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Rapamycin Induces Autophagy in Islets: Relevance in Islet Transplantation

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ABSTRACT

Islet transplantation can provide insulin independence in patients with type 1 diabetes mellitus. However, islet allograft recipients exhibit a gradual decline in insulin independence, and only 10% do not require insulin at 5 years. This decline may reflect drug toxicity to islet β cells. Rapamycin, a central immunosuppressant in islet transplantation, is a mammalian target of rampamycin inhibitor that induces autophagy. The relative contributions of autophagy in transplanted islets are poorly understood. Therefore, in the present study we sought to evaluate the effects of rapamycin on islet β cells. Rapamycin treatment of islets resulted in accumulation of membrane-bound light chain 3 (LC3-II) protein, an early marker of autophagy. In addition, rapamycin treatment of isolated islets elicited not only reduction of viability but also downregulation of in vitro potency. To further examine the occurrence of autophagy in rapamycin-treated islets, we used GFP (green fluorescent protein)–LC3 transgenic mice that express a fluorescent autophagosome marker. The GFP-LC3 signals were markedly increased in rapamycin treated islets compared with control islets. In addition, to show improvement by blockade of autophagic signaling, islets were treated with rapamycin in the presence of 3-methyladenine, which inhibits autophagy. Thereafter, both islet viability and islet potency were dramatically improved. The number of GFP-LC3 dots clearly increased after 3-MA treatment. Thus, rapamycin treatment of islets induces autophagy in vitro. This phenomenon may contribute to the progressive graft dysfunction of transplanted islets. Therapeutically targeting this novel signaling may yield significant benefits for long-term islet survival.

CLINICAL ISLET TRANSPLANTATION in patients with type 1 diabetes mellitus has recently increased because of the results of the Edmonton protocol, a rapamycin-based, glucocorticoid-free, immunosuppressive regimen that led to insulin independence at 1 year in 90% of treated patients.[1] However, long-term follow-up indicated marked reduction in graft function; only 10% of islet recipients maintained insulin independence at 5 years.[2] While the causes of decline in insulin independence rates seen in

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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-cannabalistic 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).

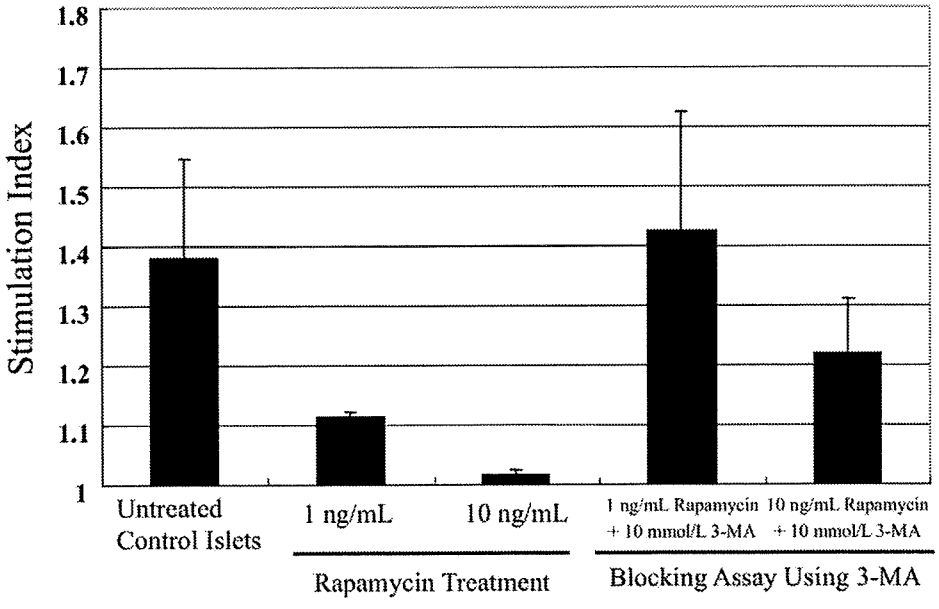

Fig 1. In vitro potency assessments of rapamycin-treated islets. Islet potency was assessed using static glucose challenge. Islet potency, expressed as stimulation index, was markedly reduced by rapamycin treatment. However, islet potency dramatically recovered with rapamycin plus 3-MA treatment. Values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

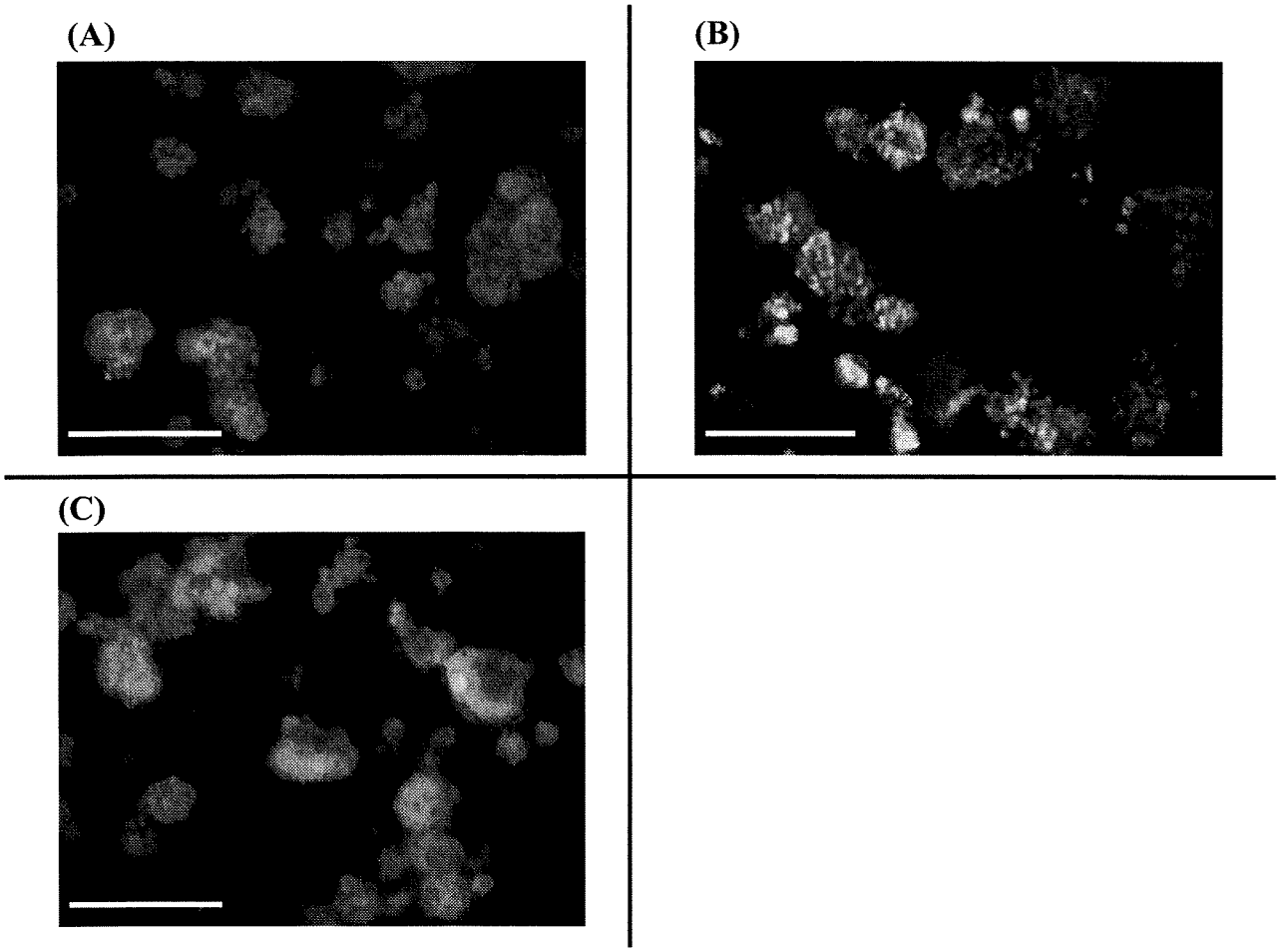

Fig 2. Islet autophagy in response to rapamycin treatment. Islet samples were prepared from GFP-LC3 transgenic mice. A, Untreated control islets. B, Islets treated with 1 ng/mL of rapamycin. C, Islets treated with rapamycin plus 3-MA. Bars indicate 100 μm.

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.

