属病院，国立病院機構千葉東病院，京都大学医学部附属病院，大阪大学医学部附属病院，神戸大学医学部附属病院，福岡大学医学部附属病院）である．移植の機会を増やすために，患者は7施設のうち複数の施設に登録することが可能である．

(3) 膵島移植の工程

ドナーから膵臓を摘出し，二層法の溶液に浸して膵島分離実施施設に運ぶ．膵島分離実施施設のクリーンルームにて，膵臓をコラゲナーゼを用いて消化し，その後純化行程を経て膵島を回収する．分離膵島が，新鮮膵島移植基準（表2）を満たす場合に，膵島は輸血用バッグに詰められ，経皮経肝的に門脈に挿入されたカテーテルを通して，点滴の要領で移植される．

移植手技自体の合併症では，穿刺部位からの出血と，門脈内血栓が報告されている．

なお，リベレース®（コラゲナーゼ）に狂牛病の原因プリオンが混入している可能性が完全には否定できないため，膵島移植は2007年3月下旬より一旦中断している．新たなコラゲナーゼが開発され，効果や安全性が確認されており，安定供給できるようになれば再開が可能となる．

(4) 膵島移植後の管理

移植された膵島は，門脈の末梢部にとどまり，肝臓からの新生血管が，膵島の血管網と吻合することで生着する．

免疫抑制薬は，当初エドモントンプロトコールに従って，IL-2レセプターのモノクローナル抗体（basiliximab）による免疫抑制の導入と，sirolimus, tacrolimusによる維持療法が行われていた．しかし，エドモントンプロトコールの血中濃度では，sirolimusの副作用として，口腔内潰瘍，下肢の浮腫，高コレステロール血症，卵巣嚢腫，蛋白尿などが高頻度に生じることが判明し，最近では，sirolimusを減量あるいは削除し，mycophenolate mofetil (MMF) を使用している施設が多い．

糖毒性で移植膵島が障害されないように，移植直後から厳格に血糖コントロールを行う．京都大学医学部附属病院では食前血糖100 mg/dl以下，食後血糖120 mg/dl以下になるようにインスリン量を調節している．また，移植後約1週間は，移植膵島に少しでも酸素を供給するために，ベッド上にいる間は酸素投与が行われている．免疫抑制薬の血中濃度が安定化し，インスリンの必要量が漸減して一定となる移植後約1カ月後に退院となる．

(5) 結果

本邦初の膵島移植は，京都大学医学部附属病院で2004年4月7日に施行された[5]．

現在までに心停止ドナーからの膵島分離が65回行われ，このうち34回で移植の条件を満たしていたため18名（男性5名，女性13名）に対して膵島移植が施行されている．

京都大学医学部附属病院では，心停止ドナー膵からも膵島が確実に分離できるKyoto Islet Isolation Method[6]を開発し，膵島分離24例のうち20例が移植基準を満たし，膵島分離成功率は83%と上昇した．そのうち，19例を1型糖尿病患者9名に移植できた．移植後血糖値は安定化し，第三者の介助を必要とする重症低血糖は消失した（図3a）．また必要インスリン量も徐々に減少し（図3b），このうち2回移植および3回移植を受けた3症例では，インスリン注射が不要となった．

また，1回の膵島移植により，たとえインスリン注射が必要でも，全症例で，基礎インスリン分泌に相当する補充量が減少し，血糖の不安定性の定量的な指標であるM値（日内の血糖値が100あるいは120 mg/dlなどの基準値からどれぐらいかけ離れているかを示す）やMAGE（日内の血糖値の変動幅の大きさを示す）は著明に低下し（図4），重症低血糖が消失し，血糖値の安定化が得られた[7]．

また我々は，膵島移植後に生着した膵島量を反映する指標として，SUIT（secretory unit of insulin in transplantation）指数を開発した（図5）[8]．

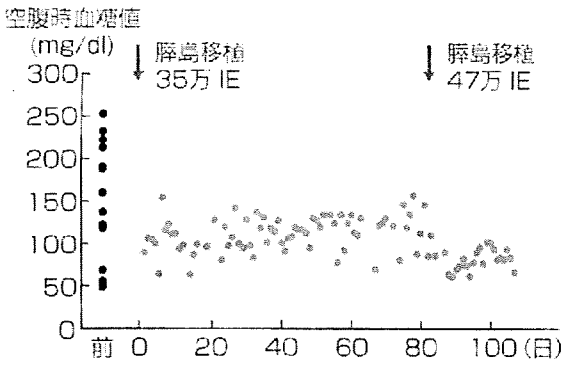


図 3a. 膵島移植前後の空腹時血糖値の推移(第 1 症例)

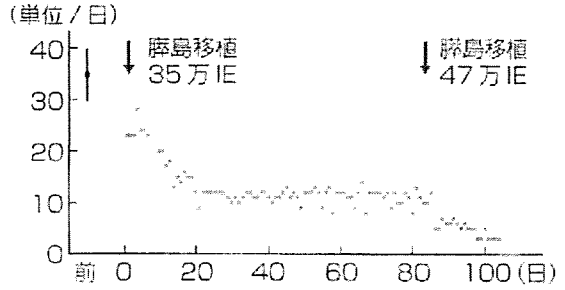


図 3b. 膵島移植前後のインスリン必要量(第 1 症例)

$$\text{SUIT 指数} = 1{,}485 \times \frac{\text{空腹時 C-peptide 値 (ng/ml)}}{\text{空腹時血糖値} - 61.8\ (\text{mg/dl})}$$

健常人の膵島を 100 として，大体どれくらいの膵島が生着しているかを表している．SUIT指数は，比較的早期から移植の成否を判定でき，インスリン治療中でも可能であり，移植した膵島に負荷を与えず，早朝 1 回の採血で評価可能である．京都大学医学部附属病院の結果から，SUIT 指数がおよそ 25 以上となると，インスリン離脱が可能であった．

