transplant, or membrane-bound FasL transfected PEC harvested at 3 days (upper panels) or 5 days (lower panels) post-transplantation. Bars indicate 100 μm. (B) Dual labelings with TUNEL and either vWF (upper panels), CD8 (middle panels), or CD68 (lower panels) are shown. (C) Data represent the mean ± SD of double positive cells (green circled by red) in high-power fields of three individual mice/group. A significant difference between parental PEC-transplanted group and D4-PEC-transplanted group is indicated by asterisks ($^{\star}P < 0.05$ for $CD8^+$ cytotoxic T lymphocytes, $^{\star\star}P < 0.01$ for $CD68^+$ macrophages). White bars indicate 100 μm.

(Fig. 5B). As described above, the cytotoxicity of $CD8^+$ CTL against pig xenograft cells, including pig islets, is strong, and overexpression of either human decoy Fas or membrane-bound FasL can effectively prevent this strong killing of $CD8^+$ CTL. However, large numbers of macrophages infiltrated into pig xenografts secrete cytokines and may contribute to xenograft rejection. As shown in Fig. 6B, membrane-bound FasL overexpression in pig xenograft cells induced apoptosis for infiltrated macrophages. Therefore, membrane-bound FasL, which we developed, is effective not only for inhibition of $CD8^+$ CTL cytotoxicity, but also for prevention of macrophage killing.

The adenovirus-mediated expression of human decoy Fas or membrane-bound human FasL in pig islets effectively prevented human $CD8^+$ CTL-mediated xenocytotoxicity in vitro. Furthermore, prolonged survival of pig islet xenografts was elicited by the overexpression of these molecules in an in vivo study. Moreover, we monitored post-transplant blood glucose level. The post-transplant blood glucose levels in rats receiving both Ad-EGFP-FD2- and Ad-DsRed-D4-transfected islets was significantly lower than the blood glucose values obtained after the transplant of either non-transfected or Ad-EGFP-transfected pig islets at 12 and 18 hr post-transplant (data not shown). However, the improvement of blood glucose levels in transplanted rats was transient. This discrepancy between glycemic control and histological survival is seen often in islet transplant models. At the 5-year follow-up of the Edmonton protocol, EA Ryan and colleagues observed that the majority (~80%) of islet transplant recipients have C-peptide present (insulin staining positive), but only a minority (~10%) maintain insulin independence (keep normal blood glucose level) [31]. These results indicate that sufficient numbers of islets are required to maintain normal blood glucose levels. We judged the effectiveness of gene modification by histological survival. Another possible reason is that β-cells may be directly destroyed by adenovirus-induced cytolysis, or alternatively, the transfection process may induce a non-specific inflammatory reaction [33]. Additionally, the reason that the DNA fragments transfected by adenovirus were not integrated into the genome of pig islets may be that adenoviral gene expression is transient. Therefore, our final goal is to generate a transgenic pig that expresses these inhibitory molecules.

When measured by in vitro cytotoxicity assay, membrane-bound human FasL was more effective than human decoy Fas in the inhibition of CTL killing. However, the viability of pig islets expressing membrane-bound FasL was downregulated, as judged by TMRE staining. The reasons for this finding are as follows: (i) membrane-bound FasL-expressing pig islets grafts may kill other pig islets via endogenous Fas antigen in pig islets [34]; and (ii) FasL expression in islets may provoke inflammation and destructive insulitis [35]. These findings indicate that overexpression of FasL in islets plays a dual role as a cytoprotective molecule for $CD8^+$ CTL and as a mediator for islets graft injury [36]. Therefore, high expression of membrane-bound FasL in pig islets may not be beneficial for the protection of pig islets grafts.

These findings lead us to hypothesize that the double expression of both decoy Fas and membrane-bound FasL in pig islets may be more effective for the inhibition of CTL cytotoxicity than high expression of FasL in pig islets. Our previous data demonstrated that strong inhibition of $CD8^+$ CTL cytotoxicity can be elicited by the stable double expression of these molecules in PEC [11]. Others have also reported that the adenoviral-mediated double expression of vascular endothelial growth factor and interleukin-1 receptor antagonist improved the function of human islets [37]. Unfortunately, we cannot demonstrate the synergistic effects of double expression in pig islets in this study because of adenovirus-mediated toxicity against pig islets themselves (data not shown). In the future, double transgenic expression of these molecules in pigs may be useful for the long-term survival of pig islets.

