

clinical islet transplantation are currently not fully understood, this decline may reflect chronic toxicity of immunosuppressive drugs on islet β cells.

Rapamycin is widely used both as an induction and maintenance immunosuppressant in islet transplantation as part of the original Edmonton protocol.¹ Rapamycin may have deleterious effects on islet β cells. The immunosuppressive mechanism of rapamycin is based on blockade of mammalian target of rapamycin (mTOR), a molecule with a pivotal role in cell cycle progression from late G1 into S phase in response to T-cell growth factor stimulation.³ The mTOR, which is ubiquitously expressed in various cell types, is a serine/threonine protein kinase that regulates important cellular process including growth, proliferation, motility, survival, protein synthesis, and transcription.⁴ Furthermore, mTOR activity inhibits autophagy in cells ranging from yeast to human.⁵ Accordingly, the ability of rapamycin to inhibit mTOR activity may induce autophagy.

Autophagy, meaning to eat oneself, is one of the main mechanisms for maintaining cellular homeostasis. Although this pathway is not directly a death pathway, it is a self-cannibalistic pathway. Mediated via lysosomal degradation, autophagy is responsible for destroying cellular proteins and degrading cellular organelles, recycling them to ensure cell survival. Although altered autophagy has been observed in various diseases, including neurodegenerative diseases, cancers, and cardiac myopathies,⁶⁻⁸ its role is not known; the crux of the problem is whether the response is cell protective or a mechanism of death.

The relative contributions of autophagy are poorly understood in transplanted islets. The objective of the present study was to evaluate the effects of rapamycin on islet β -cells, including autophagy induction, viability, and insulin secretion, factors that may strongly contribute to progressive dysfunction of transplanted islets.

MATERIALS AND METHODS

Isolation of Pancreatic Islets

Anesthetized male BL6 mice underwent bile duct cannulation with pancreatic inflation using 3 mL of extracellular-type trehalose-containing Kyoto (ET-Kyoto) solution containing 1 mg/mL of collagenase. The inflated pancreas was excised; cleaned of lymph nodes, fat, and bile duct; and digested with collagenase VIII, followed by purification using a discontinuous Ficoll gradient. Isolated islets were cultured in RPMI (Roswell Park Memorial Institute) 1640 medium.

Western Blot Analysis

Western blot analysis was performed to detect the accumulation of LC3-II, an LC3-phosphorylated conjugate, which is an early marker of autophagy. Fresh islets (30 per well) were incubated for 24 hours in culture medium in the absence or presence of either 1 or 10 ng/mL of rapamycin. Protein samples from lysed cells underwent electrophoresis with 15% sodium dodecylsulfate-polyacrylamide gel and were transferred to polyvinylidene fluoride membranes. Lysate LC3-II was recognized by immunoblotting with an anti-LC3 monoclonal antibody (MBL International Corp, Woburn, Massachusetts).⁹ As the loading control for the samples, we also detected

protein expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Protein expression levels of both LC3-II and GAPDH, expressed in arbitrary units, were quantified using an image analyzer (Fluor-Chem; Bio-Rad Laboratories, Inc, Hercules, California). The relative protein expression of LC3-II in islets was normalized to that of GAPDH and expressed as the ratio of LC3-II to GAPDH.

Islet Viability Assay

Thirty cells of fresh mice islets were cultured for 24 hours with complete medium in the absence or presence of either 1 or 10 ng/mL of rapamycin. Subsequently, islet viability was determined using the colorimetric methyl tetrazolium salt (MTS) Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega Corp, Madison, Wisconsin).¹⁰ The colorimetric reagent was added to each well and incubated for 2 hours before absorbance values were read at 490 nm.

Blocking Assay of Autophagic Signaling

To determine whether rapamycin-treated islets recovered their viability, they were assessed using the MTS assay in the absence or presence of 10 mmol/L of 3-methyladenine (3-MA), an inhibitor of class 3 phosphatidylinositol 3-kinase, an inhibitor of autophagy.¹¹

Glucose-Stimulated Insulin Release and Stimulation Index

To further determine the *in vitro* potency of rapamycin-treated islets, static glucose challenge was performed in the absence or presence of 10 mmol/L of 3-MA.¹² After overnight culture, islets were incubated with either 2.8 or 20 mmol/L of glucose in culture medium for 2 hours at 37°C. The collected supernate was stored at -80°C for measurement of insulin with an enzyme-linked immunosorbent assay. Glucose-stimulated insulin release was expressed as the stimulation index, that is, the ratio of insulin release during exposure to high glucose (20 mmol/L) incubation compared with low glucose (2.8 mmol/L) incubation.

Generation of GFP-LC3 Transgenic Mice

For *ex vivo* studies to monitor autophagy in rapamycin-treated islets, transgenic mice expressing GFP-LC3 under the control of the constitutive CAG (chicken β -actin) promoter were purchased from RIKEN BioResource Center, Wako, Japan.¹³ Fresh mouse islets, isolated as described above, were incubated for 24 hours in culture medium in the absence or presence of 1 ng/mL of rapamycin. In addition, rapamycin-treated islets were incubated in the presence of 10 mmol/L of 3-MA. Either untreated control islets, rapamycin-treated islets, or rapamycin plus 3-MA-treated islets were directly observed using a fluorescence microscope (Biozero; Keyence Corp, Osaka, Japan) to detect GFP-LC3 dots.

RESULTS

Endogenous LC3-II Markedly Accumulates in Islets Treated With Rapamycin

Endogenous LC3-II protein was detected in control islets (Table 1). From the results for islets treated with either 1 or 10 ng/mL of rapamycin, the expression level of endogenous LC3-II in 1 ng/mL of rapamycin-treated islets was similar to that in control islets. However, the amount of endogenous LC3-II was doubled in 10 ng/mL of rapamycin-treated islets

Table 1. LC3-II Accumulation in Rapamycin-Treated Islets

Assay	Untreated Control Islets	Rapamycin Treated Islets		Blocking Assay With 3-MA	
		1 ng/mL	10 ng/mL	1 ng/mL of Rapamycin Plus 10 mmol/L of 3-MA	10 ng/mL of Rapamycin Plus 10 mmol/L of 3-MA
LC3-II expression at Western blot analysis (LC3-II/GAPDH ratio)	0.50	0.46	1.08	1.04	0.73
Absorbance by MTS assay (islet viability, recovery of viability), %	100 (control)	56.8 (14.1)	49.0 (2.0)	68.5 (0.5)	75.8 (25.9)

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC3-II, light chain 3, membrane bound; 3-MA, 3-methyladenine; MTS, methyl tetrazolium salt.