2) 生体ドナーからの膵島移植

本邦では，ドナー不足は深刻である．京都大学医学部附属病院での膵島移植施行数が年間 10 例程度に対して，膵島移植を希望して受診する患者は年間 100 人以上であるため，生体ドナー膵島移植が研究され，2005 年 1 月世界で初めて成功した[9]．

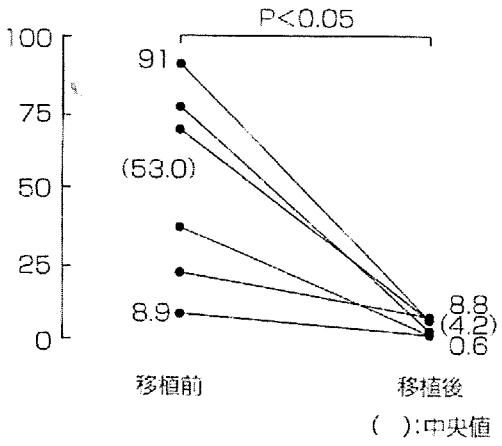


図 4a. 膵島移植前後の M 値

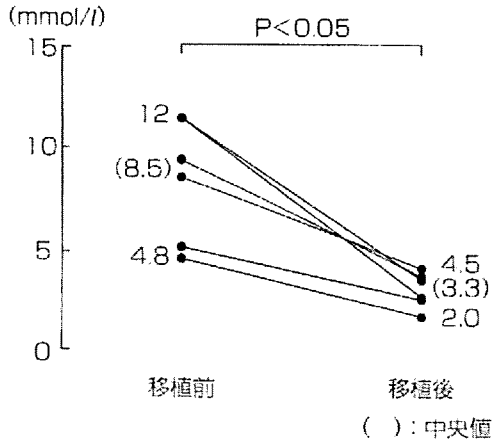
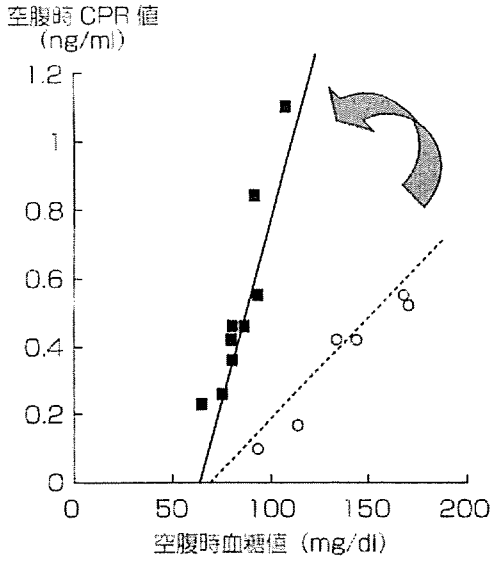


図 4b. 膵島移植前後の MAGE

生体ドナー膵島移植とは，健常なドナーの膵臓の体尾部を切除し，その膵体尾部から膵島を分離し，移植する治療法である．健常者であるドナーには，手術後の耐糖能の低下と周術期のリスクを伴う可能性があり，それを含めても移植による利益が上回ることが必要で，レシピエントの生命の危機の可能性のある重症低血糖発作の頻発などからの回避が目的となる．生体ドナー膵島移植では，脳死や心停止ドナー膵島移植と比べて死戦期の膵の傷害がなく，質の高い膵島を分離することができ，また移植時期を選べるため，レシピエントに免疫抑制薬の導入を行ったりインスリン量を最適にするなど，移植に最適な状態に準備することが可能である．



1回目の移植後（点線）ならびに2回目の移植後（実線）を示す．

図5．膵島移植後の空腹時血糖値とCPR値の相関

4．課題と展望

今後の主な課題として，ドナー不足の解消，長期成績の向上や免疫抑制薬の副作用軽減がある．

ドナー不足の解決策として，膵島分離法の改良によるmarginal donorからの移植や，新たな細胞源を用いるという方法がある．

ES細胞（embryonic stem cell）は，無限の増殖能とあらゆる細胞へ分化する多分化能をもち，膵β細胞への分化も報告されているが，奇形腫形成や免疫拒絶，倫理面などの問題がある．一方，山中らはヒトの線維芽細胞に4つの遺伝子を導入することにより，ES細胞とほぼ同等の多能性をもつiPS細胞(induced pluripotent stem cell)を作成することに成功し[10]，新たな細胞源として期待されている．また，Meltonらはマウスの膵外分泌細胞から，膵β細胞とほぼ同等の細胞にreprogramming（初期化）することに成功した[11]．

また，ドナーの供給源として，ブタの膵臓を用いた異種移植が研究されており，細胞性免疫を標的にした免疫抑制薬により，サルへの異種膵島移植における免疫拒絶反応を制御することができた[12]．ヒトへの異種膵島移植の臨床試験が米国で2，3年のうちに開始予定である．

抗胸腺細胞抗体（ATG）や抗CD20モノクローナル抗体であるrituximabによる導入療法[13]，tacrolimusを用いない新しい免疫抑制薬のプロトコールや，腸管内分泌細胞から分泌されインスリン分泌を促すホルモンであるGLP-1の長期作用アナログ（exendin-4）の使用により，膵島移植の長期成績の改善や免疫抑制薬の副作用の軽減が報告されている．また，現在は免疫抑制薬の全身投与が一生にわたって必要であるが，局所の免疫制御のみで免疫拒絶を回避できるよう研究が進められており，成功が待たれる．

おわりに

膵島移植は，血糖が極めて不安定なインスリン依存状態の糖尿病患者に対して，血糖値を安定化させ，重症低血糖を回避させる，安全で有効な治療法となり，移植を受けた患者のQOL（quality of life）は著明に改善されている．しかし，膵島移植の効果を長期に持続させたり，一人のドナーからの移植でインスリン離脱を目指すためには，慢性拒絶の回避や生着率の向上，より安全で副作用の少ない免疫抑制薬のプロトコールの開発，さらには免疫抑制薬からの離脱などが必要であり，精力的な研究が行われている．また，ドナー不足の解消のため，再生β細胞による移植や異種膵島移植が研究されており，臨床応用が期待される．これらの研究により，膵島移植はインスリン依存状態の糖尿病患者の根治療法となりうると考えられる．