Acknowledgments

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ELSEVIER

Adenovirus-Mediated Gene Expression of the Human *c-FLIP$_L$* Gene Protects Pig Islets Against Human CD8$^+$ Cytotoxic T Lymphocyte-Mediated Cytotoxicity

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ABSTRACT

Cell-mediated immunity, especially of human CD8+ cytotoxic T lymphocytes (CTLs) is believed to have an important role in the long-term survival of pig islet xenografts. Protection against human CD8+ CTL cytotoxicity may reduce the direct damage to pig islets and enable long-term xenograft survival in pig-to-human islet xenotransplantation. We have previously reported that *c-FLIP$_{S/L}$* genes, which are potent inhibitors of death receptor–mediated proapoptotic signals through binding competition with caspase-8 for recruitment to the Fas-associated via death domain (FADD), markedly suppress human CD8+ CTL-mediated xenocytotoxicity. In addition, the cytoprotective effects of *c-FLIP$_L$* seem to be significantly stronger than those of *c-FLIP$_S$*. Accordingly, in the present study, expression of *c-FLIP$_L$* was induced in intact pig islets by adenoviral transduction. Consequently, the cytoprotective capacity of the transgene in pig islets was examined in in vitro and in vivo exposure to human CD8+ CTLs. Cells from untransduced islets or mock islets were sensitive to CD8+ CTL-mediated lysis (59.3% ± 15.9% and 64.0% ± 8.9% cytotoxicity, respectively). In contrast, cells from pig islets transduced with the *c-FLIP$_L$* gene were markedly protected from lysis (30.5% ± 3.5%). Furthermore, prolonged xenograft survival was elicited from pig islets transduced with this molecule as assessed using an islet transplant model using the rat kidney capsule. Thus, these data indicate that intact pig islets can be transduced to express *c-FLIP$_L$* with adenovirus. Pig islets expressing *c-FLIP$_L$* are significantly resistant to human CTL killing and further exhibit beneficial effects to prolong xenograft survival.

THE EDMONTON PROTOCOL for human allogenic islet transplantation can successfully restore enodogenous insulin production and glycemic stability in patients with type 1 diabetes mellitus. However, insulin independence is usually not sustained despite islet infusions from two or more donors.[1] The current supply of islets from deceased human donors will almost certainly never meet

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the demand. Xenotransplantation using pig islets could potentially resolve the shortage of donor islets. Pigs are the favored donor species because of (1) their similar physiology to human beings; (2) unlimited availability owing to a short generation interval because of the high number of pregnancies; and (3) somatic cloning is possible, and, thus, production of transgenic animals can be substantially enhanced.[2] In particular, pig islets have precise glycobiologic advantages because they lack α-gal epitopes, which induce complement activation, hyperacute rejection, and acute vascular rejection of xenografts. Therefore, successful short-term xenograft survival of these islets is the result of prevention of hyperacute rejection. However, after initial survival of pig islets, infiltrating cells into pig islet xenografts, including natural killer cells, macrophages, and CD8+ CTLs, are directly cytotoxic to the islets.[3–6] In previous studies, we reported that direct cytotoxicity of human CD8+ CTLs to pig islets is mediated in major part by the Fas/FasL apoptotic pathway.[4–6] 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 the Fas-associated via death domain.[7–9] Two major c-FLIP variants result from alternative messenger RNA splicing: a short 26-KDa protein (c-FLIP$_S$) and a long 55-KDa form (c-FLIP$_L$).[7–9] We have demonstrated that the overexpression of *c-FLIP$_{S/L}$* genes markedly suppresses human CD8+ CTL-mediated xenocytotoxicity. In addition, the cytoprotective effects of c-FLIP$_L$ seem to be significantly stronger than those of c-FLIP$_S$.[6] The cytoprotective effect of c-FLIP$_L$ in pig islet cells remains controversial. Accordingly, in the present study, we evaluated the cytoprotective activities of the c-FLIP$_L$ molecule by adenovirus-mediated gene expression in pig islets.