(Table 1). Under blocking conditions of autophagic signaling by 3-MA, there was an approximately 32% reduction in the amount of LC3-II observed in rapamycin-treated islets in the presence of 10 mmol/L of 3-MA as judged by the LC3-II/GAPDH ratio (Table 1).

Rapamycin Treatment of Islets Results in Reduced Islet Viability

To assess the direct effects of rapamycin on islet viability, we performed the MTS assay. On the basis of treatment with rapamycin, there were approximately 43% and 51% reductions in viability with treatment with 1 and 10 ng/mL of rapamycin, respectively (Table 1). In contrast, the viability of rapamycin-treated islets markedly recovered in the presence of 3-MA. Approximately 69% and 76% islet viability was noted after treatment with 1 ng/mL of rapamycin plus 10 mmol/L of 3-MA and with 10 ng/mL of rapamycin plus 10 mmol/L of 3-MA, respectively (Table 1).

Rapamycin Strongly Affects In Vitro Islet Function

Islet potency was assessed using a static glucose challenge in vitro. The stimulation index (SI) of untreated control islets was 1.38 ± 0.16 (Fig 1). However, treatment of islets with

rapamycin dramatically reduced the SI. The SI was 1.11 ± 0.01 with 1 ng/mL of rapamycin, and no insulin output occurred with 10 ng/mL of rapamycin treatment (Fig 1). In contrast, the SI dramatically improved with the addition of 3-MA. Islets treated with 1 ng/mL of rapamycin plus 10 mmol/L of 3-MA, completely recovered compared with untreated control islets (Fig 1). These results indicate that rapamycin treatment of isolated islets elicited not only autophagy induction but also reduced islet viability and potency.

GFP-LC3 Signal is Strong in Rapamycin-Treated Islets

Autophagy in response to rapamycin treatment was seen on fluorescence photomicrographs of islet samples prepared from GFP-LC3 transgenic mice (Fig 2). In untreated control islets, the GFP-LC3 signal was detected diffusely in islets with few punctuate dots (Fig 2A). After 24 hours of incubation with 1 ng/mL of rapamycin, the number of GFP-LC3 dots markedly increased; most were detected as cup- or ring-shaped structures (Fig 2B). In contrast, the level of GFP-LC3 signals of rapamycin-treated islets in the presence of 10 mmol/L of 3-MA was diffuse and returned to the base level of control islets (Fig 2C).

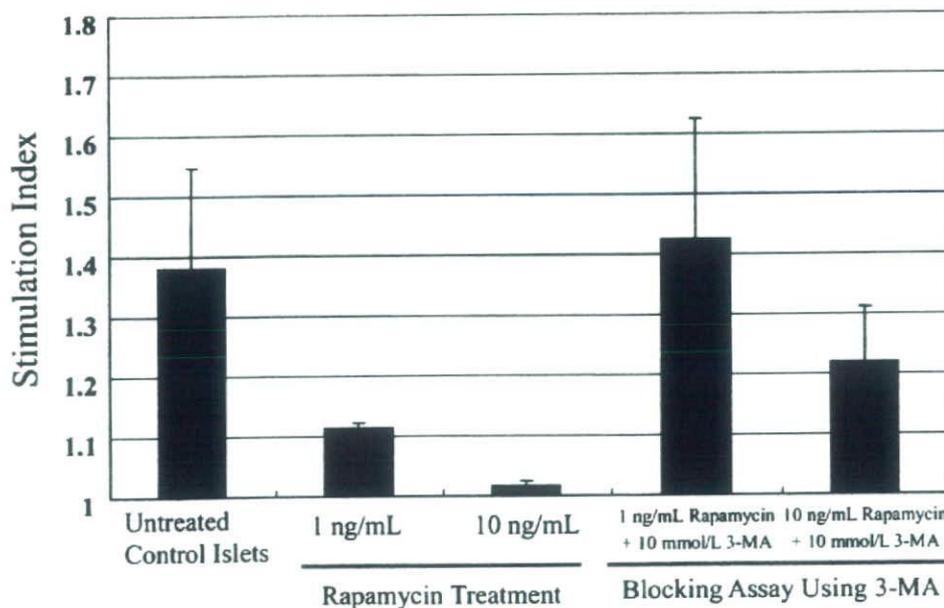


Fig 1. In vitro potency assessments of rapamycin-treated islets. Islet potency was assessed using static glucose challenge. Islet potency, expressed as stimulation index, was markedly reduced by rapamycin treatment. However, islet potency dramatically recovered with rapamycin plus 3-MA treatment. Values are expressed as the mean (SD) from three independent islets preparations.