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

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

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

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Introduction

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

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

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

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

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

Materials and Methods

Cell culture

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

Gene constructs

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

Gene expression

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

RT-PCR analyses

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

Western blot analysis

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

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

Preparation of human CD8$^+$ CTL

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

In vitro *cytotoxicity assay*

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

Assay for apoptosis

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

Assay of caspase activity in vitro

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

Transplant studies and immunohistochemical analysis

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

Double labeling

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

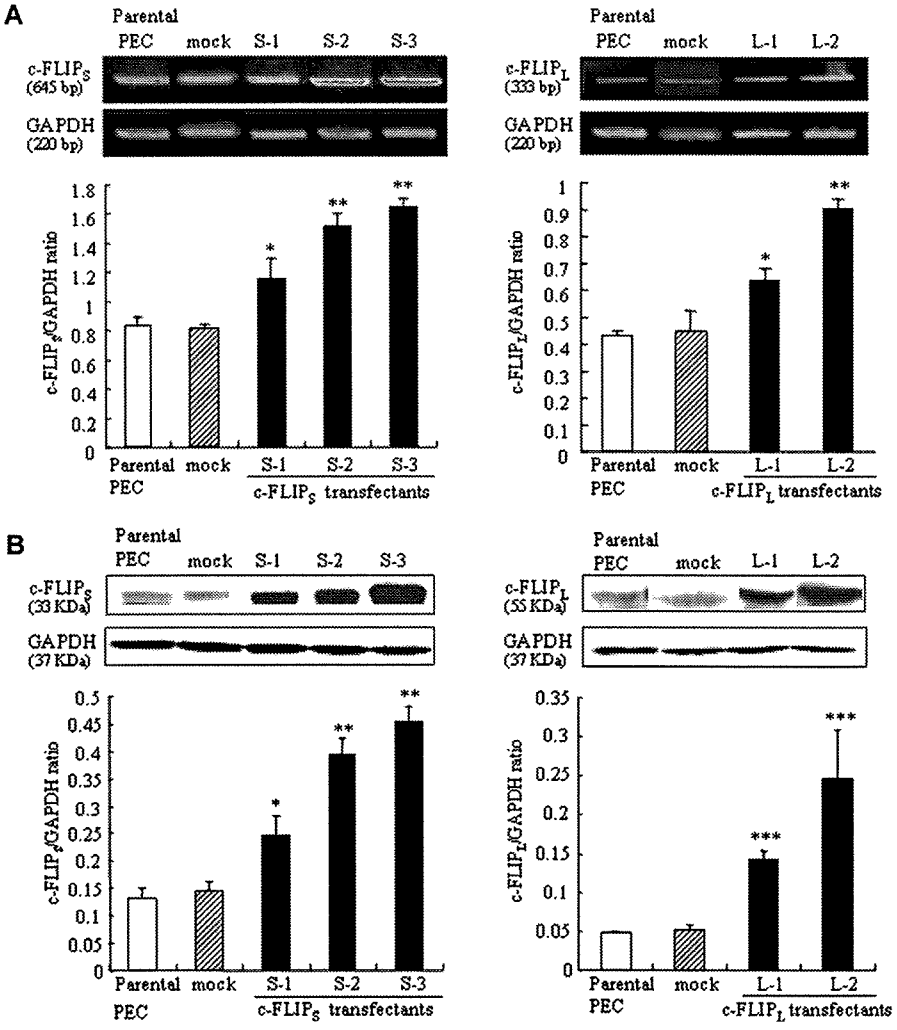

Figure 1: Changes in the expression levels of c-FLIP$_{S/L}$ mRNAs or proteins in PEC transfectants. (A) The expression levels of either c-FLIP$_S$ or c-FLIP$_L$ mRNAs were examined by RT-PCR. As an intrinsic control, GAPDH mRNA expression was measured in the same samples. Representative photographs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

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

Statistical analysis

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

Results

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

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

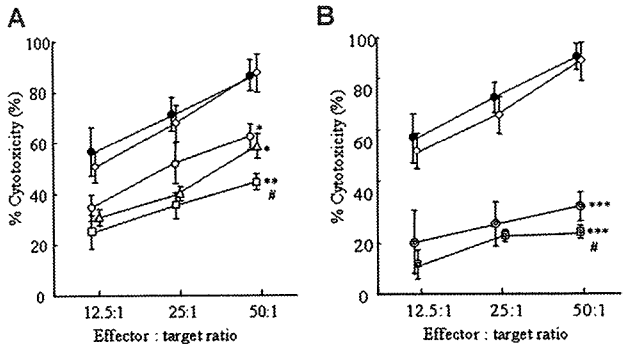

Figure 2: *In vitro* cytotoxicity assay of PEC transfectants with pig c-FLIP$_{S/L}$ genes. (A) Amelioration of human CD8$^+$ CTL-mediated cytotoxicity by c-FLIP$_S$ transfectants, control parental PEC and mock transfectants were assessed by ^{51}Cr release assay. Parental PEC (●), mock (◇), S-1 (○),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