MATERIALS AND METHODS

Pig Islet Isolation

Adult pig pancreases were removed at a slaughterhouse that handles young market-weight pigs (Large White–Landrace X–Duroc; age, 2 years; weight, 200–300 kg). Pig islets isolated using the modified Ricordi method as previously described[10,11] were maintained in complete medium 199 containing 10% heat-inactivated pig serum. Pig islet purity was assessed using the percentage of dithizone-positive cells.

Construction of Adenovirus Vector

The pAdex1CAwt adenovirus vector, regulated by the CAG (chicken β-actin) promoter, containing the open reading frame of the human *c-FLIP$_L$* gene, was purchased from RIKEN BioResource Center, Wako, Japan. The adenovirus was propagated by infection of human embryonic kidney 293 cells. Subsequently, adenovirus was purified using a Cesium/Tris gradient, separated into aliquots, and stored at −80°C until use. The titer of recombinant adenoviruses (multiplicity of infection [MOI]) was measured using the 50% tissue culture infectious dose method.[12] The "empty" control adenovirus, which lacks the human c-FLIP$_L$ insert, was also amplified in the same manner.

Transduction of Pig Islets by Adenovirus Vector

Freshly obtained adult pig islets represented in 500 μL of serum-free medium 199 were exposed to adenovirus encoding human c-FLIP$_L$ at a MOI of either 10 or 30 for 1 hour at 37°C. The transfected pig islets were then rinsed with serum-free RPMI (Roswell Park Memorial Institute) medium and resuspended in complete medium 199. Pig islets transfected with empty control adenovirus, which lacks the complementary DNA fragments of human c-FLIP$_L$ (ie, mock islets), were used as the vehicle control. Western blot analysis was performed to identify the expression of this molecule in pig islets.

Generation of Human CD8+ CTLs

To generate human CD8+ CTLs, peripheral blood mononuclear cells, freshly obtained from the blood of healthy volunteers were separated. Then 10 to 15 $\times$ 10^6 cells of separated peripheral blood mononuclear cells were cocultured for 14 days with irradiated pig endothelial cell monolayers in the presence of 50 U/mL of recombinant human IL-2 as previously described.[5,6] Subsequently, human CD8+ CTLs were positively selected using magnetic beads (Dynal Biotech AS, Oslo, Norway) and subjected to an in vitro cytotoxicity assay.

In Vitro Cytotoxicity Assay

The cytotoxic activity of human CD8+ CTLs against pig islets was assessed using a chromium 51 (^{51}Cr) release assay.[13] Either parental pig islets, mock islets, or transfected pig islets were incubated with Na_2-$^{51}CrO_4$ for 24 hours (1 μCi of ^{51}Cr per 100 islets). Subsequently, ^{51}Cr-labeled pig islets were plated in 96-well plates as target cells for admixture with human CTLs isolated using magnetic beads at various effector-to-target ratios. The ^{51}Cr released from dead islet cells was measured in the supernatants.

Transplant Studies and Immunohistochemical Analysis

To prove the in vivo effectiveness of this molecule to prolong xenograft survival, parental, mock, or transfected pig islets were transplanted under the kidney capsule in 8- to 10-week-old Lewis rats (Oriental Yeast Co, Ltd, Tokyo, Japan). The animals were randomly distributed between the three experimental groups. Rats preimmunized intraperitoneally with 250 mg of pig kidney membranes three times at 1-week intervals were the recipients.[5,6] In each case, 3000 IEQ of either parental, mock, or transfected pig islets were transplanted under the kidney capsule in the absence of immunosuppression. Transplant recipient rats were monitored until the time of harvest at day 3 or day 5 posttransplantation. Each grafted kidney was analyzed at immunohistochemistry. Kidney specimens were cut into small blocks, fixed in formalin, and embedded in a single paraffin block. After quenching endogenous peroxidase activity by exposure to 3% hydrogen peroxide–methanol, paraffin sections were stained with anti-pig insulin antibody (DAKO, Glostrup, Denmark) to detect surviving pig islet xenografts. The sections were rinsed and incubated with link antibody, followed by incubation of with horseradish peroxidase–conjugated streptavidin. Immunostaining was visualized with 0.02% diaminobenzidine (Sigma-Aldrich Corp, St Louis, Missouri) as the chromogen.

