

isotonic 33% Percoll solution containing heparin (67 U/mL) and centrifuged at 2000g at 4°C for 15 min. The resulting pellet was suspended in a 0.83% ammonium chloride solution to lyse erythrocytes. After counting, these MNCs were washed twice in PBS and used for further analysis.

Antibody and Flow Cytometry Analysis

The antibodies (Abs) used for the flow cytometry analysis were as follows: Fc block (anti-mouse Fc γ III/II mAb, 2.4G2), fluorescein isothiocyanate-conjugated anti-CD3 ϵ (clone 145-2C11, American Hamster IgG1 κ), phycoerythrin-CY5-conjugated rat anti-mouse CD4 mAb (clone RM4-5, Rat IgG2a), allophycocyanin-conjugated anti-IFN- γ mAb (clone XMG1.2, Rat IgG1), fluorescein isothiocyanate-conjugated anti-CD11b (M1/70), PerCP-conjugated anti-Gr-1 (RB6-8C5), and their isotype control (clone R3-34, Rat IgG1) were purchased from Pharmingen (San Diego, CA). Phycoerythrin-conjugated α -GalCer-loaded CD1d tetramers were prepared as described (18). For intracellular staining, cells were incubated with anti-Fc γ III/II and neutravidin (Invitrogen), surface stained, fixed, permeabilized, stained with mAbs, and analyzed on a flow cytometer (FACS Calibur; Becton Dickinson). A total of 10,000 viable cells were analyzed.

Statistical Analysis

The statistical significance with respect to the rate of euglycemia in streptozotocin-induced diabetic mice after transplantation and to the plasma glucose levels during IPGTT was determined by Fisher's exact test and Student's *t* test, respectively. Differences were considered significant when the *P* values were less than 0.05.

RESULTS

Early Loss of Transplanted Syngenic Islets in the Liver of Mice is Prevented by Adenosine

First, we determined whether adenosine has any beneficial effect on preventing early loss of transplanted syngenic islets in the liver of STZ-induced diabetic mice. Previously, we have shown that hyperglycemia of diabetic recipient mice is ameliorated after the transplantation of 400 syngenic islets into the liver, but not of 200 islets (6), the number of islets isolated from a single mouse pancreas. In this study, we determined whether hyperglycemia of diabetic mice that received 200 syngenic islets into the liver is ameliorated by adenosine. When diabetic mice received 200 islets and were treated with saline as a control, all the recipient mice (*n*=5) did not become normoglycemic after the transplantation (Fig. 1, upper panel). When diabetic mice received the same number of islets and were treated with 0.5 or 5 mg/kg adenosine once at the time of islet transplantation, 0 of 4 or 5 of 5 recipient mice became normoglycemic after the transplantation (Fig. 1, middle and lower panels). A histologic study revealed that intact or degenerated islets with well or poorly granulated β cells were seen in the liver of the normoglycemic or hyperglycemic recipient mice, respectively (histology not shown). The findings show that the amount of insulin produced from islet grafts was sufficient to ameliorate hyperglycemia of the diabetic recipient mice treated with 5 mg/kg