REFERENCES

1. Shapiro AM, Lakey JR, Ryan EA, et al: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343:230, 2000

2. Ryan EA, Paty BW, Senior PA, et al: Five-year follow-up after clinical islet transplantation. Diabetes 54:2060, 2005

3. Kirken RA, Wang YL: Molecular actions of sirolimus: sirolimus and mTor. Transplant Proc 35(3 suppl):227S, 2003

4. Hay N, Sonenberg N: Upstream and downstream of mTOR. Genes Dev 18:1926, 2004

5. Schmelzle T, Hall MN: TOR, a central controller of cell growth. Cell 103:253, 2000

6. Ravikumar B, Vacher C, Berger Z, et al: Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet 36:585, 2004

7. Paglin S, Hollister T, Delohery T, et al: A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res 61:439, 2001

8. Nakai A, Yamaguchi O, Takeda T, et al: The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med 13:619, 2007

9. Tanida I, Minematsu-Ikeguchi N, Ueno T, et al: Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy 1:84, 2005

10. Woods DC, Alvarez C, Johnson AL: Cisplatin-mediated sensitivity to TRAIL-induced cell death in human granulosa tumor cells. Gynecol Oncol 108:632, 2008

11. Kim KW, Mutter RW, Cao C, et al: Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. J Biol Chem 281:36883, 2006
12. Ranuncoli A, Cautero N, Ricordi C, et al: Islet cell transplantation: in vivo and in vitro functional assessment of nonhuman primate pancreatic islets. Cell Transplant 9:409, 2000
13. Mizushima N, Yamamoto A, Matsui M, et al: In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell 15:1101, 2004
14. Huang S, Bjornsti MA, Houghton PJ: Rapamycins: mechanism of action and cellular resistance. Cancer Biol Ther 2:222, 2003

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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 α1, 3-galactosyltransferase gene knockout animals (GT-KO) that bear deletion of the major xenoantigen, α-gal epitopes (Galα1-3Galβ1-4GlcNAc-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 killing[6] 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) encording 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 1α 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 manufacture'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 arbitary 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 × 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 (Dynal, 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 ^{51}Cr 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 ^{51}Cr for target cells, human CTLs isolated using magnetic beads were added to the wells. ^{51}Cr 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 × 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% H_2O_2/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 (Arbitary 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

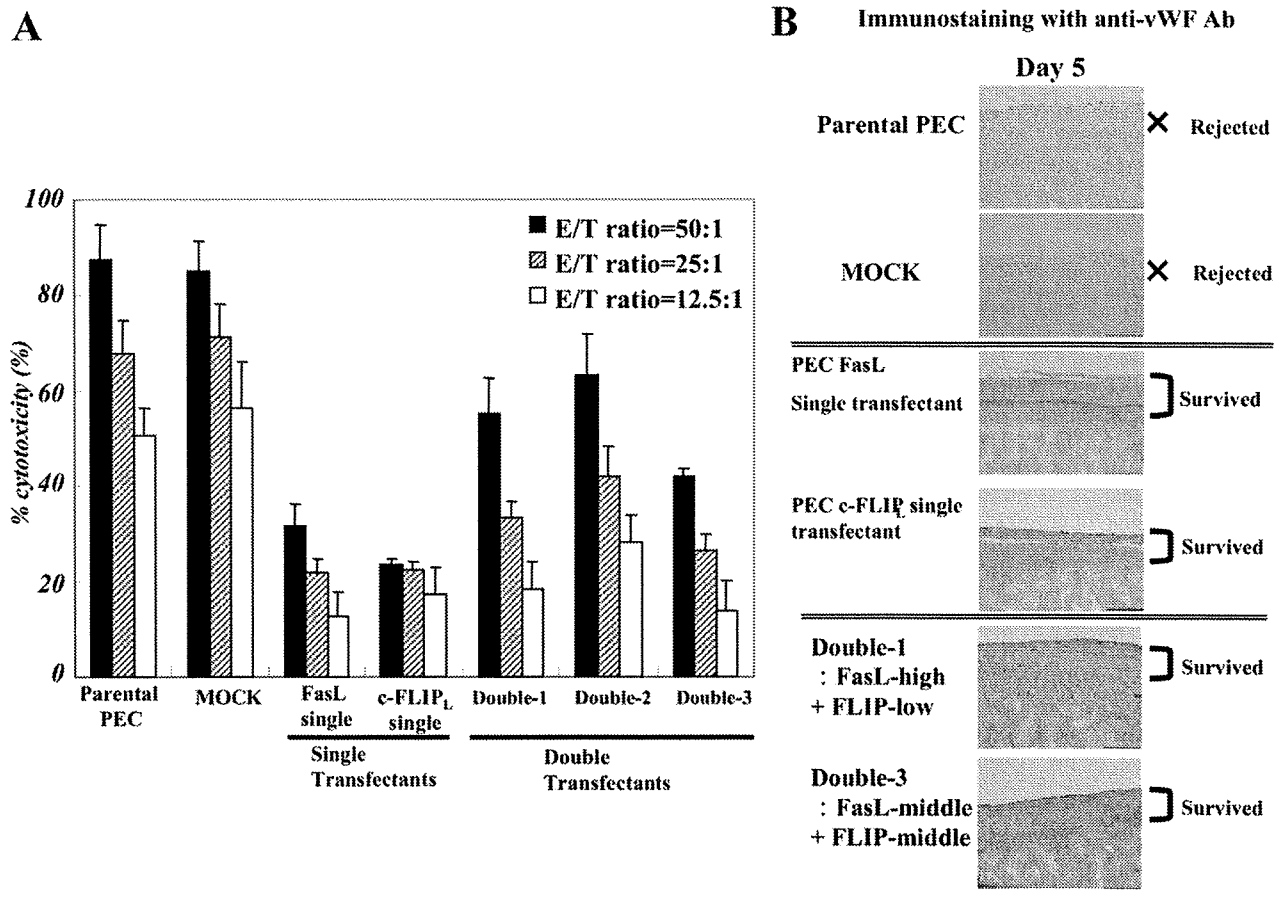


Fig 1. ^{51}Cr release assay of PEC transfectants and immunohistological findings of PEC xenografts transplanted under rat kidney capsule. Amelioration of human CD8$^+$ CTL-mediated cytotoxicity by the PEC transfectants, MOCK, and control parental PEC was estimated at the effector:target ratio of either 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 transfected 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 cooperative 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.

REFERENCES

1. Galili U: Interaction of the natural anti-Gal antibody with α-galactosyl epitopes: a major obstacle for xenotransplantation in humans. Immunol Today 14:480, 1993
2. Yamada K, Yazawa K, Shimizu A, et al: Marked prolongation of porcine renal xenograft survival in baboons through the use of α 1,3-galactosyltransferase gene-knockout donors and the cotransplantation of vascularized thymic tissue. Nat Med 11:32, 2005
3. Tanemura M, Chong AS, DiSesa VJ, et al: Direct killing of xenograft cells by CD8+ T cells of discordant xenograft recipients. Transplantation 74:1587, 2002
4. Itescu S, Kwiatkowski P, Artrip JH, et al: Role of natural killer cells, macrophages, and accessory molecule interactions in the rejection of pig-to-primate xenografts beyond the hyperacute period. Hum Immunol 59:275, 1998
5. Kawamoto K, Tanemura M, Ito T, et al: Significant inhibition of human CD8$^+$ cytotoxic T lymphocyte-mediated xenocytotoxicity by overexpression of the human decoy Fas antigen. Transplantation 81:789, 2006
6. Tanemura M, Saga A, Kawamoto K, et al: In vitro and in vivo prevention of human CD8$^+$ CTL-mediated xenocytotoxicity by pig c-FLIP expression in porcine endothelial cells. Am J Transplant 8:288, 2008
7. Miyagawa S, Shirakura R, Iwata K, et al: Effects of transfected complement regulatory proteins, MCP, DAF, and MCP/DAF hybrid, on complement-mediated swine endothelial cell lysis. Transplantation 58:834, 1994
8. Goto Y, Matsuda-Minehata F, Inoue N, et al: Porcine (Sus scrofa) cellular FLICE-like inhibitory protein (cFLIP): molecular cloning and comparison with the human and murine cFLIP. J Reprod Dev 50:549, 2004

報告

本邦膵移植症例登録報告(2008)

膵・膵島移植研究会膵臓移植班

The Registry of Japanese Pancreas Transplantation 2008

The Japan Society for Pancreas and Islet Transplantation

【Summary】

As of the end of 2007, 44 cases of pancreas transplantation from deceased and non-heartbeating donors have been performed in 10 institutions in Japan since April 2000. The following donor-and recipient-related factors were analyzed: age and sex of donor and recipient, cause of death, history of diabetes and dialysis, waiting period, cold ischemic time, operative procedure, immunosuppression and survival rates of patient and graft. Fourteen cases of pancreas transplantation from living donors also were done during the same period.

In spite of donor poor conditions, which are mostly marginal in Japan, the outcome of pancreas transplants is considered comparable to that seen in the USA and Europe.