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

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

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

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

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

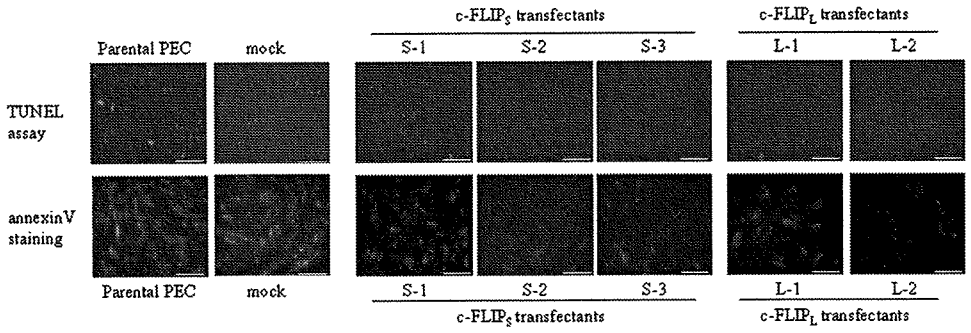

Figure 3: Detection of cell apoptosis by either TUNEL assay or annexin V staining. Parental PEC, mock and PEC transfectants were cocultured with human CD8$^+$ CTLs to induce apoptotic signals. Subsequently, cells were washed by PBS, then the reaction mixtures of either TUNEL or annexin V staining were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

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

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

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

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

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

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

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

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

Discussion

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

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

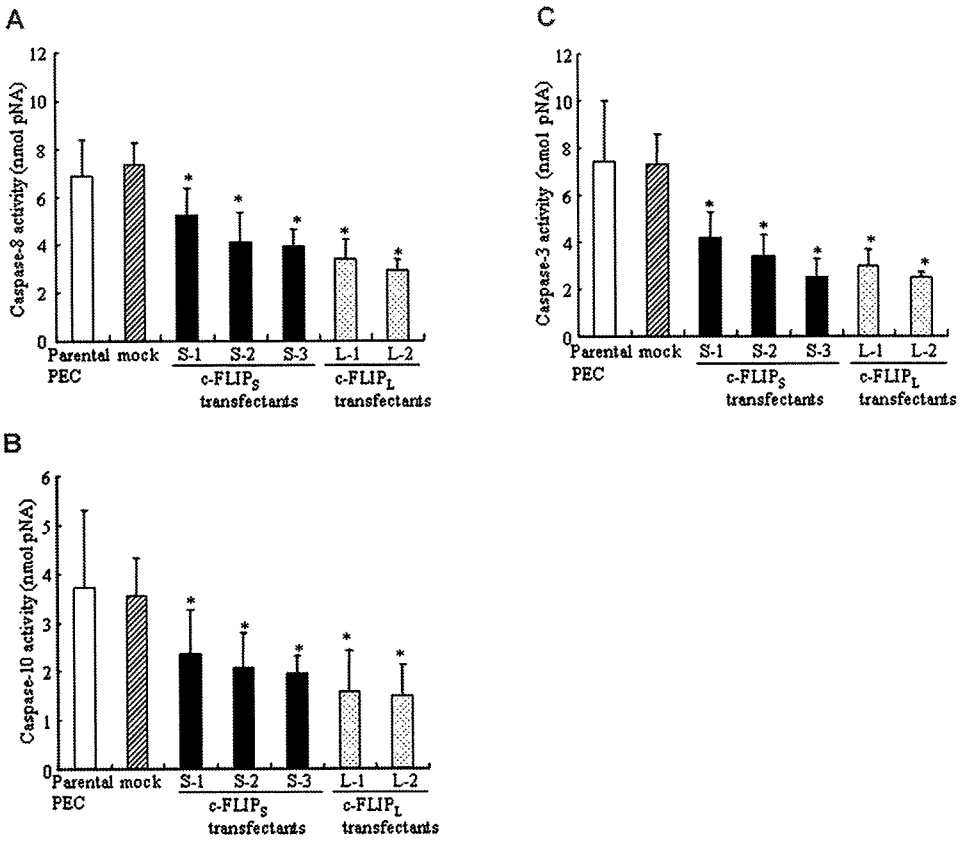

Figure 4: Inhibitory effects on caspase-8, -10 and -3 enzyme activities in PEC transfectants. Parental PEC, mock and PEC transfectants were cocultured with human $CD8^+$ CTLs. Subsequently, cells were recovered and then incubated with caspase-8, -10 and caspase-3 substrates, respectively. Caspase enzyme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

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

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

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

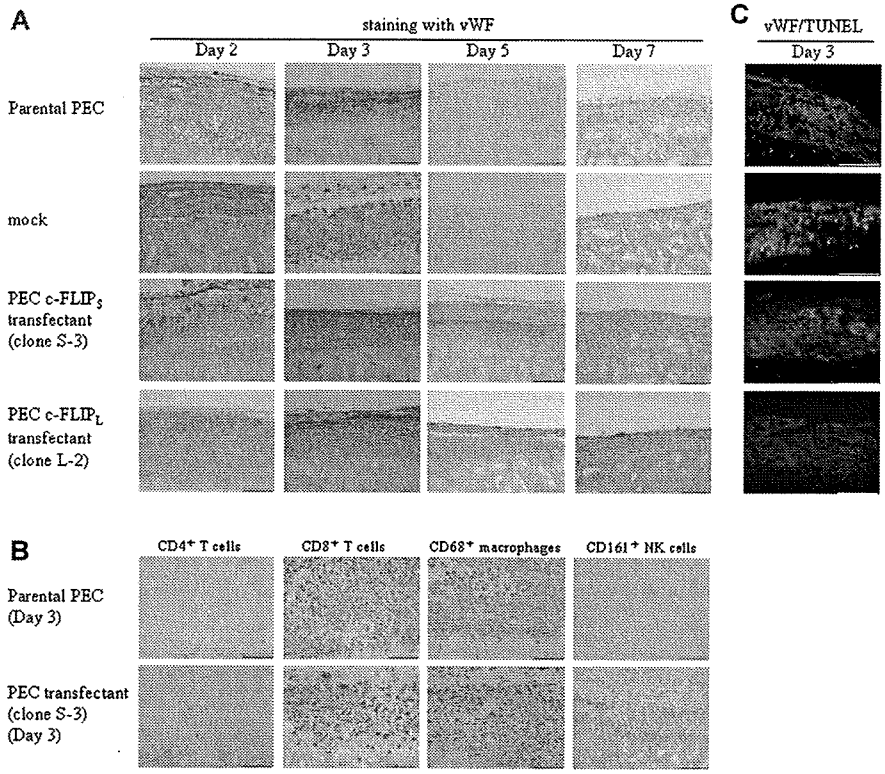

Figure 5: Histological appearance of rat kidney tissue of parental PEC, mock or PEC transfectants transplanted rats. (A) Immunostaining with anti-vWF Ab for transfected PEC of kidney specimens obtained at day 2, day 3, day 5 and day 7 posttransplant. To assess the efficacy of overexpression of c-FLIP$_{S/L}$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