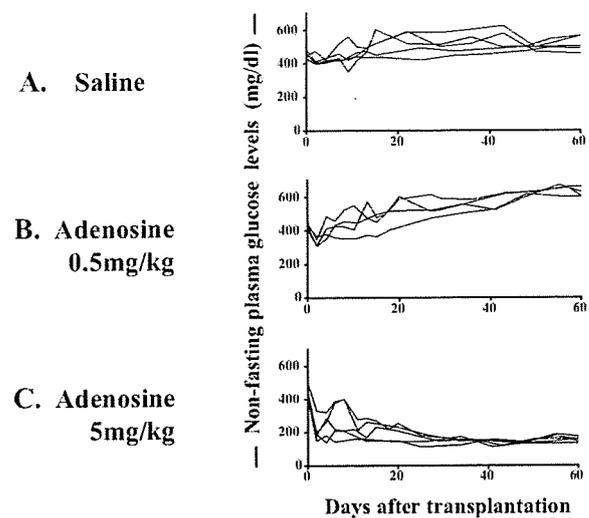


FIGURE 1. Plasma glucose levels of streptozotocin-induced diabetic mice receiving 200 syngenic islets into the liver. Diabetic mice receiving 200 syngenic islets were treated with saline (A), 0.5 mg/kg (B), or 5 mg/kg adenosine (C) once at the time of islet transplantation. Individual line represents the nonfasting plasma glucose levels of each animal.

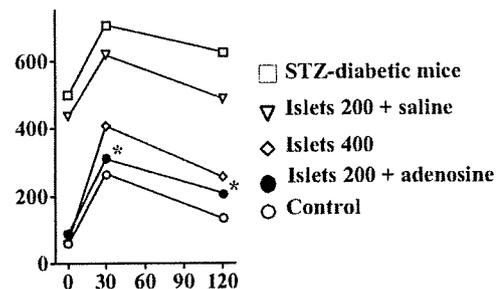


FIGURE 2. Intraperitoneal glucose tolerance test of diabetic mice receiving syngenic islets at 60 days after transplantation. Recipient mice were fasted for 8 hr before the examination, and plasma glucose levels were determined at 0, 30, and 120 min after the glucose injection (intraperitoneally, 1 g/kg). Experimental groups included diabetic mice without islet transplantation (\square , *n*=4), those receiving 400 islets without treatment (\diamond , *n*=5), 200 islets treated with adenosine (5 mg/kg) (\bullet , *n*=5), or saline (∇ , *n*=4). Untreated mice served as control (\circ , *n*=4). The plasma glucose levels at 30 and 120 min after the glucose injection between the mice receiving 400 islets without treatment and those receiving 200 islets and treated with adenosine were significantly different ($*P < 0.05$ by the Student's-*t* test).

adenosine but not that of the mice treated with saline or 0.5 mg/kg adenosine, indicating that early loss of transplanted islets is prevented by 5 mg/kg adenosine. Therefore, 5 mg/kg adenosine was used for the following studies.

Increase in Functional Islet Mass in the Liver of Recipient Mice by Adenosine

To evaluate the extent that adenosine improves the efficiency of islet transplantation, IPGTT was performed in appropriate groups of recipient mice. As shown previously (19), the plasma glucose levels of diabetic mice (*n*=5) receiving