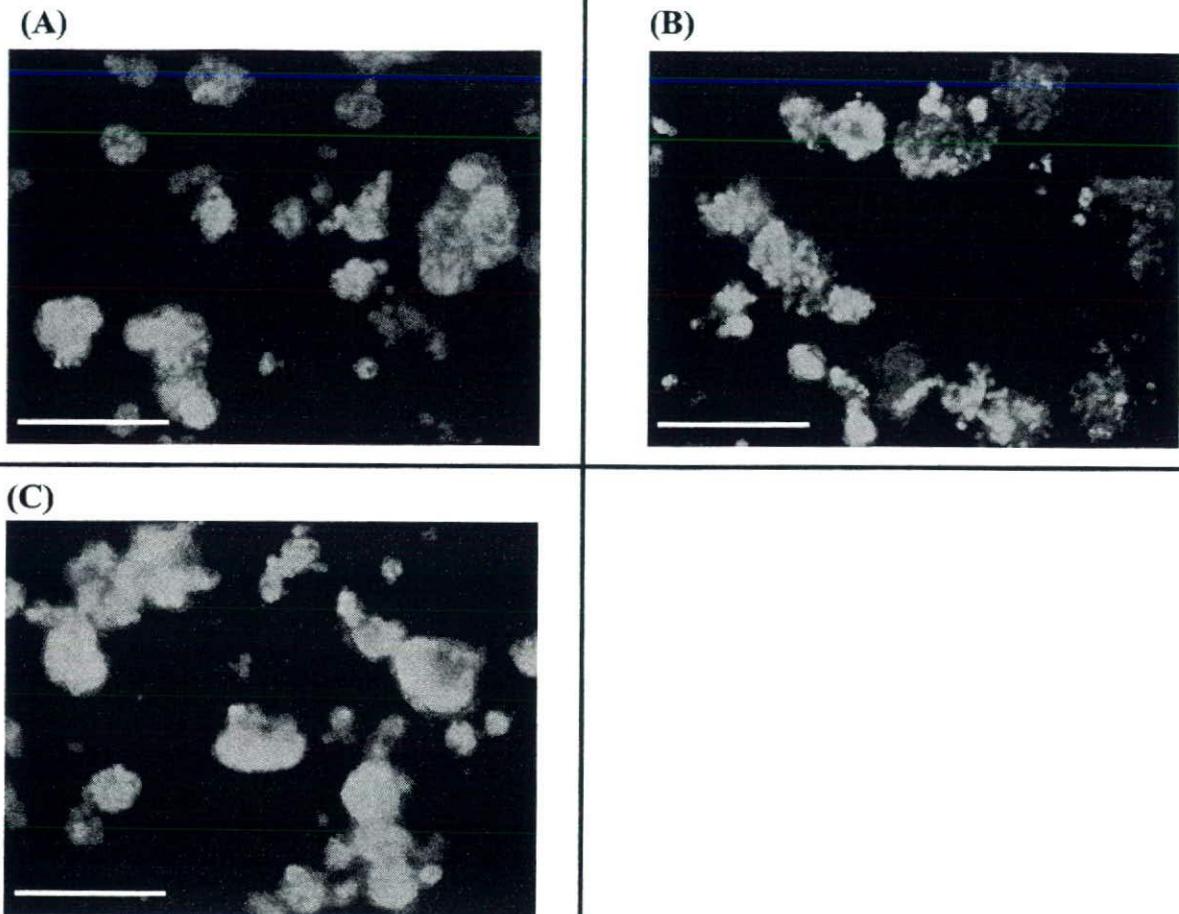


Fig 2. Islet autophagy in response to rapamycin treatment. Islet samples were prepared from GFP-LC3 transgenic mice. A, Untreated control islets. B, Islets treated with 1 ng/mL of rapamycin. C, Islets treated with rapamycin plus 3-MA. Bars indicate 100 μ m.

DISCUSSION

Rapamycin, which is a natural bacterial product that inhibits mTOR by association with an intracellular receptor FKBP12,¹⁴ is widely used as the central immunosuppressant in islet transplantation. As is well known, mTOR is a kinase that regulates important cellular processes such as inhibition of autophagy. Our results demonstrate that rapamycin treatment of isolated islets induced autophagy. This phenomenon impaired both islet viability and potency. These deleterious effects of rapamycin on islet β cells were markedly improved by the addition of 3-MA, which is an inhibitor of autophagy. Accordingly, therapeutically targeting this novel pathway may yield significant benefits, preventing the progressive islet graft dysfunction observed in transplant recipients.

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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 endogenous 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 glyco-biologic 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_{SL} 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 μ L 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 10⁶ 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 (⁵¹Cr) release assay.¹³ Either parental pig islets, mock islets, or transfected pig islets were incubated with Na₂⁵¹CrO₄ for 24 hours (1 μ Ci of ⁵¹Cr per 100 islets). Subsequently, ⁵¹Cr-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 ⁵¹Cr 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