Keywords: simultaneous pancreas and kidney transplantation (SPK), pancreas after kidney transplantation (PAK), pancreas transplantation alone (PTA), deceased donors, non-heartbeating donors, living-related donors, marginal donor, bladder drainage, enteric drainage, tacrolimus, anti IL-2 receptor monoclonal antibody, mycophenolate mofetil (MMF)

I. はじめに

膵・膵島移植研究会は症例登録のための委員会を置き，本邦における膵臓移植の全症例の把握，移植成績ならびに問題点を解析することを目的として，昨年度より登録作業を開始した。昨年は，1997年に「臓器の移植に関する法律」が実施されて以降，2006年末までに本邦で実施された脳死下，心停止下での膵臓移植ならびに生体膵臓移植全41症例につき報告した[1]。

今回は，昨年1年間に実施された17例をさらに追加して，新たに解析を行った。

II. 対象と方法

2007年末までに，本邦で実施された脳死下，心停止下での膵臓移植ならびに生体膵臓移植を受けた58症例につき，患者数の推移，ドナー・レシピエント関連因子（ドナーの性差と年令，ドナーの死亡原因，レシピエントの性差と年令，透析歴と糖尿病歴，待機期間，総冷阻血時間，手術術式，免疫抑制法），移植成績（生存率，移植膵・移植腎生着率）を解析した。なお，累積生存率，膵および腎の生着率はKaplan-Meier法で算出した。

1. 膵臓移植認定施設

現在，認定施設は北海道大学（2），東北大学（1），福島県立医科大学（2），新潟大学（2*），東京女子医科大学（7），東京医科大学八王子医療センター（0），国立病院機能千葉東病院（11*），名古屋第二赤十字病院（0），京都府立医科大学（2），大阪大学（11＋1*），奈良県立医科大学（0），神戸大学（2），広島大学（0），九州大学（17），以上14施設である（括弧内は2007年末までの膵臓移植の実施数で，*は生体膵移植数である）。

2. 膵臓移植実施体制

本邦における膵臓移植は中央調整委員会の下に，認定13施設（現在は14施設）の代表からなる実務者委員会が組織され，そこで作成された実施のためのガイドライン『膵臓移植に関する実施要綱，2001年版』[2]（2009年3月に改訂予定）に従って運用されている。とりわけ，膵臓移植の特徴は，他の臓器と異なり，national teamを編成してall Japanで対応している点であ

る。実務者委員の中で特に経験の多いエキスパート（幹事）が中心となり，手術ならびに術後管理に対応している。

3. レシピエントカテゴリーと登録システム

膵臓移植には3つのレシピエントカテゴリーがある。すなわち，腎不全がある場合に膵臓と腎臓を同時に移植する膵腎同時移植（simultaneous pancreas and kidney transplantation：SPK）と，先に腎臓移植を先行させ後に膵臓移植を行う腎移植後膵移植（pancreas after kidney transplantation：PAK）とがあり，もうひとつは腎不全のない方に対する膵単独移植（pancreas transplantation alone：PTA）である。

膵臓移植の適応基準[2]に従い，レシピエント候補者の主治医が地域の膵臓移植適応評価委員会にデータを添えて申請して，その結果が中央調整委員会へ送付される。最終的に中央調整委員会から移植施設に対して，移植可能の是非が確認され，日本臓器移植ネットワークへ登録となる。

ドナー（脳死下，心停止下）発生時には，登録されたレシピエントの中から，選択基準[2]に従って選択される。

III. 結果と考察

1. 膵移植新規登録患者数

膵移植の日本臓器移植ネットワークへの登録は1999年10月より開始され，それ以降の新規登録患者数の推移を**図1**に示した。2007年末までに，日本臓器移植ネットワークに新規登録された患者数は計224名である。2001年以降は毎年30名弱の新規患者が登録されている。なお，登録後，糖尿病性合併症の増悪などにより20名が死亡した。また，10人が糖尿病性合併症の進行などの理由で登録を取り消している。

2. 膵移植症例数

1997年10月「臓器の移植に関する法律」の施行後，2007年末までの脳死下での臓器提供は63例あり，その内，膵臓が提供に至ったのは42例（66.7%）であった。その内訳はSPKが34例，PAKが5例に加えて，2007年には新たに3例のPTAが行われた。なお，提供されなかった21例の内訳は医学的理由が9例，未登録時期（～1999年9月）での提供が4例，意思表示カード上での未承諾が4例，適合者不在が3例，クロスマッチ陽性が1例であった。また，同期間中に2例の心停止下での膵臓移植（SPK）が行われた。さらに，生体ドナーからの膵臓移植も14例行われた。移植症例数の年次推移が示されている（**図2**）。ここ数年は増加傾向を認めている。

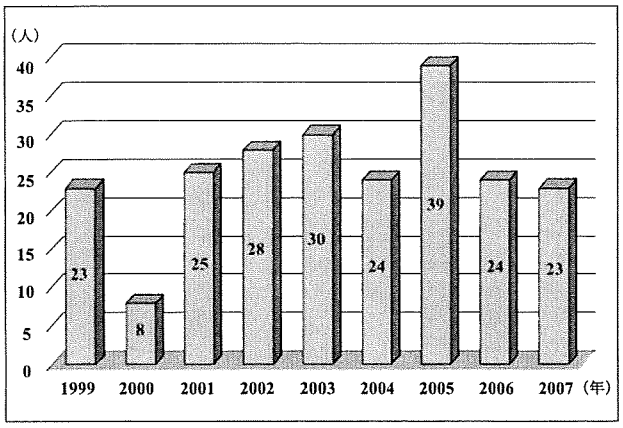


図1　新規登録患者の年次推移

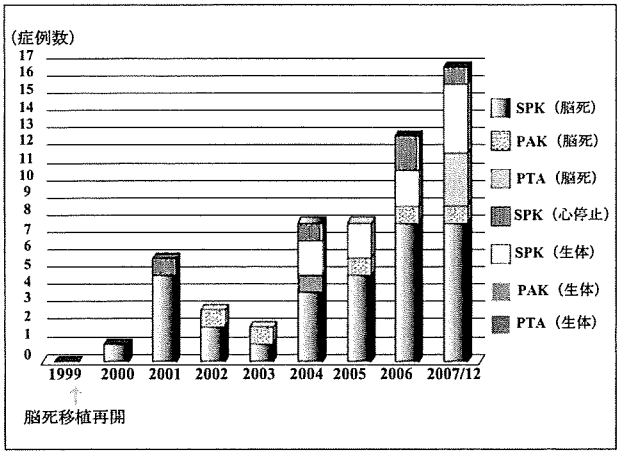


図2　膵移植症例数の推移

3. ドナー・レシピエント関連因子（脳死下・心停止下）

脳死・心停止下で行われた膵臓移植症例44例のドナー・レシピエント関連因子について，解析した。

1）ドナーの年齢・性差

男女比は19：25と女性が多く，年齢は50歳代が15名と最も多く，40歳代の13名に続き，30歳代，20歳代，10歳代がそれぞれ，8名，7名，1名であった（**図3**）。本邦では40歳以上の高齢ドナーが28名（63.4%）と2/3弱を占めている。

2）ドナーの死亡原因

死因は脳血管障害が24名（54.5%）と最も多く，なんらかの動脈硬化性変化が否定できない。他に，低酸素血症が10名，外傷が7名，その他が3名であった（**図4**）。