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

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

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

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

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ELSEVIER

Xenotransplantation

Adenoviral-Mediated Overexpression of Either Membrane-Bound Human FasL or Human Decoy Fas Can Prolong Pig Islet Xenograft Survival in a Rat Transplant Model

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ABSTRACT

The success of pancreatic islet transplantation is limited because of the severe shortage of allogeneic pancreas donors. Accordingly, pig islets are considered to be an attractive, promising alternative. However, cell-mediated immunity, especially $CD8^+$ cytotoxic T lymphocyte (CTL)-mediated cytotoxicity, remains a formidable barrier to prevent long-term islet survival in xenograft recipients. Therefore, it is particularly important to explore methods to specifically prevent cell-mediated immunity against pig islets. Our group previously demonstrated that the overexpression of either membrane-bound human FasL or human decoy Fas antigen in pig endothelial cells prevented CTL xenocytotoxicity. In this study, we assessed the cytoprotective effects of adenoviral-mediated overexpression of either membrane-bound human FasL or human decoy Fas antigen in pig islets to inhibit CTL xenocytotoxicity. The CTL-mediated killing of pig islets infected with an adenoviral vector carrying either membrane-bound human FasL or human decoy Fas was significantly reduced compares with that of control pig islets transfected with adenoviral vector encoding enhanced green fluorescent protein (EGFP). Moreover, we transfected pig islets with these molecules to confirm their cytoprotective effects in in vivo studies. The significant long-term survival of pig islets expressing these molecules was elicited through days 3 to 5 posttransplantation. Thus, these results demonstrated that the remodeling of either death receptor or death ligand on pig islets by adenoviral gene transfer prevented innate cellular immunity against xeno-islet grafts facilitating long-term xenograft survival.

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PIG ISLETS are considered to be an attractive, promising alternative to treat type 1 diabetes mellitus. However, antibody-mediated immunity due to the interactions between the natural anti-Gal antibody and the α-gal epitopes on pig cells results in rejection of pig vascularized organs transplanted into humans. Fortunately, this immunological barrier may not occur in the case of pig-to-human islet xenotransplantation, because pig islets do not express α-gal epitopes. However, pig islet xenografts are destroyed by cell-mediated immunity. Recently, several groups have achieved prolonged functional survival of pig islet xenografts in immunosuppressed nonhuman primates.[1,2] However, the high level of immunosuppression used in these studies may be difficult to employ in 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 $CD8^+$ cytotoxic T lymphocyte (CTL), is highly detrimental toward xenografts.[3] We have explored methods to prevent this killing by overexpression of either membrane-bound human FasL or human decoy Fas antigen.[4] In the present study, we have assessed the effectiveness of these molecules to protect pig islet xenografts from human $CD8^+$ CTL-mediated cytotoxicity.

MATERIALS AND METHODS

Preparation of Pig Islets

Pig pancreata were harvested from a slaughterhouse that handles young market weight pigs (Large White/Landrace × Duroc, 6–12 months old, 200–300 kg). Pig islets were isolated by the modified Ricordi method.[5] The pig pancreas distended with Liberase HI solution (Roche Diagnostics, Indianapolis, Ind, United States) was cut into several pieces, which were put into a sterile chamber for clinical islet isolation (Umihira, Kyoto, Japan) and digested as previously described.[5] Pig islets were purified with a continuous density gradient of iodixanol in an apheresis system (COBE2991 cell processor, Gambro Laboratory, Denver, Colo, United States).

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 μg/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 membrane-bound human FasL gene[7] or human decoy Fas antigen[8] were subcloned into the SwaI cloning site of the cytomegalovirus promoter-containing adenovirus-based cosmid vector (Ad-), pAxcwit (Takara Bio, Otsu, Japan).[9] All virus stocks were purified by cesium chloride density gradient centrifugation. Non-functioning enhanced green fluorescent protein (EGFP)-adenoviral vector was used as a control. Freshly isolated pig islets were exposed to these adenovirus vectors at a multiplicity of infection (MOI) of 30 for 1 hour. The expression of these molecules in pig islets was assessed by fluorescence-activated cell sorting (FACS) analysis.

Gene Expression in PEC Line

PEC transfectants, stably expressing either human decoy Fas antigen or membrane-bound human FasL gene, were generated by lipofectoAMINE (Invitrogen) as previously described.[4] The expression of these molecules in PEC was assessed by FACS analysis.

Transplant Studies and Immunohistochemical Analysis

Lewis rats (8–10 weeks old) were purchased from Oriental Yeast (Tokyo, Japan). Recipient rats were preimmunized with 250 mg of pig kidney membranes as previously described.[10] Either 3000 islet equivalents (IEQ) of adenoviral transduced pig islets or 2×10^6 PEC stable transfectants were transplanted under the kidney capsule. The grafted kidney retrieved at day 3 or 5 posttransplantation was subjected to immunohistochemical analysis (IHC), using anti-pig insulin antibody or anti-human von Willebrand factor (vWF) polyclonal antibody (Dako, Glostrup, Denmark). To identify the phenotype of graft-infiltrating rat lymphocytes we performed IHC for rat $CD4^+$ T cells, $CD8^+$ T cells, $CD68^+$ macrophages, and $CD161^+$ NK cells. Briefly, tissue sections were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed using Pascal (Dako) except for insulin staining. Endogenous peroxidase

Table 1. Gene Expression and Xenograft Survival

Cells or Pig Islets	Mean Fluorescence Intensity Determined by FACS Analysis		Survived PEC or Islets Identified by Anti-vWF or Anti-insulin Abs	
	Fas Antigen	FasL	Day 3	Day 5
Mock-PEC	15 (endogenous)	Not detected	+	Rejected
Decoy Fas-PEC	342	Not detected	++	+
Membrane-bound FasL-PEC	20 (endogenous)	127	+++	++
Ad-EGFP pig islets	25 (endogenous)	Not detected	Rejected	Rejected
Ad-decoy Fas pig islets	198	Not detected	+	+
Ad-membrane-bound FasL pig islets	22 (endogenous)	75	++	+

Results are averages of 5 experiments.