Statistical Analysis

Data were evaluated using the t test, with $P < .05$ considered significant. Data are presented as means (SD).

RESULTS

Protein Expression of Human c-FLIP$_L$ in Pig Islets

No protein expression of c-FLIP$_L$ was observed in parental pig islets (Fig 1A). The MOCK pig islets transduced with

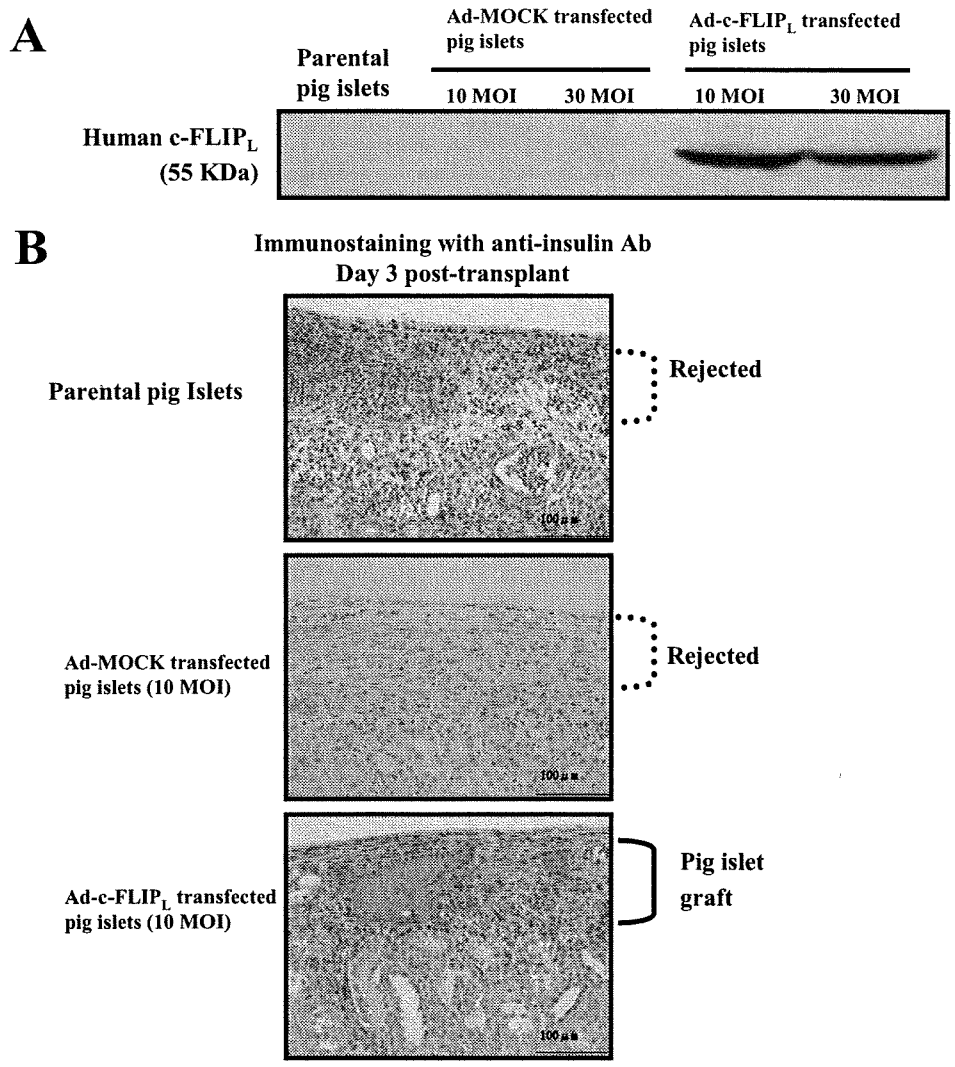

Fig 1. **(A)** The intracellular expression of c-FLIP$_L$ protein. Adenovirus-mediated gene expression of human c-FLIP$_L$ protein was assessed by Western blot analysis. **(B)** Immunohistological findings of rat kidney tissue of pig islets transplanted rats. Immunostaining with anti-pig insulin Ab for transplanted pig islet xenografts obtained at day 3 post-transplant. The black bars in each picture indicated 100 μm.

control adenovirus vector did not exhibit protein expression of c-FLIP$_L$ at adenovirus concentrations of 10 and 30 MOI. In contrast, transduction with adenovirus vector containing complementary DNA of c-FLIP$_L$ resulted in distinct expression of this molecule at 10 and 30 MOI. The expression level of c-FLIP$_L$ was similar compared with adenovirus transduction of 10 and 30 MOI (Fig 1A).