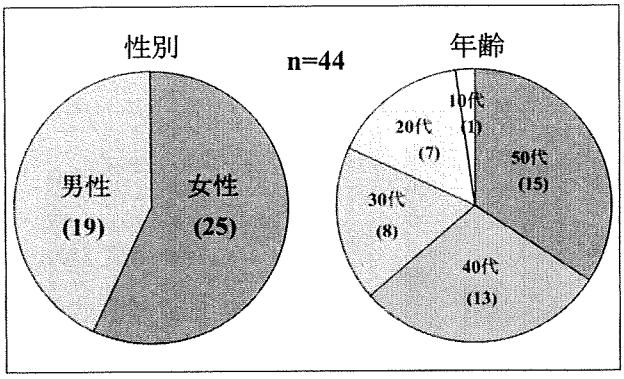


図3　ドナーの性と年齢

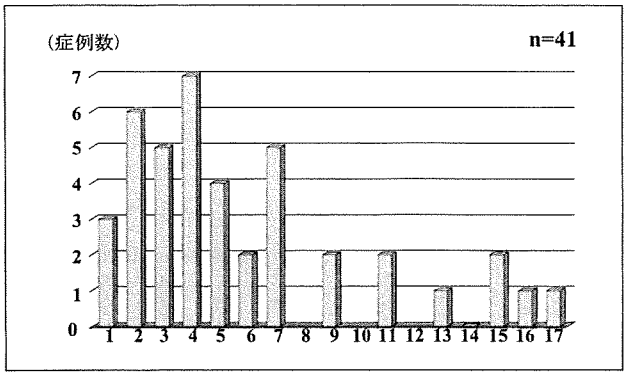


図6　透析歴

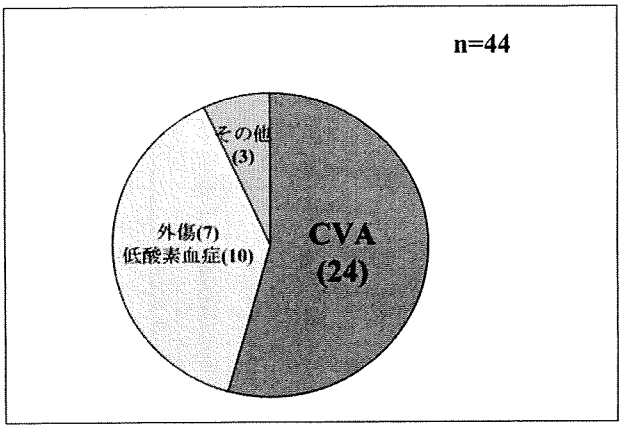


図4　ドナーの死亡原因

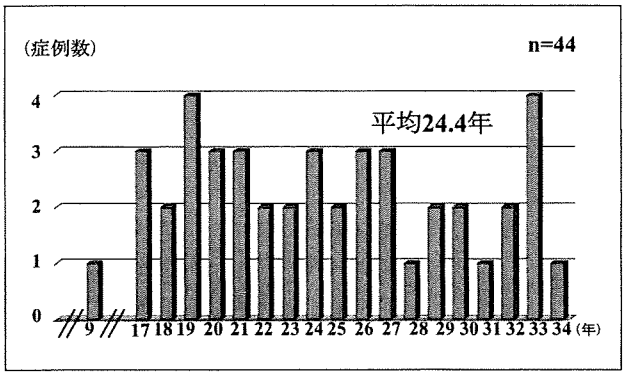


図7　糖尿病歴

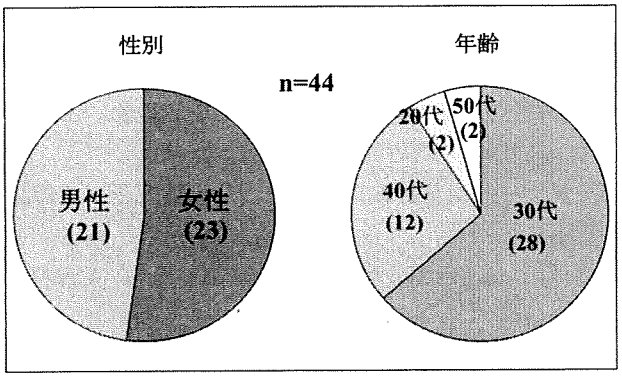


図5　レシピエントの性と年齢

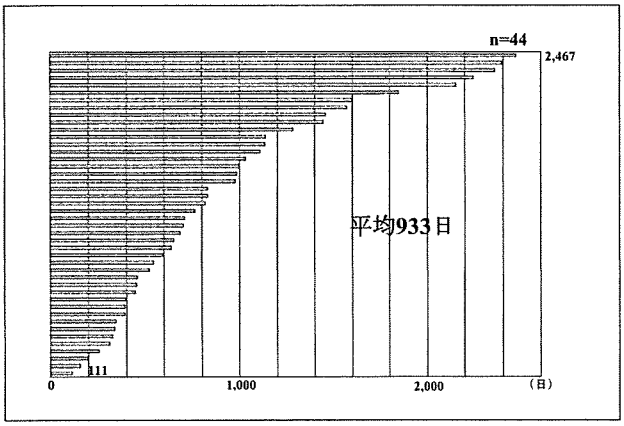


図8　待機期間

3) レシピエント年齢・性差

男女比は21：23でほぼ同数，年齢は30代が28名と最も多く，ついで40代が12名で50代，20代がそれぞれ2名ずつであった（**図5**）。

4) 透析歴と糖尿病歴

透析歴は平均5.9(1～17)年で，糖尿病歴は平均24.4(9～34)年であった（**図6，7**）。

5) 待機期間

移植を受けたレシピエントの平均待機期間は933(111～2,467)日と年々増加しており，約2年半であった（**図8**）。

6)　総冷阻血時間（TCIT）

膵のTCITは平均11時間47分であった。腎のTCITは平均13時間10分であり，2峰性を示し両臓器とも

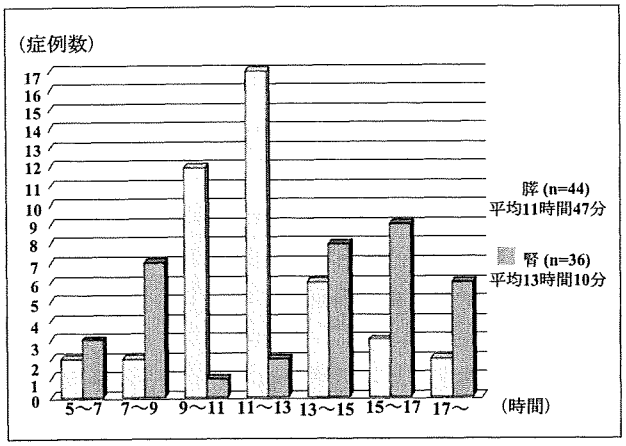


図 9　総冷阻血時間

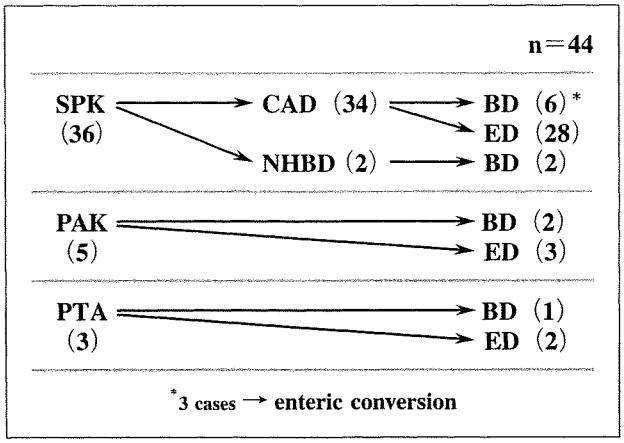


図 11　手術術式

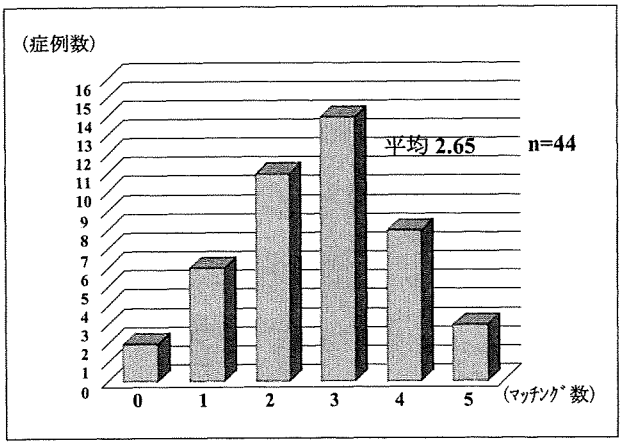


図 10　HLA ミスマッチ

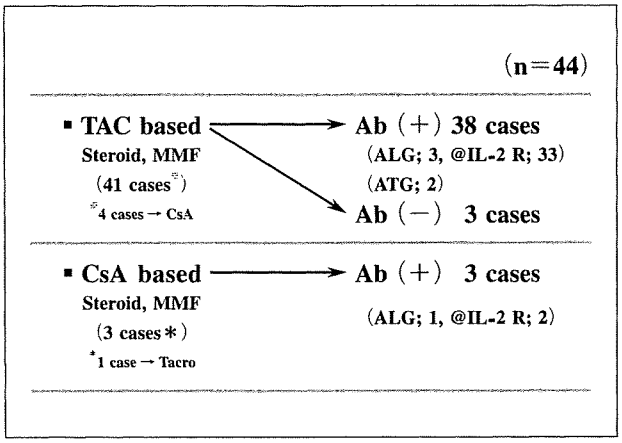


図 12　免疫抑制法

に十分許容範囲であった。これは SPK の場合，腎移植を先行させる場合と膵移植を先行させる場合があることによると考えられた（**図 9**）。なお，臓器搬送に要する時間は平均 4 時間 05 分であった。

7）HLA ミスマッチ

HLA ミスマッチ数は平均 2.65 であった（**図 10**）。

8）移植術式

脳死下での SPK　34 例では当初は安全性を考慮して，膀胱ドレナージ（bladder drainage：BD）が行われたが，最近ではもっぱら腸管ドレナージ（enteric drainage：ED）が 28 例と 80% 以上を占めている。なお，BD　8 例の内，尿路感染症や逆行性グラフト膵炎などの理由で 3 例は enteric conversion（EC）となった。また，心停止下での場合や PAK・PTA 症例ではグラフトの膵液をモニターする必要性から，10 例中 5 例に BD が用いられた（**図 11**）。

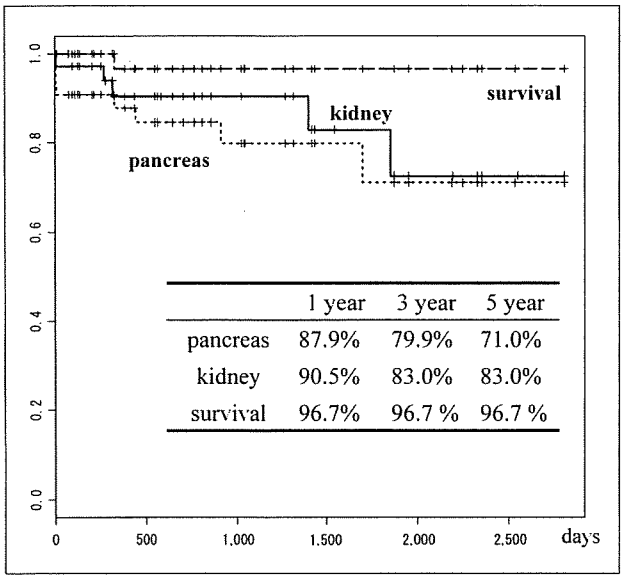
9）免疫抑制法

タクロリムス（TAC）をベースとして，ステロイド，ミコフェノール酸モフェチル（MMF），抗 IL-2R 抗体（basiliximab）の 4 剤併用療法が 76.7% と最も多く用いられている。うち，4 例が毒性のため TAC からシクロスポリン（CsA）へ変更となっている。一方，CsA をベースとして，4 剤併用療法が 3 例に行われ，うち 1 例は TAC へ変更となった（**図 12**）。

4. 移植成績（脳死下・心停止下）

脳死・心停止下での移植症例 44 例のうち，1 例（SPK）が移植後 11 カ月原因不明の心肺停止があり，その後蘇生後脳症にて死亡したが，他の 43 例は全例生存している。