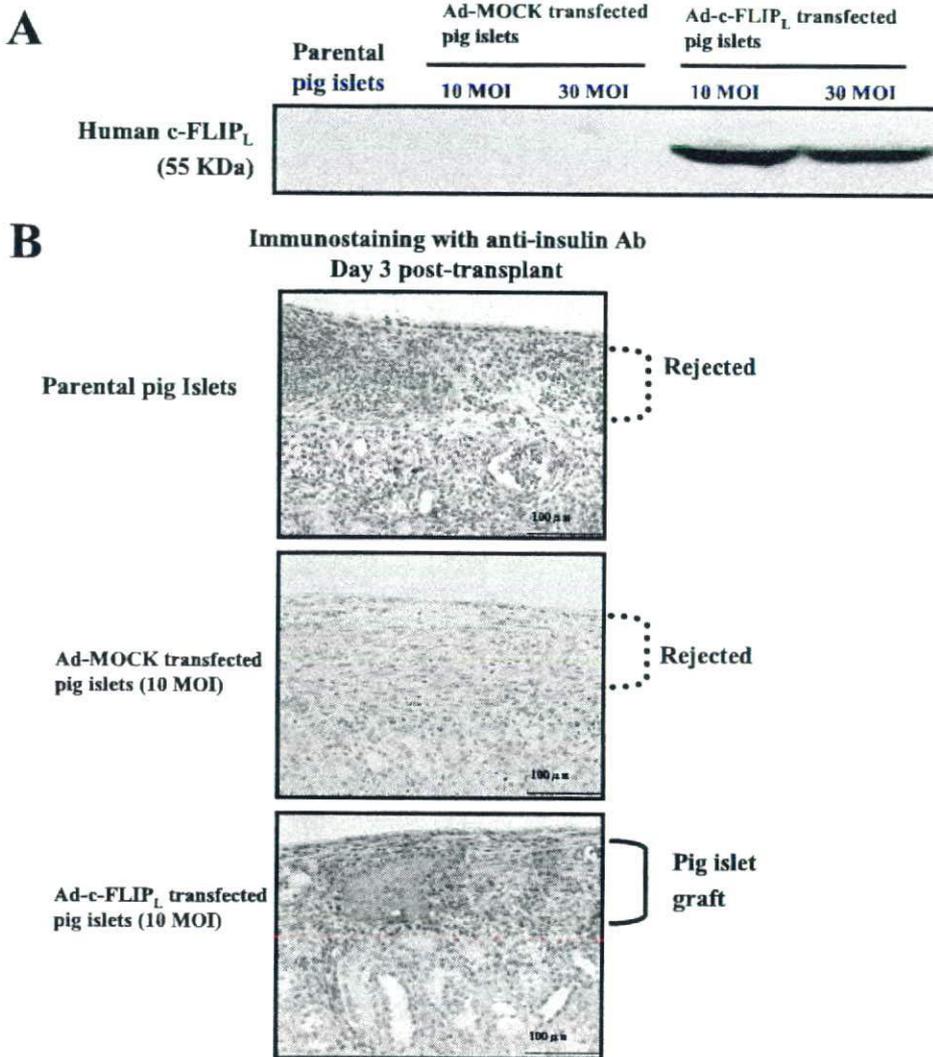


Fig 1. (A) The intracellular expression of c-FLIP_L protein. Adenovirus-mediated gene expression of human c-FLIP_L protein was assessed by Western blot analysis. (B) Immunohistological findings of rat kidney tissue of pig islets transplanted rats. Immunostaining with anti-pig insulin Ab for transplanted 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)	10	64.0 (8.9)	48.7 (14.8)
transfected pig islets)	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 ^{51}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 secrete inflammatory cytokines including IL-1 β , 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-1 β , 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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Intracellular and Extracellular Remodeling Effectively Prevents Human CD8⁺ Cytotoxic T Lymphocyte-Mediated Xenocytotoxicity by Coexpression of Membrane-Bound Human FasL and Pig c-FLIP_L in Pig Endothelial Cells

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ABSTRACT

Human CD8⁺ cytotoxic T lymphocyte (CTL)-mediated cytotoxicity, which participates in xenograft rejection, is mediated mainly by the Fas/FasL apoptotic pathway. We previously developed methods to inhibit human CTL xenocytotoxicity by extracellular remodeling using overexpression of membrane-bound human FasL on pig xenograft cells, and by intracellular blockade of death receptor-mediated apoptotic signals, such as the Fas/FasL pathway using the pig c-FLIP_L molecule. To investigate the cooperative effects of both membrane-bound FasL and pig c-FLIP_L, we cotransfected both genes into pig endothelial cells (PEC). The double remodeling with these molecules effectively prevented CD8⁺ CTL killing. Although double transfectants and single high transfectants of either membrane-bound FasL or c-FLIP_L gene displayed similar inhibition of CTL cytotoxicity, the expression levels of these 2 molecules in double transfectants were almost half the expression levels of single transfectants. Furthermore, to show *in vivo* prolongation of xenograft survival, we transplanted PEC transfectants under the rat kidney capsule. Prolonged survival was displayed by PEC double transfectant xenografts whereas those from either parental PEC or MOCK (vehicle control) were completely rejected by day 5 posttransplantation. These data suggested that intracellular and extracellular remodeling by coexpression of membrane-bound FasL and pig c-FLIP_L in xenograft cells may prevent an innate cellular response to xenografts. The gene compatibility of these molecules to generate transgenic pigs may be sufficient to create a window of opportunity to facilitate long-term xenograft survival.

OVERCOMING hyperacute rejection (HAR) by $\alpha 1, 3$ -galactosyltransferase gene knockout animals (GT-KO) that bear deletion of the major xenoantigen, α -gal epitopes (Gal $\alpha 1$ -3Gal $\beta 1$ -4GlcNAc-R), has been a significant step toward successful pig-to-primate organ xenotransplantation.^{1,2} However, long-term xenograft survival has yet to be achieved because a xenograft may be rejected directly by cell-mediated immunity, including natural killer (NK) cells, macrophages, and CD8⁺ cytotoxic T lymphocytes (CTLs).³⁻⁵ Therefore, overcoming cell-mediated immunity, especially mediated by human CD8⁺ CTLs, is an important strategy for long-term successful xenograft survival. Our previous studies have reported that the highly detrimental cytotoxic activity of human CD8⁺ CTLs against pig endothelial cells (PEC) is mediated in major part by the

Fas/FasL apoptotic pathway.⁵ To inhibit this strong CTL killing, we have exploited the weapon of extracellular overexpression of a membrane-bound human FasL that carries a deletion at the metalloproteinase cleavage site.⁵ In addition, we have demonstrated that intercellular overexpression of pig c-FLIP_{long} (c-FLIP_L), which is a potent inhibitor of death receptor-mediated pro-apoptotic signals

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protects PEC from human CTL-mediated killing⁶ by virtue of blocking the signaling pathway more upstream (before caspase-8 activation and release).

The present study addressed the question regarding human CD8⁺ CTL-mediated xenocytotoxicity—will double overexpression of both membrane-bound human FasL and pig c-FLIP_L on pig xenograft cells display cooperative effects to prevent CTL-mediated xenocytotoxicity? Furthermore, we examined the *in vivo* prolongation effects of xenograft survival by double remodeling of these molecules, using transplantation studies.

MATERIALS AND METHODS

Cell Culture

A PEC line, MYP-30,⁷ was maintained in DMEM (Sigma-Aldrich, St Louis, Mo United States) supplemented with 10% FBS (Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1 mmol/L nonessential amino acids (Invitrogen, Carlsbad, Calif, United States).

Gene Construction

Complementary DNA (cDNA) encoding the membrane-bound human FasL, which cannot be cleaved with metalloproteinase, was subcloned into the site of pEF-BOS expression vector, which carried the promoter of the human elongation factor 1 α chromosomal gene.⁵ cDNA of pig c-FLIP_L was subcloned into the EcoRI site of pCR3.1 expression vector, which carried a cytomegalovirus (CMV) promoter.⁸

Transfection of Plasmids

Each 20 µg of these plasmids was cotransfected into the PEC line (MYP-30) using lipofectamine (Invitrogen), according to the manufacturer's instructions. PEC that had been stably transfected with pEF-BOS and pCR3.1 expression vectors, which lack the cDNA fragments of either membrane-bound FasL or pig c-FLIP_L, was also established (ie, MOCK) as a vehicle control. The expression of membrane-bound human FasL on the PEC surface was assessed using FACS analysis, as previously described.⁵ The intracellular protein expression of pig c-FLIP_L was detected using Western blot analysis, as previously described.⁶ Protein expression levels of pig c-FLIP_L in PEC transfectants were quantified using Fluor-chem image analyzer (BioRad) as expressed by arbitrary units. As the loading control for each sample, protein expression of pig GAPDH in either parental, MOCK, or PEC transfectants was detected using a goat anti-pig GAPDH monoclonal antibody (mAb; Santa Cruz Biotechnology, Santa Cruz, Calif, United States). The relative protein expression of pig c-FLIP_L in PEC transfectants was normalized to that of pig GAPDH as expressed by the c-FLIP_L/GAPDH ratio.