Adenovirus Expression of c-FLIP$_L$ Effectively Inhibits Cytotoxicity of Human CD8+ CTLs Against Pig Islet Cells

Human CD8+ CTLs generated by in vitro culture exhibited strong direct killing against parental and mock islets. Approximately, 60% lysis of both parental and mock islets was evident in these human CTLs at an effector-to-target ratio of 50:1 (Table 1). In contrast, the cytotoxicity was significantly reduced against pig islet cells transduced with the c-FLIP$_L$ adenovirus vector, that is, 52% inhibition at an effector-to-target ratio of 50:1 (Table 1).

Prolonged Xenograft Survival Was Elicited From c-FLIP$_L$-Transfected Pig Islet Cells

To determine whether adenovirus expression of c-FLIP$_L$ in pig islets can prolong xenograft survival, we transplanted pig islets under the kidney capsule in rats. The results of immunohistochemical analysis are shown in Fig 1B. At day 2 posttransplantation, parental, MOCK, and transfected pig islet xenografts survived under the kidney capsule (data not shown). At day 3 posttransplantation, parental and MOCK pig islet xenografts were completely rejected (Fig 1B). In contrast, pig islet xenografts expressing c-FLIP$_L$ survived intact as judged by insulin staining (Fig 1B). At day 5 posttransplantation, pig islet xenografts expressing c-FLIP$_L$ still exhibited insulin staining despite reduced graft size (data not shown). These findings demonstrate the beneficial effects of both in vitro and in vivo cytoprotection of pig islet xenografts expressing c-FLIP$_L$.

Table 1. ^{51}Cr Release in Pig Islets

Pig Islets	Adenovirus Concentration, MOI	Percent Cytotoxicity at ^{51}Cr Release Assay, Mean (SD) E/T Ratio 50:1	E/T Ratio 25:1
Parental		59.3 (15.9)	47.6 (8.2)
Mock (control adenovirus transfected pig islets)	10	64.0 (8.9)	48.7 (14.8)
	30	59.0 (1.4)	43.3 (5.5)
c-FLIP$_L$ transfected pig islets	10	30.5 (3.5)*	24.3 (1.6)*
	30	23.6 (11.6)*	21.0 (11.0)*

Abbreviations: ^{51}Cr, chromium 51; E/T, effector-target; cFLIP$_L$, cellular FLICE-like inhibitory protein, long form; MOI, multiplicity of infection.

Amelioration of human CD8+ cytotoxic T lymphocyte–mediated cytotoxicity by transduced pig islets was assessed by ^{5}Cr release assay Control parental and mock pig islets were estimated at the E/T Ratio of either 25:1 or 50:1. Values are given as the mean (SD) from five independent experiments.

*Difference statistically significant ($P < .05$, c-FLIP$_L$-transfected pig islets vs parental and mock pig islets).

DISCUSSION

In the present study, we determined that the expression of human c-FLIP$_L$ can be induced in pig islet cells using adenovirus vectors. Pig islet xenografts expressing this molecule were markedly protected from direct human CD8+ CTL-mediated lysis. Furthermore, beneficial effects of in vivo prolongation of pig islet xenografts with adenoviral expression of c-FLIP$_L$ were demonstrated.

It is generally thought that the adenoviral vector is not able to penetrate more than a few cell layers. In a previous study, we demonstrated that the virus vector used was able to infect more than 80% of islet cells, as assessed using fluorescence-activated cell sorting, and that protein expression in big islets was restricted to the outer cell layers.[13,14] In addition, because the new DNA is not integrated into the genome of the infected cells, the gene expression is only transient. The strategy of adenovirus-mediated expression in pig islet cells may have only restricted application to clinical islet xenotransplantation. Another strategy would be to generate transgenic pigs expressing the c-FLIP$_L$ molecule in the islet cells. However, in the study in which islets isolated from transgenic pigs expressed high levels of human decay-accelerating factor on endothelial cells, no or only minimal levels of this factor were detected on the islet cells.[15] Therefore, these findings indicate that transgenic pigs, in which the gene constructs containing c-FLIP$_L$ may be regulated by, for example, the insulin promoter, will have to be created to provide sufficient cytoprotection against CD8+ CTL cytotoxicity in pig islet xenotransplantation.