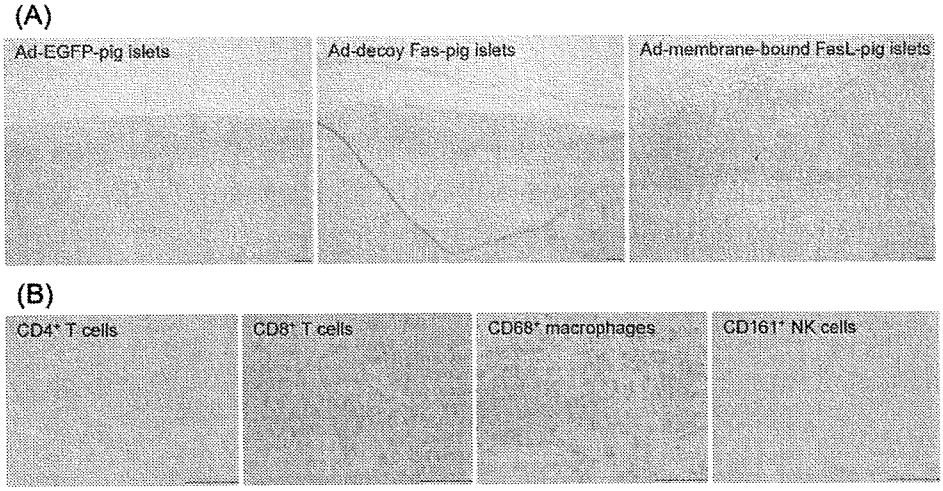

Fig 1. **(A)** Immunohistochemical analysis of tissue sections from rat kidney transplanted with either Ad-EGFP pig islets, Ad-decoy Fas pig islets, or Ad-membrane-bound human FasL pig islets (day 3). Representative sections are shown. **(B)** Phenotype of the graft-infiltrating rat lymphocytes into nontransfected pig xeno-islets at day 3 posttransplantation.

was blocked with 3% H_2O_2 methanol. After blocking with 10% BSA Tris buffered saline (TBS) containing 0.1% Tween 20, the sections were incubated with either guinea pig anti-pig insulin polyclonal antibody (pAb; Dako) or rabbit anti-human vWF pAb (Dako). Then the sections were visualized with 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 in which the primary antibody was omitted.

RESULTS

The expression levels of these molecules in PEC or pig islet transfectants were significantly increased as judged by mean fluorescence intensity, as summarized in Table 1. The survival of either pig islets or PEC xenografts was evaluated by IHC. Long-term survivals were observed using both decoy Fas-PEC and membrane-bound FasL-PEC xenografts, whereas control mock-PEC were completely rejected through days 3 to 5 posttransplantation (Table 1). Similarly, prolongation of xenografts with Ad-decoy Fas pig islets or Ad-membrane-bound FasL pig islets was also observed compared with EGFP-transfected (Table 1). Representative sections for insulin staining for the kidneys transplanted with pig islets are shown in Fig 1A. To assess the phenotype of graft-infiltrating lymphocytes, we performed IHC for rat CD4, CD8, CD68, and CD161. The majority of infiltrating cells belonged to $CD8^+$ CTL or $CD68^+$ macrophage elements (Fig 1B).

DISCUSSION

In this study, we demonstrated that both membrane-bound human FasL and human decoy Fas antigen prolonged pig islet or PEC xenograft survivals using an in vivo transplant model. These results suggested that pig islets isolated from transgenic pigs with either membrane-bound human FasL or human decoy Fas antigen genes may achieve long-term xenograft survival with minimal immunosuppression.

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ELSEVIER

Pig Cellular FLICE-like Inhibitory Protein (c-FLIP) Overexpression in Pig Xenograft Cells Induces Resistance to Human CD8$^+$ Cytotoxic T Lymphocyte-Mediated Xenocytotoxicity

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ABSTRACT

Although the use of organs from α1,3-galactosyltransferase gene knockout pigs may prolong xenograft survival, resulting in overcoming antibody-mediated hyperacute rejection, pig xenografts will be destroyed directly by cell-mediated immunity, such as NK cells, macrophages, and CD8$^+$ cytotoxic T lymphocytes (CTLs). Therefore, conquering cell-mediated immunity, especially of human CD8$^+$ CTLs, is of particular importance to the success of long-term xenograft survival. We have previously reported that the cytotoxicity of human CD8$^+$ CTLs is strong against pig endothelial cells (PEC) and mediated in major part by the Fas/FasL apoptotic pathway. Cellular FLICE inhibitory protein (c-FLIP) was originally identified as a potent inhibitor of death-receptor signaling through binding competition with caspase-8 for recruitment to Fas-associated via death domain (FADD). Two major c-FLIP variants result from alternative mRNA splicing: a short, 26-kDa protein (c-FLIP$_S$) and a long, 55-kDa form (c-FLIP$_L$). The present study demonstrated that overexpression of c-FLIP$_{S/L}$ genes in PEC markedly suppressed human CD8$^+$ CTL-mediated xenocytotoxicity; moreover, the cytoprotective effects of c-FLIP$_L$ appeared to be significantly stronger than those of c-FLIP$_S$. Furthermore, to prove the in vivo prolongation effects of xenograft survival, we transplanted PEC transfectants with c-FLIP$_{S/L}$ genes under the rat kidney capsule. Prolonged survival was displayed by xenografts of FLIP$_{S/L}$ PEC transfectants, whereas xenografts of parental PEC were completely rejected by day 5 posttransplantation. Thus, intracellular blocking of death receptor-mediated apoptotic signals by overexpression of c-FLIP$_{S/L}$ in xenograft cells may prevent innate cellular attacks against xenografts opening the window of opportunity for long-term xenograft survival.