Preparation of Human CD8⁺ CTL

To generate human CD8⁺ CTLs, 10 to 15 $\times 10^6$ separated PBMCs were cocultured for 14 days with irradiated PEC as stimulator cells in the presence of recombinant human interleukin (IL)-2, as previously described.^{5,6} Subsequently, human CD8⁺ CTLs positively isolated by magnetic beads (Dyna, Oslo, Norway) coated with anti-human CD8 mAb (RPA-T8, BD Biosciences Pharmingen, San Jose, Calif, United States) were examined using an *in vitro* cytotoxicity assay.

In Vitro Cytotoxicity Assay

The cytotoxic activity of human CD8⁺ CTLs incubated under various conditions was assessed using a ⁵¹Cr release assay as previously described.^{5,6} Parental PEC, MOCK, and PEC transfectants with either membrane-bound FasL, pig c-FLIP_L, or both genes were plated at 5000 cells/well in 96 well plates as target cells. After labeling with ⁵¹Cr for target cells, human CTLs isolated using magnetic beads were added to the wells. ⁵¹Cr released from the dead cells was measured in the supernates. The cooperative effects of FasL and pig c-FLIP_L double expression on human CTL-mediated xenocytotoxicity were determined by comparisons with PEC single transfectants and parental PEC.

Transplantation Studies and Immunohistochemical Analysis

Lewis rats (8 to 10 weeks old) purchased from Oriental Yeast (Tokyo, Japan) were distributed randomly between experimental groups (n = 5 rats per group) to receive either parental PEC, MOCK, or PEC transfectants. Rats immunized 3 times intraperitoneally with pig kidney membranes (250 mg) with a 1-week interval between injections were used as recipients. In each case, 2.5 $\times 10^6$ cells of either parental PEC, MOCK, or PEC transfectants were transplanted under the kidney capsule of rats in the absence of immunosuppression. Transplanted rats were monitored until the time of harvest at day 2, day 3, or day 5 posttransplantation. Each grafted kidney was analyzed using immunohistochemistry. Kidney specimens cut into small blocks and fixed in formalin were embedded in a single paraffin block. After quenching endogenous peroxidase activity by exposure to 3% H₂O₂/methanol, paraffin sections were stained with a rabbit anti-human Von Willebrand Factor (vWF) polyclonal antibody (DAKO) to specifically detect endothelial cells. The rinsed sections were then incubated with link antibody, followed by incubation of horseradish peroxidase-conjugated streptavidin. Immunostaining was visualized with 0.02% diaminobenzidine (DAB, Sigma-Aldrich) as the chromogen. The specificity for the primary vWF antibody was verified by control sections in which we omitted the primary antibody.

RESULTS

Establishment of PEC Transfectants Overexpressed Either Membrane-Bound FasL, Pig c-FLIP_L, or Both Genes

Two single positive clones were isolated: one had a high expression of membrane-bound human FasL, and other had a high expression of pig c-FLIP_L. Additionally, we also established three double-positive clones. Of the double transfectants, double-1 had a high expression of membrane-bound FasL and a low level of pig c-FLIP_L protein; whereas, double-2 had a low expression of FasL and a moderate protein expression of pig c-FLIP_L, and double-3 showed moderate expression levels of both molecules with almost half of the levels compared with the single high expression clones of each molecule, respectively (Table 1).

Double-Overexpression of FasL and c-FLIP_L Effectively Prevents CD8⁺ CTL-Mediated Cytotoxicity Against PEC

Human CD8⁺ CTLs generated by *in vitro* culture displayed strong killing against parental PEC and MOCK, namely, a lysis of >80% at an effector to target ratio of 50:1 (Fig 1A).

Table 1. Changes in the Expression Levels of Either Membrane-Bound FasL or c-FLIP_L Molecules

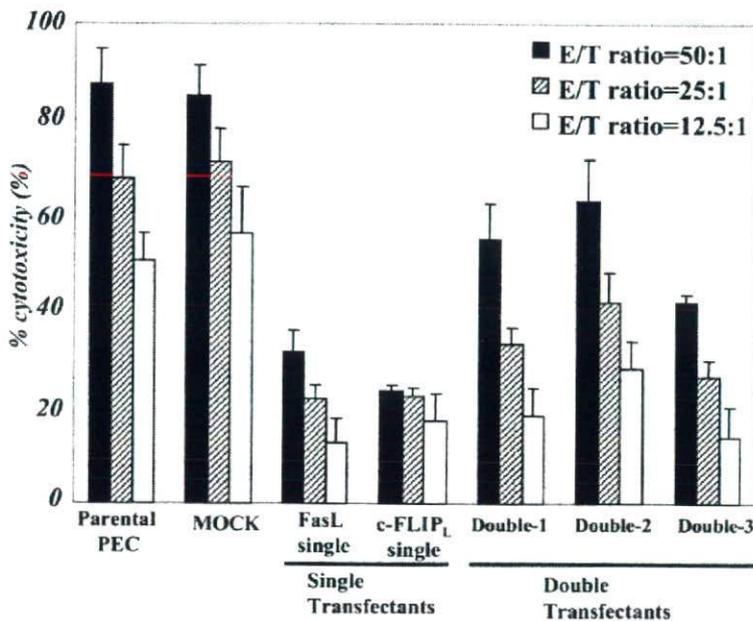
Cells	Protein Expression in PEC Transfectants	
	Membrane-Bound FasL (Mean Fluorescence Intensity by FACS)	c-FLIP _L /GAPDH Ratio (Arbitrary Units)
Parental PEC	Not detected	0.4 (endogenous)
MOCK	Not detected	0.38 (endogenous)
PEC-FasL (single, high)	127	0.35 (endogenous)
PEC-FLIP _L (single, high)	Not detected	1.8
Double 1: FasL (high) + FLIP _L (low)	80.6	0.8
Double 2: FasL (low) + FLIP _L (middle)	33.1	1.2
Double 3: FasL (middle) + FLIP _L (middle)	62.0	1.1

Note: The expression level of membrane-bound FasL on the PEC surface was examined using FACS analysis. The intracellular expression of c-FLIP_L protein was assessed using Western blotting. Image analyzer profiles were used to quantify the expression level of c-FLIP_L protein in parental PEC, MOCK, and PEC transfectants. The relative protein expression of c-FLIP_L in PEC transfectants was normalized to that of pig GAPDH and expressed as the c-FLIP_L/GAPDH ratio.