In this pig islet transplant model, large infiltrations of both CD8+ T cells and macrophages were detected. A large number of macrophages infiltrating pig islet xenografts secret inflammatory cytokines including IL-1β, tumor necrosis factor–α, and interferon-γ, which may induce β-cell damage through activation of several intracellular stress-signaling pathways.[16] Our preliminary data suggest that pig islet cells expressing c-FLIP$_L$ induce resistance against cytokine exposure containing 100 U/mL of IL-1β, 1000 U/mL of tumor necrosis factor–α, and 1000 U/mL of interferon-γ, as assessed using both the tetramethylrhodamine ethyl ester assay and the colorimetric methyl tetrazolium salt Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega Corp, Madison, Wisconsin). Future experiments will be required to further confirm the role of c-FLIP$_L$ expression in pig islet cells.

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ELSEVIER

In Vivo Controlling of Cellular Response to Pig Islet Xenografts by Adenovirus-Mediated Expression of Either Membrane-Bound Human FasL or Human Decoy Fas

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ABSTRACT

The critical problem with clinical islet transplantation for patients with type 1 diabetes is the severe shortage of human donors. Pig islet xenotransplantation has the potential to provide a virtually unlimited source of donor pancreata. However, our previous studies demonstrated that cell-mediated rejection, especially human $CD8^+$ cytotoxic T lymphocyte (CTL)–mediated cytotoxicity, remains a major obstacle for long-term islet xenograft survival. Moreover, we have demonstrated that the overexpression of either membrane-bound human FasL (mFasL) or human decoy Fas antigen (decoy Fas) in pig islets not only prevented CTL xenocytotoxicity in vitro, but also prolonged histological survival of pig islet xenografts in vivo. Therefore, the aim of the present study was to determine whether adenoviral transfer of these genes into pig islets ex vivo prior to transplantation had a beneficial effect on posttransplantation glycemic control of diabetic recipients. Isolated pig islets were transfected with adenovirus vector carrying complementary DNA (cDNA) of either mFasL or decoy Fas. The transfected islets were transplanted under the kidney capsule of diabetic recipient rats. Rats transplanted with either mFasL- or decoy Fas-transfected pig islet grafts showed significantly suppressed blood glucose levels from 12 hours to 18 hours posttransplantation compared with control groups transplanted with empty vector-transfected pig islets. Unfortunately, blood glucose levels of these groups were increased, with no significant difference observed at 24 hours posttransplantation. However, transgenic expression of these molecules with clinically tolerable amount of immunosuppressants may be more effective to achieve islet xenograft survival in the future.

PIG ORGANS are considered to be an attractive, promising alternative for the severe shortage of human donors. However, both humoral and cellular immune responses remain formidable barriers preventing vascularized xenograft survival, with the former predominantly consisting of the interactions between natural anti-Gal antibody and the α-gal epitopes on pig cells, and the latter including $CD4^+$ T cells, $CD8^+$ cytotoxic T lymphocytes (CTLs), macrophages, and natural killer (NK) cells. Fortunately, the humoral immunologic barrier may not occur in the case of pig-to-human islet xenotransplantation, because pig islets fail to express these α-Gal epitopes. However, pig islet xenografts are rejected by cellular immunity. Therefore, it is rational to target cellular immunity for successful pig islet xenotransplantation. Recently, several groups have shown that prolonged xenograft survival of functional adult pig islets may be achieved in immunosuppressed nonhuman primate recipients.[1,2] However, the high level of immunosuppression in these studies may be difficult to use clinically for human diabetic patients. Therefore, specific immunosuppression, consisting of local expression of cytoprotective molecules on pig islets, may be required to realize pig-to-human islet xenotransplantation. Our group previously demonstrated that cell-mediated xenocytotoxicity, especially human $CD8^+$ CTL, is highly detrimental to pig cells.[3] We have explored methods to prevent killing by overexpression of either membrane-bound human FasL (mFasL) or

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human decoy Fas antigen (decoy Fas).[4] In this study, we determined whether overexpression of these molecules into pig islets ex vivo prior to transplantation showed a beneficial effect to improve islet xenograft survival and function.