THE CLINICAL APPLICATION of pig-to-human xenotransplantation has great potential to solve the current shortage of human organs and tissues for transplantation. However, meaningful benefits of xenotransplantation are severely restricted by antibody-mediated immunological barriers, consisting of human natural anti-Gal antibody that binds to α-gal epitopes (Galα1–3Galβ1–4GlcNAc-R) which are abundantly expressed on pig cells, inducing complement activation, hyperacute rejection, and acute vascular rejection of xenografts.[1] To avoid this immunological obstacle, α1,3-galactosyltransferase gene knockout (GT-KO) pigs have recently been

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produced not to express α-gal epitopes.[2] Although overcoming hyperacute rejection, xenografts are rejected by cell-mediated immunity, including NK cells, macrophages, and $CD8^+$ cytotoxic T lymphocytes (CTLs).[3,4] Our group previously demonstrated that human $CD8^+$ CTLs display strong cytotoxicity against xenograft cells, and that the Fas/FasL apoptotic pathway is a major contributor to this CTL-mediated xenocytotoxicity.[5] Therefore, human CTL cytotoxicity in xenograft recipients represents another immunological barrier to long-term xenograft survival.[3–5]

Fas-associated death domain-like interleukin-1-β-converting enzyme-inhibitory protein (FLIP) is a potent inhibitor of death receptor-mediated pro-apoptotic signals by blocking the signaling pathway more upstream before caspase-8 activation and release.[6–8] The mammalian cellular homologue is called c-FLIP.[6–8] Only 2 c-FLIP proteins are detected: a 26-kDa short form ($c\text{-}FLIP_S$) and a 55-kDa long form ($c\text{-}FLIP_L$).[6–8]

The present study addressed the following question regarding human $CD8^+$ CTL-mediated xenocytotoxicity: Will overexpression of $c\text{-}FLIP_{S/L}$ on pig xenograft cells block death receptor-mediated apoptotic signals and offer cytoprotection? Furthermore, using transplant studies we showed in vivo prolongation effects on xenograft survival by overexpresson of $c\text{-}FLIP_{S/L}$ molecules.

MATERIALS AND METHODS

Cell Culture

A pig endothelial cell (PEC) line, MYP-30,[9] was maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St Louis, Mo, United States) supplemented with 10% fetal bovine serum (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 Constructs

The full-length DNA fragments encoding either pig $c\text{-}FLIP_S$ or pig $c\text{-}FLIP_L$ were subcloned into the EcoR1 site of pCR3.1 expression vector (Invitrogen), which carried a CMV promoter and the SV40 replication origin with a neomycin-resistant gene, respectively.[8]

Gene Expression

Twenty micrograms of each of the plasmids ($pCR\text{-}FLIP_S$, $pCR\text{-}FLIP_L$) were introduced into the PEC line (MYP-30) by lipofectoAMINE (Invitrogen) according to the manufacturer's instructions. To generate stable PEC transfectants expressing pig $c\text{-}FLIP_{S/L}$ genes, the transfected PECs were cultured for 3 weeks in selection medium, namely, DMEM complete medium containing 1 mg/mL of G-418 (Sigma-Aldrich).

Western Blot Analysis

To identify the protein expression of either pig $c\text{-}FLIP_S$ or pig $c\text{-}FLIP_L$ in parental PEC or PEC transfectants, we performed Western blot analysis. Protein samples from parental or PEC transfectants were obtained using lysis buffer, consisting of 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L EDTA, 125 mmol/L NaCl, 10% (w/v) glycerol, 0.1% (w/v) NaN_3, 1% (w/v) NP-40, 5 μg/mL aprotinin, and 0.1 mmol/L phenylmethanesulfonyl fluoride (PMSF). For each sample, 10 μg of protein applied to the gel was then separated by electrophoresis in 8% or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, and transferred onto a PVDF membrane (Millipore, Bedford, Mass, United States). After transfer, the immunoblots were blocked overnight at 4°C by incubation with phosphate buffered saline (PBS) containing both 3% bovine serum albumin (BSA) and 0.1% Tween 20. Next, the blots were incubated with mouse anti-human c-FLIPmAb (Santa Cruz Biotechnology, Santa Cruz, Calif, United States). After washing, the blots revealed with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (DAKO, Glostrup, Denmark) were subsequently developed with an enhanced chemiluminescence (ECL) plus Western blotting detection system (GE Healthcare Bio-sciences KK, Piscataway, NJ, United States). Image scanner profiles were employed to evaluate expression levels of pig $c\text{-}FLIP_{S/L}$ protein in PEC transfectants, expressed as arbitrary units.

Generation of Human $CD8^+$ CTLs

To generate human $CD8^+$ CTLs, 10 to 15 × 10^6 cells of separated peripheral blood mononuclear cells (PBMCs) were cocultured for 14 days with irradiated PEC in the presence of recombinant human interleukin-2 (IL-2) as previously described.[5] Subsequently, human $CD8^+$ CTLs positively isolated using magnetic beads (Dynal, Oslo, Norway) coated with anti-human CD8 mAb (RPA-T8, BD Biosciences Pharmingen, San Jose, Calf, United States) were subjected to an in vitro cytotoxicity assay.