移植膵の生着については，移植後急性期に 4 例が血栓症にて移植膵が摘出され，移植後 2 年目に 1 例がイレウスからグラフト十二指腸穿孔により摘出された。他に，3 年目に 1 例が慢性拒絶反応にて，4 年目に 1



	1 year	3 year	5 year
pancreas	87.9%	79.9%	71.0%
kidney	90.5%	83.0%	83.0%
survival	96.7%	96.7 %	96.7 %

図 13　生存率とグラフト生着率（Kaplan-Meier 法）

例が原因不明にて，また 1 例は移植膵が機能するも死亡にて，計 8 例が機能喪失となっている（**図 13**）。1 年，3 年，5 年生着率はそれぞれ 87.9%，79.9%，71.0% であった。

一方，移植腎の生着については，SPK 36 症例中，1 例が primary　non-function（PNF），他の 4 例がそれぞれ，10 カ月および 2 年（8 カ月で透析再導入）で再移植，3 年と 5 年で透析再導入となっている。1 年，3 年，5 年生着率はそれぞれ 90.5%，83.0%，83.0% であった。なお，PAK の 4 例の移植腎機能は膵臓移植によって一部増悪する場合も見られた。

5. 生体膵臓移植について

生体ドナーから行われた膵臓移植症例 14 例における上記関連因子について解析した。

ドナーは全例，両親のどちらか(母親；9 例，父親；5 例）からであり，平均年齢は必然的に 61.7（55～72）歳と高齢であった。一方，レシピエントは男性，女性ともに 7 例ずつで，平均年齢は 35.6(29～46)歳であった。カテゴリ別では，SPK が 10 例と最も多く，ついで PTA の 3 例，PAK が 1 例であった。術式別では，脳死・心停止下とは異なり，大半が BD（11 例）であり ED は 3 例であった。免疫抑制療法は脳死・心停止下の場合と同様であった。

移植成績：1 例が移植 1 年後，脳梗塞にて死亡した。これは PAK の 1 例で，移植膵は機能するも，膵移植後 2 カ月で移植腎の機能が増悪して透析再導入となった症例である。PAK の場合には，膵臓移植前に移植腎の機能を慎重に評価しなければならないと考えられた。移植膵機能については，移植後 6 カ月以内に 2 例がインスリン再導入となっている。

IV. まとめ

以上，2007 年末までの本邦における膵臓移植症例 58 例について，ドナー，レシピエント関連因子を解析し，治療成績を報告した。本邦では marginal case が多く，ドナーの条件は良くないにもかかわらず，移植成績は欧米と遜色のない結果であると考えられた。また，「臓器の移植に関する法律」が実施されて 10 年以上が経過した。近々に同法律の改正を是非とも実現させて，糖尿病の根治療法としての膵臓移植のさらなる飛躍を期待したい。

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膵臓移植症例登録委員会事務局
伊藤壽記，石橋道男

文　献

1）本邦膵症例登録報告 2007　移植 42（5）；433-438, 2007
2）膵・膵島移植研究会編．膵臓移植に関する実施要綱 2001 年版. 東京: 膵・膵島移植研究会, 2001.

Effects of Cyclosporin A on the Activation of Natural Killer T Cells Induced by α-Galactosylceramide

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Background. Natural killer T (NKT) cells play crucial roles in preventing autoimmune diseases and inducing transplantation tolerance. We investigated whether cyclosporin A (CsA), which is generally used in clinical transplantation and autoimmune disease therapy, could modulate the NKT cell activation induced by α-galactosylceramide (α-GalCer) treatment.

Methods. C57BL/6 (B6) mice were given daily intraperitoneal injections of CsA (30 or 50 mg/kg) from day −1 and injected intravenously with α-GalCer (2 μg/mouse) on day 0. The kinetics of $NK1.1^+CD3^+$ or $NK1.1^+Thy1.2^+$ cells in the liver and spleen were analyzed by flow cytometry. Apoptosis of $NK1.1^+CD3^+$ cells, cytokine levels (interleukin [IL]-2, IL-4, IL-10 and interferon [IFN]-γ) in the recipient serum and changes in dendritic cell activation in the spleen were analyzed.

Results. In B6 mice treated with α-GalCer, $NK1.1^+CD3^+$ cells rapidly decreased in both the liver and spleen, and repopulated to their normal levels by day four, while $NK1.1^+Thy1.2^+$ cells rapidly decreased, expanded by day four and reduced to their normal level by day 15. When B6 mice were treated with α-GalCer plus 30 or 50 mg/kg CsA, $NK1.1^+CD3^+$ or $NK1.1^+$ $Thy1.2^+$cells were similarly decreased and then expanded via extensive proliferation by day seven or four, respectively. When B6 mice were treated with α-GalCer, substantial amounts of IL-2, IL-4 and IFN-γ were produced, and the surface markers of dendritic cells were upregulated. However, these cytokine productions and maturation of dendritic cells were profoundly suppressed after treatment with α-GalCer and CsA. Apoptosis of $NK1.1^+CD3^+$ cells was not affected in mice treated with α-GalCer or α-GalCer and CsA.

Conclusions. CsA suppresses α-GalCer-induced cytokine productions and dendritic cell maturation of mouse NKT cells but does not decrease $NK1.1^+CD3^+$ cells on day one. The modulation of NKT-mediated immunoregulatory functions by CsA requires careful consideration in clinical transplantation and autoimmune disease therapy.

Keywords: Natural killer T cells, Cyclosporin A, α-galactosylceramide.

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Natural killer T (NKT) cells have been characterized as cells that coexpress the natural killer (NK) cell marker NK1.1 and the T-cell receptor (TCR) (*1–4*). Although their natural ligands have not been well characterized, NKT cells recognize and are strongly stimulated by a glycolipid antigen, α-galactosylceramide (α-GalCer), presented by the major histocompatibility complex (MHC) class I-like molecule CD1d (*5*). By using flow cytometry (FCM) and RNA extraction assays, initial studies have revealed that α-GalCer quickly activates NKT cells, which then become undetectable. This finding is correlated with increased apoptosis and increased expression of Fas and FasL by NKT cells (*6–8*). Another study reported a similar disappearance of NKT cells in vivo after stimulation with an anti-CD3 mAb or IL-12 (*9*). This demise of NKT cells after treatment with an anti-CD3 mAb or interleukin (IL)-12 was usually followed by repopulation within two to three days after the stimulation due to homeostatic proliferation in the bone marrow. On the other hand, more recent studies have suggested that receptor down-regulation is the primary cause of the NKT cell disappearance and reappearance following α-GalCer treatment (*10–12*).

After recognition of α-GalCer, NKT cells activate and rapidly secrete large amounts of both Th1 and Th2 cytokines, such as IL-4 and interferon (IFN)-γ (*5*). The activation of NKT cells has been considered to develop their immunoregulatory functions through Th1 and/or Th2 cytokines. NKT cells activated by α-GalCer have been shown to play important roles in preventing autoimmune diseases and enhancing anti-tumor cytotoxicity (*13–16*). In transplant immunity, NKT cells play vital roles in the induction of not only allograft tolerance but also xenograft tolerance, although the precise mechanisms for these effects have not yet been clarified (*17, 18*).

Cyclosporin A (CsA) is a popular immunosuppressive drug that is widely used in organ transplantation and autoimmune disease therapy. In mice, a middle dose (30 mg/kg) of CsA suppresses IL-2 production by $CD4^+$ helper T cells, whereas a high dose (75 mg/kg) suppresses that by both $CD4^+$ and $CD8^+$ helper T cells (*19*). Paradoxically, however, CsA can cause autoimmune diseases (*20, 21*) and a graft-versus-host (GVH)-like syndrome in syngeneic bone marrow transplantation (*22, 23*), and interfered with the induction of allograft tolerance in rodents (*24*). Because NKT cells play

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essential roles in the maintenance of tolerance and prevention of autoimmune diseases, breakdown of the transplantation tolerance or autoimmune disease prevention induced by CsA may be caused via the suppression of NKT cell functions.

Therefore, in the present study, we investigated whether middle and high doses of CsA (30 and 50 mg/kg, respectively) could modulate the activation of NKT cells following treatment with α-GalCer on day 0. After treatment with α-GalCer and CsA, a similar rapid disappearance of $NK1.1^+CD3^+$ cells was observed on day one, but these cells subsequently increased to a higher level than that after treatment with α-GalCer alone. Cytokine productions were completely suppressed and $CD11c^+$ dendritic cells did not become mature after treatment with α-GalCer and CsA. These results indicate that CsA could completely suppress the cytokine productions by NKT cells, but did not down-regulate their surface markers. Therefore, the results of the present study suggest that suppression of the immunoregulatory functions of NKT cells by CsA may be one of the causes of autoimmune disease development and interfere with tolerance induction.

MATERIALS AND METHODS

Animals

Inbred female mice of the C57BL/6 SnSlc (B6; H-2^b) strain were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan), and used at 8–16 weeks of age. All animals received humane care in compliance with both the Guidelines for Animal Experiments of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese government.