MATERIALS AND METHODS

Preparation of Pig Islets

Pig pancreata were harvested from a slaughterhouse that handles young market weight pigs (Large White/Landrace×Duroc, 200–300 kg). The gland was shipped to our laboratory using 2-layer methods. Pig islets were isolated by the modified Ricordi method.[5] Briefly, the pig pancreas distended with Liberase HI solution (Roche Diagnostics, Indianapolis, Ind, United States) was cut into several pieces, which were placed into a sterile chamber for clinical islet isolation (Umihira, Kyoto, Japan) by digestion as previously described.[5] The pig islets were purified with a continuous density gradient of iodixianol-based solution in an apheresis system (COBE2991 cell processor, Gambro Laboratory, Denver, Cdo, United States), which was cooled with special equipment (Umihira).

Pig Endothelial Cell Culture

A pig endothelial cell (PEC) line, MYP-30,[6] was cultured in Dulbecco's Modified Eagle's Medium (DMEM), including 10% fetal bovine serum (FBS), 100 U/mL Penicillin, 100 μ g/mL streptomycin, and 0.1 mmol/L nonessential amino acids (Invitrogen, Carlsbad, Calif, United States).

Construction of Adenoviral Vectors and Gene Expression in Pig Islets

cDNAs encoding either mFasL gene[7] or decoy Fas[8] were subcloned into the SwaI cloning site of the cytomegalovirus (CMV) promoter-containing adenovirus-based cosmid vector (Ad-), pAxcwit (Takara Bio, Otsu, Japan).[9] All virus stocks were purified using cesium chloride density gradient centrifugation. Nonfunctioning enhanced green fluorescent protein (eGFP)–adenovirus vector was used as a vehicle control (ie, MOCK). Freshly isolated pig islets were exposed to these adenovirus vectors at a multiplicity of infection of 30 for 2 hours. The expression of these molecules in pig islets was assessed using FACS analysis.

In Vitro Cytotoxicity Assay

The cytotoxicity of cultured human $CD8^+$ CTL toward pig islets was assessed with a ^{51}Cr release assay, as previously described.[4]

Transplant Studies and Immunohistochemical Analysis

Lewis rats of 8 to 10 weeks old were purchased from Oriental Yeast (Tokyo, Japan). Recipient rats were preimmunized with 250 mg of pig kidney membranes as previously described.[10] Then, the recipients were rendered diabetic by a single intraperitoneal injection of streptozotocin (60 mg/kg) at day 7 prior to islet transplantation. Diabetes was confirmed by blood glucose levels >300 mg/dL on 2 individual days. We transplanted 3000 islet equivalents (IEQ) of adenoviral transfected pig islets under the left kidney capsule of the recipient rats and monitored blood glucose levels. The grafted kidney was retrieved at day 3 or 5 posttransplantation for immunohistochemical analysis (IHC), using anti-pig insulin antibody (Dako, Glostrup, Denmark).[11] Briefly, deparaffinized and rehydrated tissue sections had endogenous peroxidase blocked with 3% H_2O_2 methanol. After blocking with 10% BSA-Tris buffered saline containing 0.1% Tween 20, the sections were incubated with either guinea pig anti-pig insulin polyclonal antibody (Dako). Then the sections were visualized using Dako LSAB+/HRP kit (Dako) with 0.02% diaminobenzidine as the chromogen. After washing, the sections were counterstained with hematoxylin. The specificity of the primary antibodies was verified by control sections omitting the primary antibody. The apoptotic cells were visualized using TUNEL staining at day 3 posttransplantation as previously described.[11]