The single overexpression of either membrane-bound FasL or pig c-FLIP_L in PEC resulted in marked cytoprotection from CD8⁺ CTLs (Fig 1A). Inhibition of cytotoxicity by 64% to 73% was observed among single high expression

clones of either FasL or c-FLIP_L transfectants at effector-to-target ratios of 50:1. The double-overexpression of these molecules in PEC effectively reduced CD8⁺ CTL-mediated cytotoxicity (Fig 1A). From the result for the double-3

A



B

Immunostaining with anti-vWF Ab

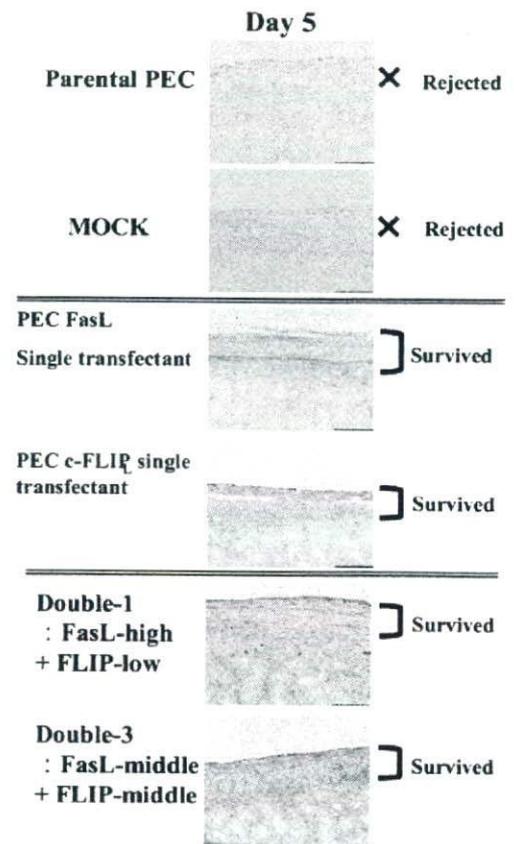


Fig 1. ⁵¹Cr release assay of PEC transfectants and immunohistological findings of PEC xenografts transplanted under rat kidney capsule. Amelioration of human CD8⁺ CTL-mediated cytotoxicity by the PEC transfectants, MOCK, and control parental PEC was estimated at the effector:target ratio of either 12.5:1, 25:1, or 50:1. (A) The percentages of CTL-killing by PEC transfectants. Each value is expressed as the mean ± SD from 5 independent experiments. (B) Immunohistological findings of rat kidney tissue of PEC transplanted rats. Immunostaining with anti-vWF Ab for transfectant PEC of kidney specimens obtained at day 5 posttransplantation. Pictures are representative of immunostaining of kidney sections obtained from 5 animals per each transfectant group. The black bars in each picture indicated 100 μm.

transfectant, the inhibition level of CTL killing by this clone was similar to that of either the FasL or the c-FLIP_L single high expression clones at an effector-to-target ratio of either 25:1 or 12.5:1. In contrast, the expression levels of these 2 molecules were almost half that of the single high transfectants, as judged by either c-FLIP_L/GAPDH ratio of western blot analysis or mean fluorescence intensity of FACS analysis. These findings indicated that additional effects to inhibit CD8⁺ CTL-mediated xenocytotoxicity can be obtained by double-expression of both FasL and c-FLIP_L in PEC.

Double-Overexpression of FasL and c-FLIP_L can Prolong Xenograft Survival

To prove that the double-overexpression of both FasL and c-FLIP_L molecules in PEC was effective to prolong xenograft survival, we transplanted PEC transfectants under the rat kidney capsule. The results of the immunohistochemical analysis are summarized in Fig 1B. At day 3 posttransplantation, large numbers of well-preserved both parental PEC, MOCK, and PEC transfectants were observed under the kidney capsules (data not shown). By day 5 posttransplantation, parental PEC and MOCK had been completely rejected (Fig 1B). In contrast, both single high and double transfected PEC xenografts survived intact at day 5 posttransplantation (Fig 1B). Accordingly, we observed beneficial effects for *in vivo* prolongation of xenografts with double PEC transfectants.

DISCUSSION

Cellular immunity toward xenograft rejection, including human NK cells, macrophages, and CD8⁺ CTLs, seems to be an important obstacle to prolonged graft survival in pig-to-human xenotransplantation. We have previously developed methods to prevent immune attack of human CD8⁺ CTLs toward pig xenograft cells by means of both extracellular remodeling of the death receptor using membrane-bound human FasL and intracellular blocking of death receptor-mediated apoptotic signals, such as Fas/FasL pathway by the use of pig c-FLIP_L molecule. In the present study, we assessed the amelioration of cytoprotective effects from human CTL-mediated killing by combined overexpression of both membrane-bound human FasL and pig c-FLIP_L genes.

Both *in vitro* and *in vivo* analyses revealed the coopera-

tive effects of intracellular and extracellular remodeling with these molecules to inhibit CTL xenocytotoxicity. Our final goal was to generate a double transgenic pig with both membrane-bound FasL and c-FLIP_L genes. Consequently, pig islets obtained from this double transgenic pig may prove to be beneficial to prolong xenograft survival by a high resistance to the immune attack of human CTLs. However, from the embryological view, the birth of a double transgenic pig, which highly expresses both FasL and c-FLIP_L molecules, respectively, may be difficult. It takes a long time to select the high expression clone of these molecules. The cooperative effects and compatibility of multi-transgenes must be understood to efficiently generate transgenic pigs with multi-genes. Thus, our findings in the present study demonstrated that double remodeling with both membrane-bound human FasL and c-FLIP_L may well be compatible to generate transgenic pigs and can elicit cooperative effects for the inhibition of human CTL xenocytotoxicity.

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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. *Xenotransplantation* 2008; 15: 333–343. © 2008 Wiley Periodicals, Inc.