Reagents

α-GalCer (KRN7000) was kindly provided by Kirin Brewery (Takasaki, Japan), dissolved in 0.5% polysorbate 20 at a concentration of 200 μg/ml and then further diluted with 0.9% NaCl. CsA (Novartis Pharmaceuticals, Basel, Switzerland) was dissolved in 0.9% NaCl at a concentration of 2 mg/ml.

In Vivo Treatments

Mice were injected intravenously (i.v.) with 2 μg of α-GalCer. As a control, mice were injected with an equivalent amount of vehicle, namely 0.5% polysorbate 20 and 0.9% NaCl. From one day before the α-GalCer treatment, mice received daily intraperitoneal (i.p.) injections of CsA (30 or 50 mg/kg). The day of the α-GalCer injection is referred to as day 0 throughout this report.

Cell Preparation

Mice were sacrificed by decapitation, and cell suspensions were prepared from the liver and spleen. The liver was disrupted in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) by pressing the liver fragments between two glass slides, then washed, resuspended in 40% isotonic Percoll solution (Amersham Biosciences, Piscataway, NJ) and overlaid on 67.5% isotonic Percoll solution. Following centrifugation at 3000 rpm for 30 min at room temperature, liver mononuclear cells (LMNC) were isolated from the interface, washed twice with Hanks balanced salt solution (HBSS) containing 2% FCS and then resuspended in the same solution. The spleen was disrupted in RPMI 1640 medium in the same manner as the liver, and then washed with HBSS containing 2% FCS. The spleen cell (SC) suspensions obtained were filtered through cotton gauze and washed twice with HBSS containing 2% FCS. Viable nucleated cells were counted and usually adjusted to 1×10^7 cells/mL.

Thymectomy

Recipients were anesthetized by an i.p. administration of 50 mg/kg phenobarbital (Nembutal; Shionogi, Osaka, Japan). After a partial sternotomy, a thymectomy was performed via an en bloc excision using two forceps (*25*). The absence of thymic tissue was always confirmed when the thymectomized animals were sacrificed, and animals showing the presence of residual thymic tissue were excluded from the analysis.

Flow Cytometry

The surface phenotypes of the LMNC and SC were identified by two-color FCM. Cells were incubated with phycoerythrin (PE)-conjugated anti-NK1.1 or anti-CD5 (BD PharMingen, San Diego, CA), biotin-conjugated anti-CD3e or anti-NK1.1 (BD PharMingen) monoclonal antibodies (mAbs) and fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 (BD PharMingen) for 60 min at 4°C and then washed twice with HBSS containing 2% FCS. Biotin-conjugated reagents were developed with FITC- or allophycocyanin (APC)-conjugated streptavidin (SA; BD PharMingen).

To detect the NKT cells undergoing apoptosis, three-color FCM was used. LMNC were isolated at three hr after the α-GalCer injection. The cells were stained with PE-conjugated anti-NK1.1 (BD PharMingen) and biotin-conjugated anti-CD3e (BD PharMingen) mAbs. Biotin-conjugated reagents were developed with APC-SA (BD PharMingen). Cells were washed twice in annexin V binding buffer (BD PharMingen) before labeling with FITC-conjugated annexin V (BD PharMingen) for 30 min at room temperature in the dark.

To analyze the maturation of dendritic cells (DC), SC were isolated at 24 hr after the α-GalCer injection. The cells were stained with FITC-conjugated anti-CD11c (BD PharMingen) and PE-conjugated anti-I-A^b (BD PharMingen), anti-CD40 (BD PharMingen), anti-CD80 (BD PharMingen) or anti-CD86 (BD PharMingen) mAbs for 30 min at 4°C. To block nonspecific FcγR receptor binding of the labeled antibodies, 10 μL of an undiluted culture supernatant containing 2.4G2 (a rat antimouse FcγR mAb) was added to the first incubation, and then washed. All data were analyzed with a FACSCalibur (Becton Dickinson, Sunnyvale, CA). Dead cells were excluded by gating out low forward scatter and high propidium iodide-retaining cells.

Cytokine Secretion Following In Vivo α-GalCer Treatment

Mice were injected with either α-GalCer or vehicle alone and then bled after 2 or 18 hr. The cytokine levels (IL-2, IL-4, IL10 and IFN-γ) in the serum were determined using a standard sandwich enzyme-linked immunosorbent assay (ELISA) (BioSource International Inc., Camarillo, CA).

Statistics

The statistical significance of the data was determined using Students *t* test. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Kinetics of the Percentages of NK1.1$^+$CD3$^+$ Cells in the Liver and Spleen of Recipient Mice Treated with α-GalCer and CsA

When α-GalCer is administered to B6 mice, a rapid reduction followed by restoration has been shown for NK1.1$^+$CD3$^+$ cells among the LMNC and SC (*6*, *26*). Here, we examined the effects of CsA on the kinetics of NK1.1$^+$CD3$^+$ cells induced by α-GalCer. A middle or high dose of CsA (30 or 50 mg/kg, respectively) was injected i.p. daily from one day before the α-GalCer treatment. As shown in Figure 1A, the percentage of NK1.1$^+$CD3$^+$ cells in the LMNC rapidly decreased to less than 2% on day one, was restored to around 8% by day four and then remained constant until day 15 in mice treated with α-GalCer. In the LMNC of mice treated with α-GalCer plus 30 mg/kg CsA, the NK1.1$^+$CD3$^+$ cells were reduced to less than 2% on day one, restored to the normal level by day seven and then reduced to about 13% by day 15. In the LMNC of mice treated with α-GalCer plus 50 mg/kg CsA, the NK1.1$^+$CD3$^+$ cells were similarly reduced to less than 2% on day one, restored to above the normal level by day seven and then reduced to the normal level by day 15. Similar results were observed for the SC of mice treated with α-GalCer plus 30 or 50 mg/kg CsA, and representative data are shown in Figure 1A. We analyzed the early response of NKT cells after treatment with α-GalCer or α-GalCer and CsA by using other T-cell markers such as CD5 or Thy1.2. The kinetics of the NK1.1$^+$CD5$^+$ cells after treatment with α-GalCer or α-GalCer and CsA were same as those observed for the NK1.1$^+$CD3$^+$ cells (data not shown). The kinetics of the NK1.1$^+$Thy1.2$^+$ cells in the liver and spleen of recipient mice treated with α-GalCer and CsA are shown in Figure 1B. In the liver of mice treated with α-GalCer alone, NK1.1$^+$Thy1.2$^+$ cells rapidly decreased on day one and repopulated by day four. In the liver of mice treated with α-GalCer plus 30 or 50 mg/kg CsA, NK1.1$^+$Thy1.2$^+$ cells also decreased on day one and then gradually increased by days 7 to 15. In the spleen of mice treated with α-GalCer alone or α-GalCer plus 30 or 50 mg/kg CsA, NK1.1$^+$Thy1.2$^+$ cells rapidly decreased on day one, increased to above the normal level by days four and seven and then returned to the normal level on day 15.

Kinetics of the Numbers of NK1.1$^+$CD3$^+$ Cells among the LMNC and SC of Mice Injected with α-GalCer and CsA

We further examined the numbers of total cells and NK1.1$^+$CD3$^+$ cells in the liver after treatment with α-GalCer and CsA. The LMNC counts did not change significantly on day one, increased by day four and then decreased to the normal level by day 15 in mice treated with α-GalCer alone or α-GalCer plus 30 or 50 mg/kg CsA (Fig. 2a). The LMNC dramatically increased in mice treated with α-GalCer alone (~fourfold) and α-GalCer plus 30 or 50 mg/kg CsA (~six- to eight-fold) on day four. These expansions of the LMNC number on day four showed significant differences between mice treated with α-GalCer alone and those treated with α-GalCer plus 30 or 50 mg/kg CsA. Similar results were observed for the changes in the SC counts, although the LMNC were more extensively increased than the SC.

In the liver of mice treated with α-GalCer alone, the NK1.1$^+$CD3$^+$ cell counts rapidly decreased on day one, were restored to the normal level by day four and then gradually decreased by day 15 (Fig. 2b). On the other hand, the NK1.1$^+$CD3$^+$ cell counts in the liver of mice treated with α-GalCer plus 30 or 50 mg/kg CsA showed similar decreases on day one, then increased to above the normal level by day four, further increased on day seven (~fourfold) and gradually decreased by day 15. There were significant differences in the NK1.1$^+$CD3$^+$ cell numbers between mice treated with α-GalCer alone and those treated with α-GalCer plus 30 or 50 mg/kg CsA on day seven. Similar results were observed for the changes in the spleen NKT cell counts. As shown in Figure 1A, NK1.1$^+$CD3$^+$ cells in mice treated with α-GalCer plus 30 or 50 mg/kg CsA were still decreased on day four and repopulated by day seven. Therefore, the number of NK1.1$^+$CD3$^+$ cells showed the largest increase on day seven, while the total number of LMNC showed the largest increase on day four. NK1.1$^+$Thy1.2$^+$ cells in the liver and spleen of mice treated with α-GalCer alone or α-GalCer plus 30 or 50 mg/kg CsA showed rapid decreases on day one and expansion on day four (Fig. 2c).