^{51}Cr Release Assay

The cytotoxic activity of human $CD8^+$ CTLs incubated under various conditions was assessed by ^{51}Cr release assays as previously described.[3,5] Parental PEC and PEC transfectants with either pig $c\text{-}FLIP_S$ or pig $c\text{-}FLIP_L$ genes were plated as target cells. After labeling target cells with ^{51}Cr, human CTLs isolated with magnetic beads were added to the wells. The ^{51}Cr released from dead cells was measured in the supernates. The effect of

Table 1. Changes in the Expression Levels of $c\text{-}FLIP_{S/L}$ Proteins*

Cells	Arbitrary Units in Western Blots	
	$c\text{-}FLIP_S$ Expression	$c\text{-}FLIP_L$ Expression
Parental PEC	5841 (endogenous)	7155 (endogenous)
A-4 PEC $FLIP_S$	7155	Endogenous
B-7 PEC $FLIP_S$	11,049	Endogenous
B-2 PEC $FLIP_S$	14,667	Endogenous
E-10 PEC $FLIP_L$	Endogenous	19,429
F-5 PEC $FLIP_L$	Endogenous	29,166

*The expression levels of either $c\text{-}FLIP_S$ or $c\text{-}FLIP_L$ proteins were examined by Western blotting. Image scanner profiles were employed to evaluate the expression levels of $c\text{-}FLIP_{S/L}$ proteins in both parental PEC and PEC transfectants.

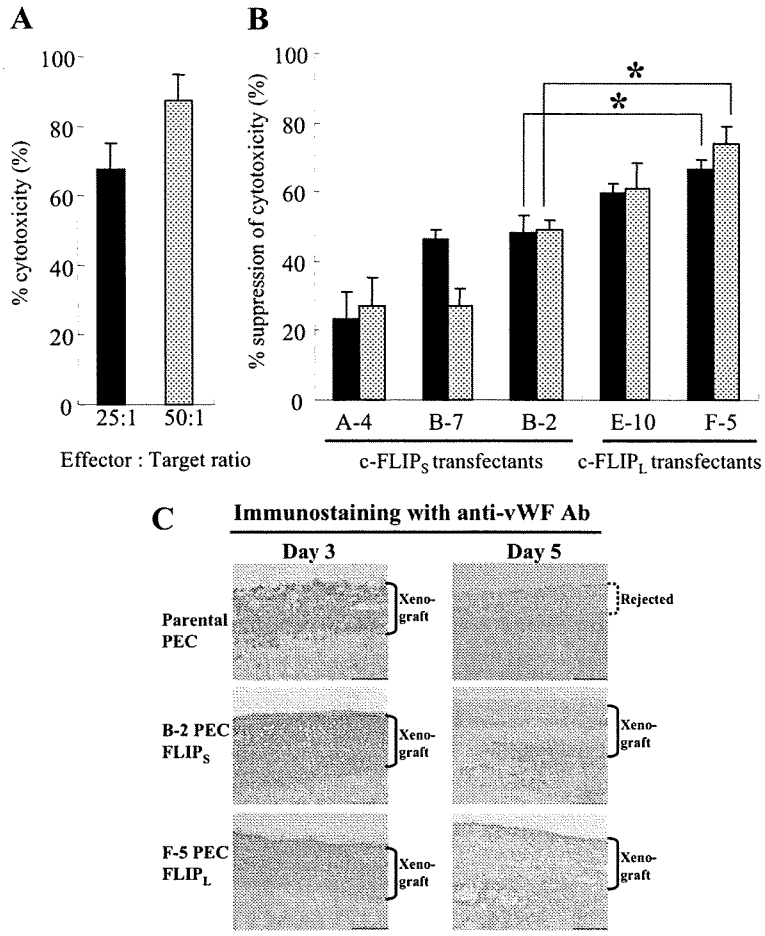

Fig 1. ^{51}Cr release assay of pig c-FLIP$_{S/L}$ transfected PEC. Amelioration of human CD8$^+$ CTL-mediated cytotoxicity by the PEC transfectants and control parental PEC was estimated at the effector-to-target ratio of either 25:1 or 50:1. **(A)** Percentages of CTL cytotoxicity against parental PEC. **(B)** Percentages of suppression of CTL killing by c-FLIP$_{S/L}$ transfectants. Closed rectangles: effector-to-target ratio = 25:1; dotted rectangles: effector-to-target ratio = 50:1. Each value is expressed as the mean ± SD from 5 independent experiments. Statistical significance: *P < .05 compared with B-2 c-FLIP$_S$ and F-5 c-FLIP$_L$ transfectants. **(C)** Immunohistological appearance of rat kidney tissue of PEC transfected rats. Immunostaining with anti-vWF antibody for transfected PEC of kidney specimens obtained at day 3 and day 5 posttransplantation. Pictures are representative of immunostaining of kidney sections obtained from 5 animals per each transfectant group. The bars in each picture indicate 100 μm.

c-FLIP$_{S/L}$ expression on human CTL-mediated cytotoxicity was determined by comparisons with PEC transfectants and parental PEC.

Transplant Studies and Immunohistochemical Analysis

Lewis rats (8–10 weeks old) were purchased from Oriental Yeast (Tokyo, Japan) and distributed randomly between experimental groups (n = 5 rats per group) to receive either parental PEC or PEC transfectants. Rats preimmunized 3 times intraperitoneally with 250 mg of pig kidney membranes with a 1-week interval between injections were employed as the recipients. In each case, 2.5 × 10^6 cells of either parental 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, 3, 5, or 7 posttransplantation. Each grafted kidney was analyzed by immunohistochemistry. Kidney specimens cut into small blocks were fixed in formalin and embedded in a single paraffin block. After quenching endogenous peroxidase activity by exposure to 3% H_2O_2 methanol, paraffin sections were stained with a rabbit anti-human von Willebrand factor (vWF) polyclonal antibody (DAKO) to detect specifically endothelial cells. The sections were then rinsed and incubated with link antibody, followed by HRP-conjugated streptavidin. Immunostaining was visualized with 0.02% diaminobenzidine (DAB; Sigma-Aldrich) as the chromogen. The specificity for the primary vWF antibody was verified with control sections in which the primary antibody was omitted.

Statistical Analysis

Data were evaluated using the Student's t test with P < .05 considered significant. Data were presented as mean values ± SD.