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 iodixanol-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 × 10⁶ 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 *Swa*I cloning site of the cosmid cassettes, pAxcwit. These cosmids contained the adenovirus genomic sequence, except for E1 and E3. *Escherichia coli* DH5 α 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 $\text{Na}_2^{51}\text{CrO}_4$ for 24 hr ($1\mu\text{Ci}^{51}\text{Cr}/100$ 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% H₂O₂ 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

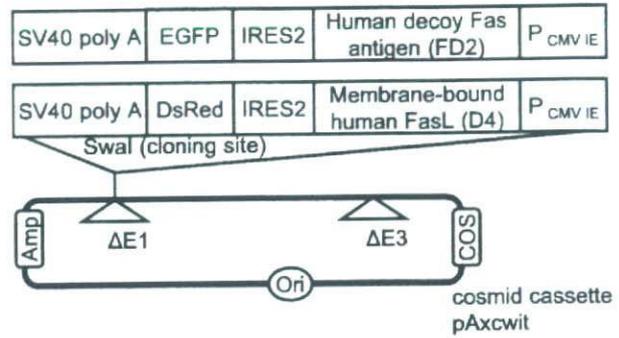


Fig. 1. Gene structures of EGFP-FD2 (human decoy Fas antigen) and DsRed-D4 (membrane-bound human FasL). These cosmids contain the adenovirus genomic sequence except for E1 and E3 (open triangles). Replication-defective adenovirus recombinants expressing either EGFP, EGFP-FD2, or DsRed-D4 were constructed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

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).

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).

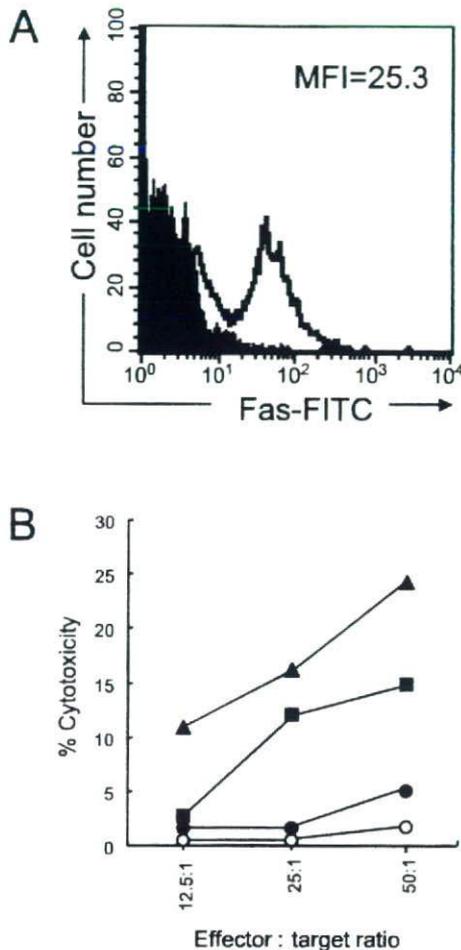


Fig. 2. (A) Flow cytometry analysis of freshly isolated pig islets. The data are from one of three representative islets. (open histogram) Single islet cells stained with anti-Fas mAb (clone 13). (closed histogram) Unstained pig islets. Mean fluorescence intensity (MFI) is expressed in the upper right 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 nM. 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