Effects of Thymectomy on the Kinetics of the NK1.1$^+$CD3$^+$ Cell Counts After Treatment with α-GalCer and CsA

Many studies have suggested that the thymus is involved in the normal development of NKT cells. Therefore, we investigated whether the thymus was involved in the repopulation of NK1.1$^+$CD3$^+$ cells after treatment with α-GalCer and CsA by using thymectomized B6 mice. In thymectomized mice treated with α-GalCer alone or α-GalCer plus 30 or 50 mg/kg CsA, the percentage of NK1.1$^+$CD3$^+$ cells in the LMNC rapidly decreased on day one, remained the same on day four and was then restored to above the normal level by day seven. Therefore, the NK1.1$^+$CD3$^+$ cells in thymectomized mice showed the same kinetics as those in nonthymectomized mice (Fig. 3).

In Vivo Cytokine Responses at 2 or 18 Hours After Injection of α-GalCer and CsA

To further examine the effects of CsA on the α-GalCer-induced activation of NKT cells, the productions of Th1 (IL-2 and γ-IFN) and Th2 (IL-4 and IL-10) cytokines were measured. At 2 and 18 hr after the administration of α-GalCer, serum was collected from the mice and the level of each cytokine was determined using a standard sandwich ELISA. Consistent with previous studies (*7*, *13*, *14*), α-GalCer treatment induced the productions of IL-2, IL-4 and γ-IFN (Fig. 4). However, these cytokine productions were almost abrogated in the serum of mice treated with α-GalCer plus 30 or 50 mg/kg CsA.

Maturation of DC After Intravenous Injection of α-GalCer and CsA

A recent report (*27*) demonstrated that α-GalCer treatment induces the maturation of splenic CD11c$^+$ DC, as indi-

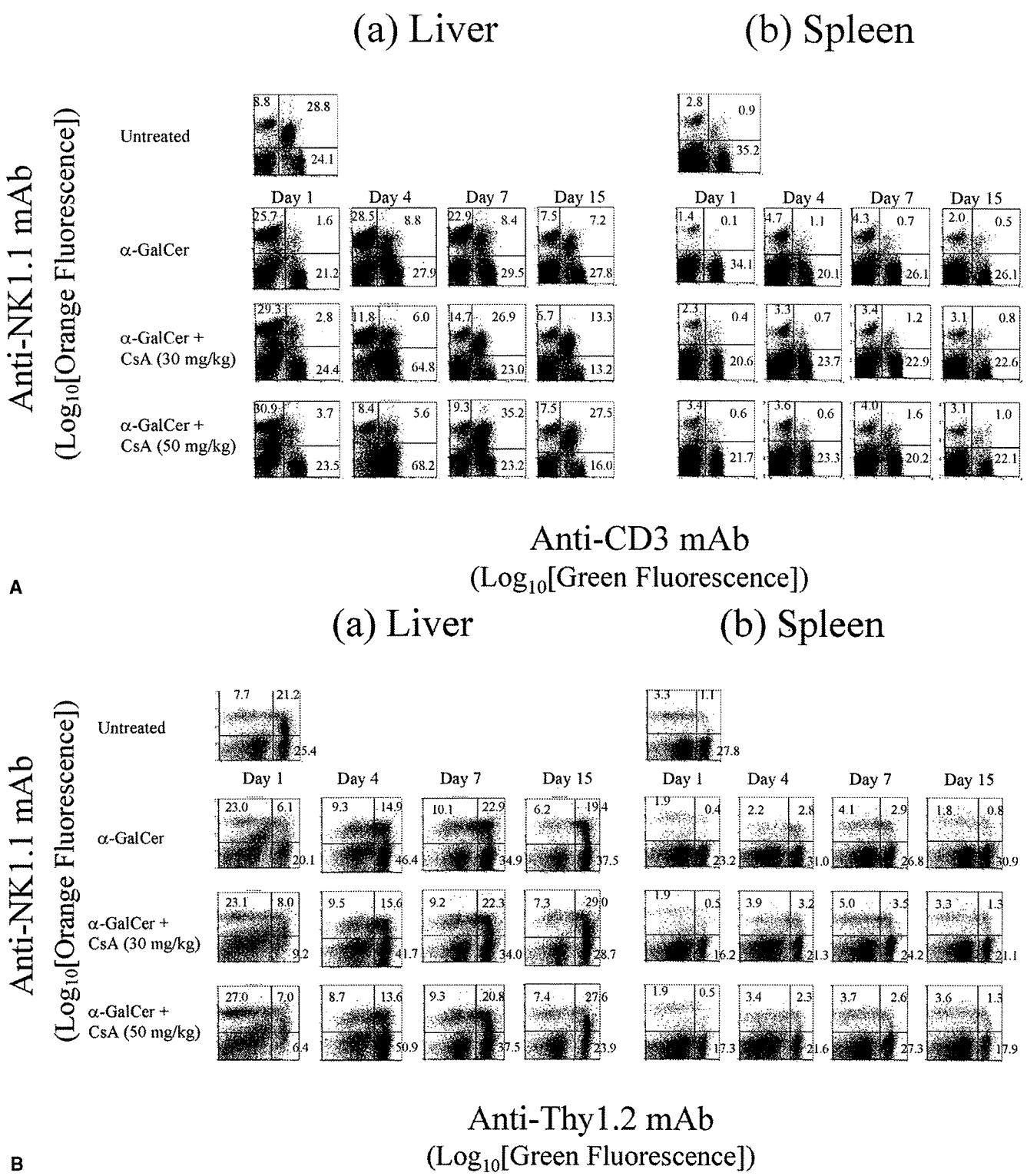

FIGURE 1. Phenotypic characterization of NK1.1$^+$CD3$^+$ cells by two-color immunofluorescence. B6 mice were injected with α-GalCer on day 0 and CsA (30 or 50 mg/kg) daily from one day before the α-GalCer treatment. The cells were labeled with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 mAbs (A) or PE-conjugated anti-NK1.1 and FITC-conjugated anti-Thy1.2 mAbs (B). Liver mononuclear cells (a) and spleen cells (b) were isolated after the indicated times and analyzed by flow cytometry. The numbers indicate the percentage of cells in the quadrant relative to the total cell population. The experiment shown is representative of four independent experiments.

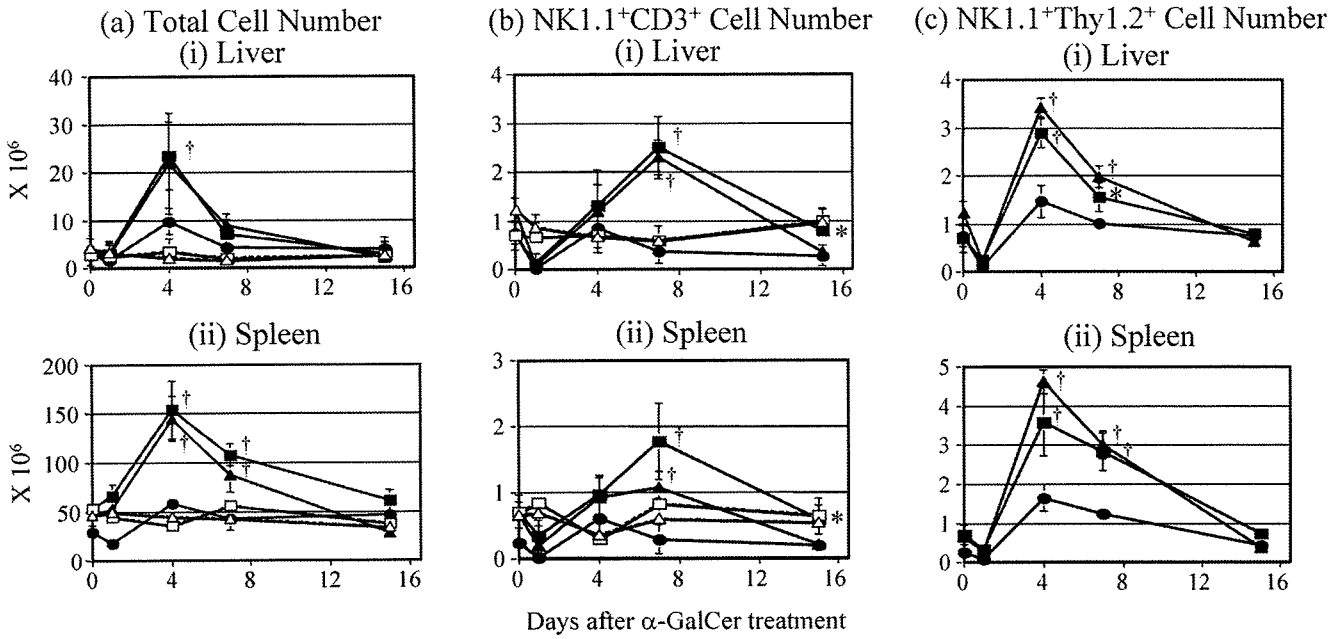

FIGURE 2. Influence of CsA on the induction of cell proliferation after α-GalCer treatment. Cells were labeled with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 mAbs or PE-conjugated anti-NK1.1 and FITC-conjugated anti-Thy1.2 mAbs. The kinetics of the total cell numbers (a), NK1.1$^+$CD3$^+$ cells (b) and NK1.1$^+$Thy1.2$^+$ cells (c) in the liver mononuclear cells and spleen cells of B6 mice treated with α-GalCer and CsA are shown. B6 mice were injected with α-GalCer on day 0 and CsA (30 or 50 mg/kg) daily from one day before the α-GalCer treatment. Liver and spleen cells were obtained from B6 mice treated with α-GalCer alone (● n=4), α-GalCer plus 30 mg/kg CsA (▲ n=4), α-GalCer plus 50 mg/kg CsA (■ n=4), 30 mg/kg CsA alone (Δ n=4) or 50 mg/kg CsA alone (□ n=4). Vertical bars represent the SD. *P<0.05, †P<0.01 vs. mice treated with α-GalCer alone.