RESULTS

Expression of these molecules in pig islet transfectants was detected as judged by mean fluorescence intensity of FACS analysis (Fig 1). The expression levels of transfected molecules in islets are summarized in Table 1. The histological survival of transplanted pig islets was evaluated using insulin staining. The prolongation of xenograft survival with either Ad-decoy Fas pig islets or Ad-mFasL pig islets was shown compared with Ad-eGFP or parental islet groups (Table 1). Apoptotic cells were clearly detected in parental pig islet xenografts at day 3 posttransplantation. In contrast, a smaller number of apoptotic cells was observed in decoy Fas islet xenografts at day 3 posttransplantation. In membrane-bound FasL islets grafts, many infiltrating cells were apoptotic (data not shown). Glycemic control among experimental groups was improved compared with the control groups through 12 to 18 hours posttransplantation. Unfortunately, blood glucose levels gradually increased in the experimental groups; no significant difference in glucose levels could be observed at 24 hours posttransplantation. This short-term improvement of glucose levels might be further prolonged by the use of immunosuppressive drugs.

DISCUSSION

We demonstrated that both mFasL and decoy Fas were effective to prolong pig islet xenograft survival in an in vivo transplantation model. Moreover, these molecules exerted

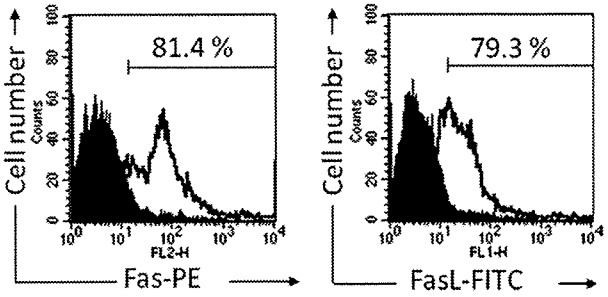

Fig 1. Adenovirus-mediated overexpression of either human decoy Fas or membrane-bound human FasL in transfected pig islets: (closed histogram) unstained islets; (open histogram) stained islets with anti-human Fas mAb (DX2) or anti-human FasL mAb (4H9). The percentages of transfected islets displaying fluorescence in channels greater than 10 in each of the preparations are indicated in the upper right of each histogram.

Table 1. Adenoviral Expression in Pig Islets and Xenograft Survival of Pig Islets in Pig-to-Rat Transplantation Model

	Mean Florescence Intensity (Transduction Efficiency by Adenovirus [%])		% Cytotoxicity by ^{51}Cr Release Assay	Xenograft Survival	Blood Glucose Levels of Recipients (mg/mL)		
Pig Islets	FasL	Fas Antigen	E:T Ratio = 50:1	d 3	12 h	18 h	24 h
Parental pig islets	Not determined	25 (endogenous)	36.7 ± 3.9	Rejected	430.5 ± 38.8	462.2 ± 52.4	478.6 ± 42.1
Mock islets	Not determined	26 (endogenous)	47.8 ± 1.0	Rejected	447.8 ± 45.0	450.3 ± 44.3	469.5 ± 33.3
Decay Fas islets	Not determined	198 (81.4)	18.8 ± 1.0	++ (survived)	186.6 ± 18.7*	272.5 ± 56.5*	458.3 ± 36.9
mFasL islets	75 (79.3%)	30 (endogenous)	5.7 ± 1.8	++ (survived)	196.4 ± 44.3*	304.3 ± 42.9*	490.0 ± 38.5

Note: The expression levels of pig islet transfectants, the glycemic control of the recipient rats, and histological islet xenograft survival were summarized. Each value is expressed as mean ± SD from 4 independent experiments.
*$P < .05$ compared with mock.

their cytoprotective effects for the metabolic outcome of recipients for the first 24 hours after transplantation. These results suggested that adenoviral transduction of cytoprotective genes into pig islets is a promising method to prolong xenograft survival. However, there were only transient positive effects on glycemic control in recipient rats. We speculated that this may be explained by several factors. Although adenovirus vectors are powerful weapons to transduce genes of interest in most tissues, including pig islets, adenoviral gene delivery may have deleterious effects upon pig islets, otherwise their chemotactic properties may restrict the efficiency of modified genes. We did not use immunosuppressants seeking to verify the pure effect of gene modification; therefore, islets might be damaged by multiple factors, including non-gal-dependent antibody-mediated, nonimmunological or immunological rejections. We expect synergistic effects between gene transduction and immunosuppressants. Therefore, our future projects seek to assess the effectiveness of transgenic pig islets bearing these molecules.

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