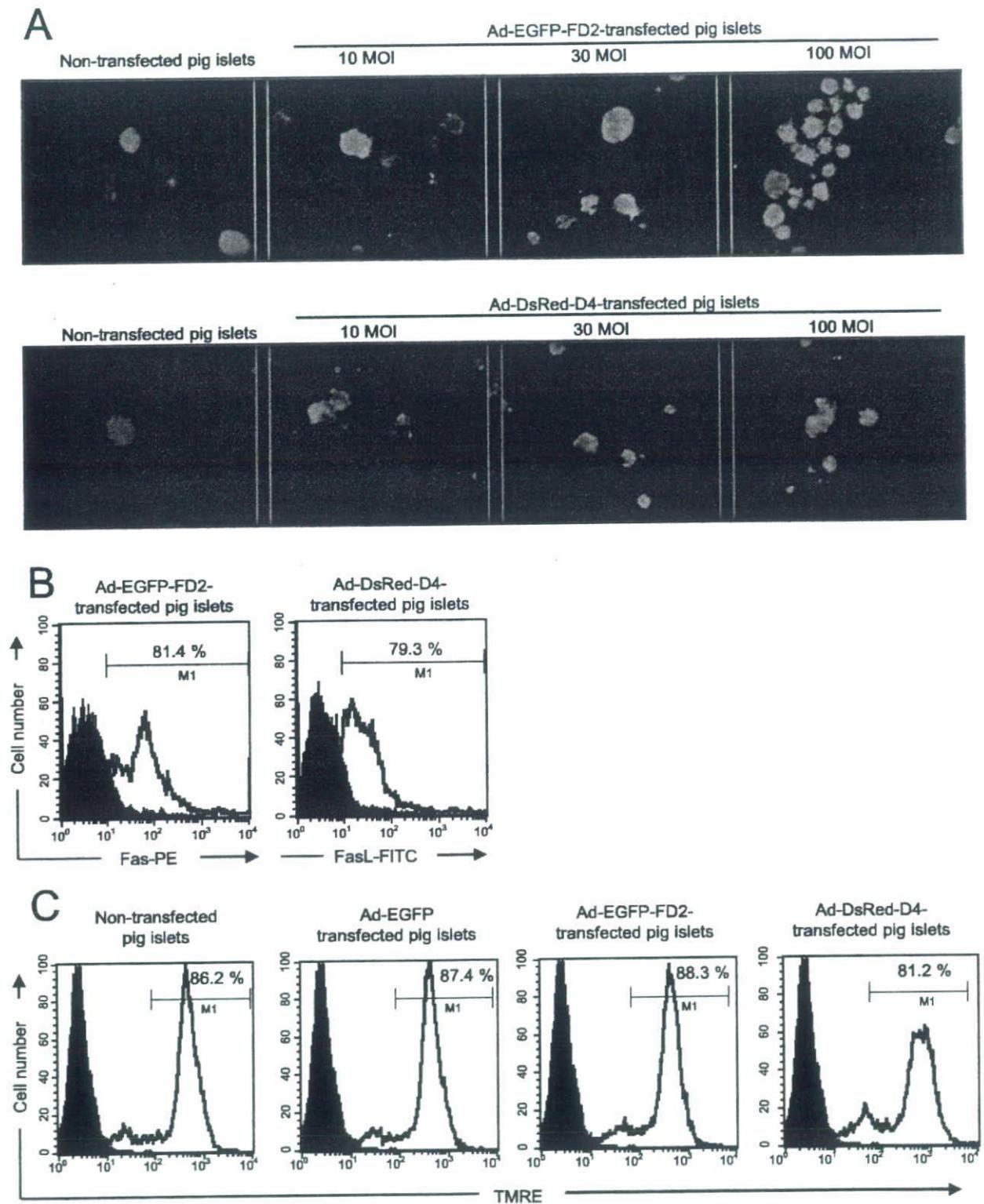


Fig. 3. Adenovirus-mediated overexpression of human decoy Fas antigen with a standard GFP-filter (upper panels) and membrane-bound human FasL with a TRITC-filter (lower panels). (B) Overexpression of human decoy Fas or membrane-bound human FasL in transfected pig islets. (closed histogram) Unstained 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).

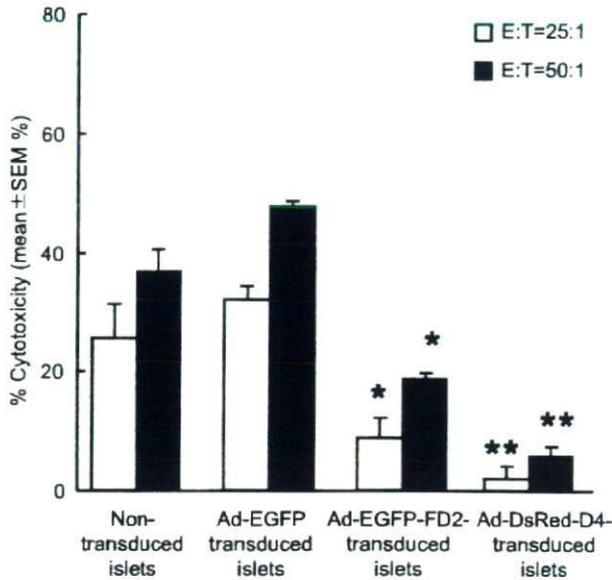


Fig. 4. Cytotoxicity assay of human decoy Fas antigen or membrane-bound human FasL transfected pig islets. Amelioration of CD8⁺ cytotoxic T lymphocytes-mediated cytotoxicity by decoy Fas or membrane-bound FasL and controls (Non-transfected and EGFP-transfected islets). White bars indicate the cytotoxicity 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 (*P < 0.01, **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) (*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) (*P < 0.01).

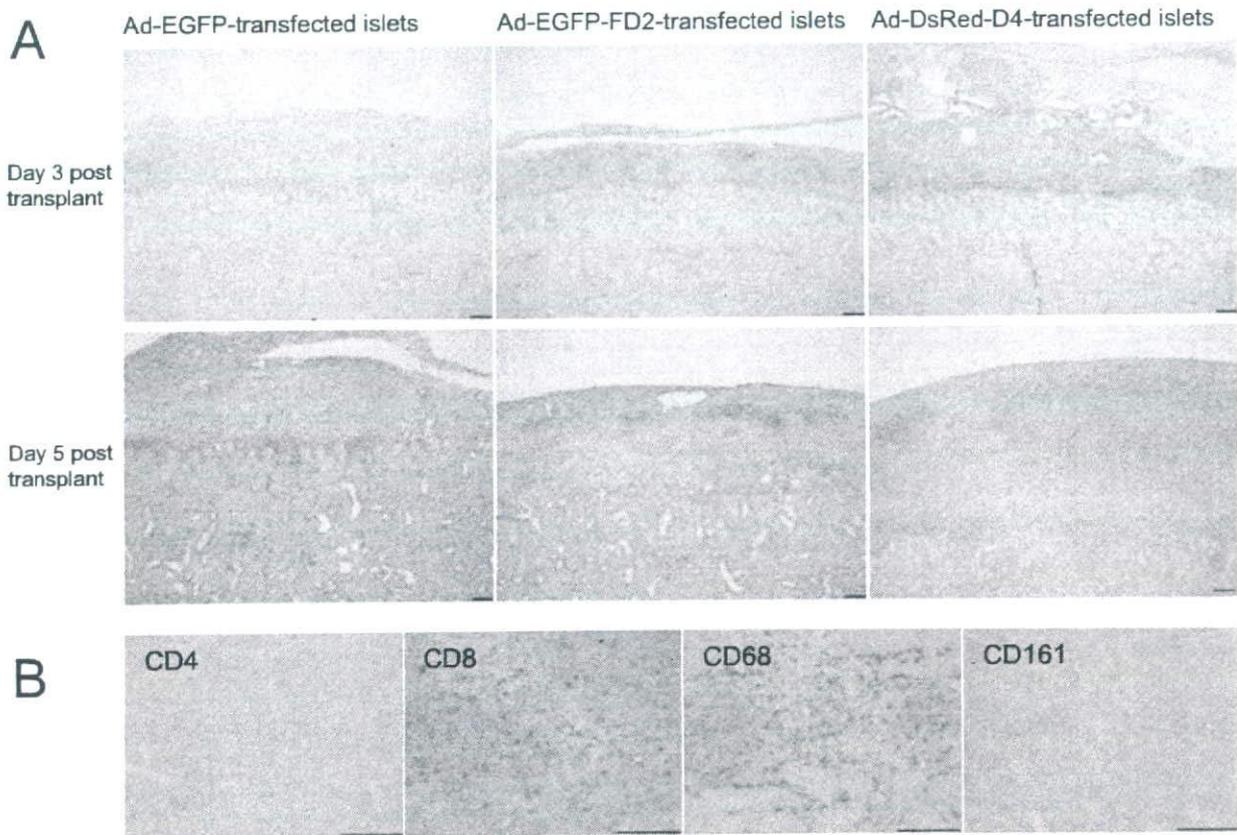


Fig. 5. Histological analysis of grafts from rats transplanted with either Ad-EGFP-, Ad-EGFP-FD2-, or Ad-DsRed-D4-transfected islets. (A) Insulin staining of representative sections from graft-bearing kidney transplanted with either Ad-EGFP, Ad-EGFP-FD2-, or Ad-DsRed-D4-transfected islets harvested at 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