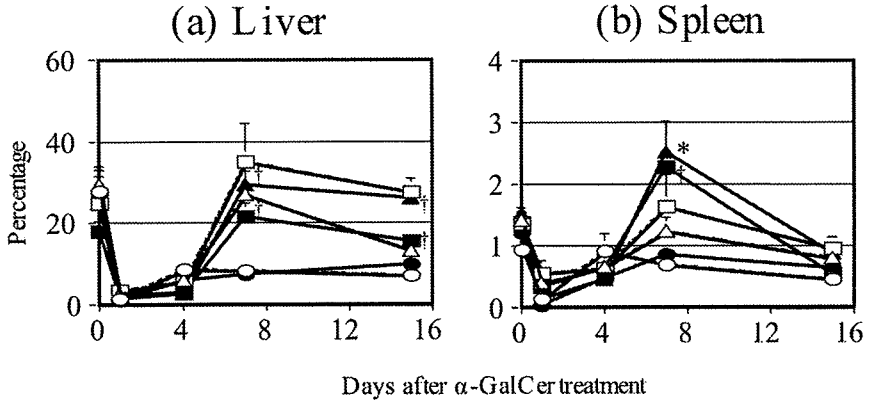

FIGURE 3. Kinetics of NK1.1$^+$CD3$^+$ cells in thymectomized mice treated with α-GalCer and CsA. The kinetics of the mean percentages of NK1.1$^+$CD3$^+$ cells in the liver mononuclear cells (a) and spleen cells (b) of thymectomized B6 mice treated with α-GalCer and CsA are shown. Cells were labeled with PE-conjugated anti-NK1.1 and FITC-conjugated anti-CD3 mAbs. B6 mice were thymectomized at four weeks before the α-GalCer treatment. The thymectomized mice were i.v. injected with α-GalCer (2 μg) on day 0 and i.p. injected with CsA (30 or 50 mg/kg) daily from one day before the α-GalCer treatment. Liver and spleen cells were obtained from non-thymectomized B6 mice treated with α-GalCer alone (○ n=4), α-GalCer plus 30 mg/kg CsA (Δ; n=4) or α-GalCer plus 50 mg/kg CsA (□ n=4), and from thymectomized B6 mice treated with α-GalCer alone (● n=4), α-GalCer plus 30 mg/kg CsA (▲ n=4) or α-GalCer plus 50 mg/kg CsA (■ n=4). Vertical bars represent the SD *P<0.05, †P<0.01 vs. mice treated with α-GalCer alone.

cated by the up-regulation of I-A^b, CD40, CD80 and CD86. At 24 hours after α-GalCer treatment, SC were stained with FITC-conjugated anti-CD11c and PE-conjugated anti-I-A^b, CD40, CD80 or CD86 mAbs, and the CD11c$^+$ cells were analyzed. As shown in Table 1, the expressions of I-A^b, CD40, CD80, and CD86 were clearly augmented in the CD11c$^+$ SC of mice treated with α-GalCer alone compared with those of untreated mice. The increases in the CD40, CD80, and CD86 expressions were lower in the CD11c$^+$ SC of mice treated with α-GalCer plus 30 or 50 mg/kg CsA than in those of mice treated with α-GalCer alone. On the other hand, the expression of I-A^b was not augmented in the CD11c$^+$ SC of mice

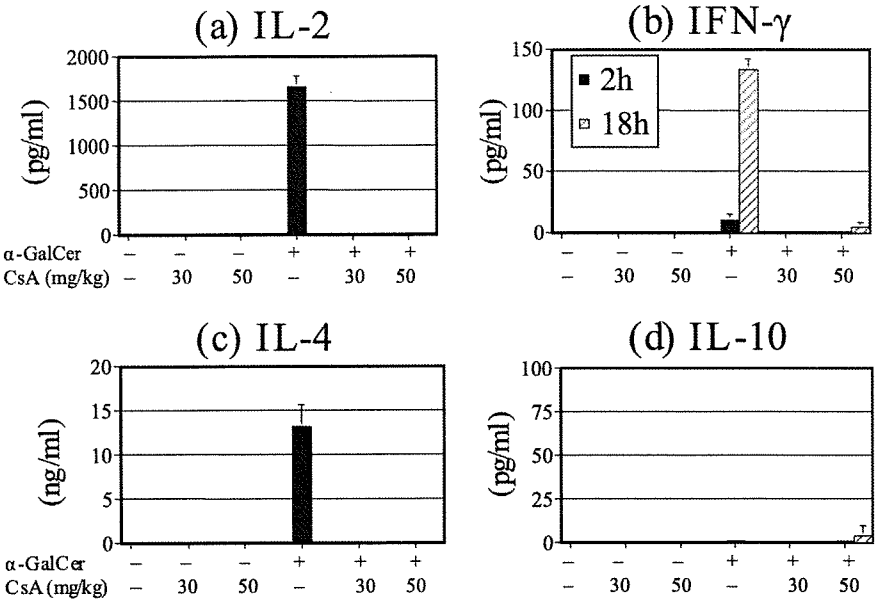

FIGURE 4. Production of Th1 and Th2 cytokines in B6 mice at 2 and 18 hr after α-GalCer treatment. B6 mice were injected with CsA (30 or 50 mg/kg) one day before the α-GalCer treatment. The serum levels of IL-2 (a), IFN-γ (b), IL-4 (c), and IL-10 (d) at 2 and 18 hr after injection of α-GalCer or vehicle were determined by ELISA. The data shown are the mean±SD of three mice for each group.

TABLE 1. Maturation of surface markers on splenic dendritic cells at 24 hours after treatment with α-GalCer and CsA

Group	Treatment[a]		No. of mice	Median fluorescence intensity (mean values±SD)			
	α-GalCer (2 μg)	CsA (mg/kg)		I-A[b]	CD40	CD80	CD86
1	–	—	4	1174.6±74.2	46.8±6.2	51.5±2.5	40.2±5.5
2	–	30	4	848.1±82.2	37.9±4.7	49.0±5.1	38.4±3.2
3	–	50	4	633.8±83.7	36.7±3.6	40.7±5.4	37.7±4.6
4	+	—	4	2714.4±51.8[b]	151.2±9.8[b]	186.8±17.5[b]	413.6±32.6[b]
5	+	30	4	865.4±310.9[e]	66.5±16.6[c,e]	59.0±6.5[e]	72.2±16.7[c,e]
6	+	50	4	681.8±91.6[e]	67.1±6.6[d,e]	53.1±4.1[d,e]	71.7±18.5[d,e]

[a] Mice received CsA on days −1 and 0 and were injected with α-GalCer on day 0.
[b] $P<0.01$ vs. Group 1.
[c] $P<0.05$ vs. Group 2.
[d] $P<0.05$ vs. Group 3.
[e] $P<0.01$ vs. Group 4.

treated with α-GalCer plus 30 or 50 mg/kg CsA compared with those of mice treated with CsA alone. However, the augmentations of all these molecules were suppressed in the $CD11c^+$ SC of mice treated with α-GalCer plus 30 or 50 mg/kg CsA compared with those of mice treated with α-GalCer alone.

α-GalCer-Induced Apoptosis of $NK1.1^+CD3^+$ Cells After Treatment with α-GalCer and CsA

Depletion of $NK1.1^+CD3^+$ cells among the LMNC and SC is already observed at three hours after the administration of α-GalCer, and most of the $NK1.1^+CD3^+$ cells become undetectable at 24 hours after the α-GalCer injection. Previous reports have shown that liver $NK1.1^+CD3^+$ cells from α-GalCer-treated mice exhibit a significant increase in annexin V binding at three hours after the injection compared to those from control mice (*6, 7*). This observation indicates that the rapid depletion of $NK1.1^+CD3^+$ cells after α-GalCer treatment is primarily due to apoptotic cell death. However, a recent study showed no significant increase in annexin V binding in liver $NK1.1^+CD3^+$ cells in α-GalCer-treated mice (*11*). Therefore, we tested whether liver NKT cells exhibited an increase in annexin V binding at three hours after the injection of α-GalCer and CsA (Table 2). The median fluorescence intensity (MFI) and percentage of annexin V-positive $NK1.1^+CD3^+$ cells in the LMNC of mice treated with CsA alone did not differ significantly from those in untreated mice. Moreover, the MFI and percentage of annexin V-positive $NK1.1^+CD3^+$ cells in the LMNC of mice treated with α-GalCer alone did not increase significantly compared with those in untreated mice. Similarly, there were no significant changes in the MFI values and percentages of annexin V-positive $NK1.1^+CD3^+$ cells of mice treated with α-GalCer plus 30 or 50 mg/kg CsA.

DISCUSSION

NKT cells have been characterized as cells that express an invariant Vα14 TCR together with the NK cell marker NK1.1. To date, a number of studies have reported on the unique characteristics and physiological functions of NKT cells (*28*). NKT cells recognize a glycolipid antigen, α-GalCer,