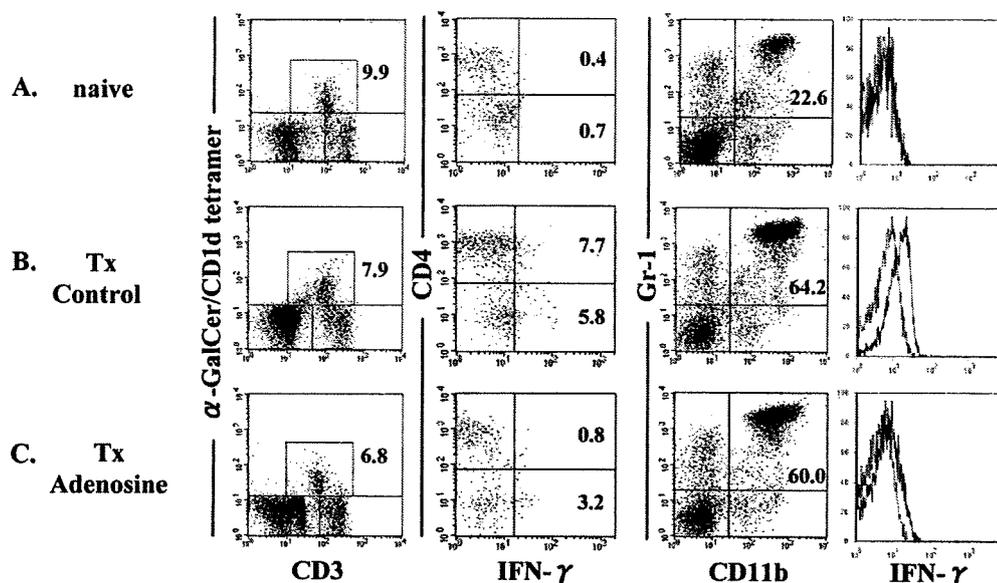


FIGURE 3. Flowcytometry of mononuclear cells in the liver of mice. Mononuclear cells in the liver of diabetic mice receiving 200 syngenic islets and treated with saline (B) or adenosine (5 mg/kg) (C) were isolated at 6 hr after islet transplantation and examined by the flow cytometry. NKT cells defined as the cells expressing α -galactosylceramide/CD1d tetramer and CD3 on their cell surface were further analyzed with respect to the CD4 expression and the interferon- γ production (second column). Gr-1⁺CD11b⁺ cells were gated to examine the interferon- γ production (4th column). The numbers in the figures represent the percentage of cells in the corresponding areas. Representative data from the three experiments are shown.

200 islets at 0, 30, and 120 min after the IP injection of glucose (1g/kg) were similar to those of diabetic hyperglycemic mice without islet transplantation ($n=5$; Fig. 2). In marked contrast, the plasma glucose levels of the normoglycemic mice received 200 islets and treated with 5 mg/kg adenosine were significantly lower in comparison with those of the normoglycemic mice receiving 400 islets at 30 and 120 min after the IP injection of glucose (1 g/kg; Fig. 2). The findings indicate that the treatment with adenosine (5 mg/kg/injection) prevented a significant decrease of the islet mass in the liver of recipient mice after transplantation.

IFN- γ Production of NKT Cells and Gr-1⁺CD11b⁺ Cells in the Liver of Mice Receiving Syngenic Islets is Suppressed by Adenosine

Previously, we have shown that the NKT cell-mediated IFN- γ production of Gr-1⁺CD11b⁺ cells (neutrophils) is an essential component of the early loss of transplanted islets in the liver of mice (6). Therefore, in this study we determined whether adenosine has any effect on the NKT cell-mediated IFN- γ production of Gr-1⁺CD11b⁺ cells in the liver receiving islets. For these purposes, MNCs in the liver of mice receiving islets and treated with adenosine or with saline were isolated and examined by flow cytometry with respect to IFN- γ production of α -GalCer/CD1d-tetramer⁺ CD3⁺NKT cells and Gr-1⁺CD11b⁺ cells (neutrophils). NKT cells are composed of two major populations, namely CD4⁺CD8⁻ and CD4⁻CD8⁻ NKT cells. It was found that the IFN- γ production of both NKT cell populations in the liver of mice receiving syngenic islets and treated with saline was upregulated at 6 hr after transplantation (Fig. 3, II, 2nd column). The IFN- γ production of CD4⁺NKT cells was significantly sup-

pressed in the liver of the mice treated with adenosine. The IFN- γ production of Gr-1⁺CD11b⁺ cells accumulated in the liver of mice receiving islets and treated with adenosine was suppressed at 6 hr after transplantation (Fig. 3, II and III, 4th column). Interestingly, the number of accumulated Gr-1⁺CD11b⁺ cells in the liver of mice receiving islets was not altered irrespective of the treatment with adenosine (Fig. 3, 3rd column). Therefore, these findings show that adenosine has an inhibitory effect on the IFN- γ production of NKT cells and Gr-1⁺CD11b⁺ cells in the liver of mice after islet transplantation.

Adenosine Prevents α -GalCer-Stimulated IFN- γ Production of NKT Cells and Gr-1⁺CD11b⁺ Cells

To confirm further an essential role of adenosine in NKT cell activation, α -GalCer, a synthetic ligand of NKT cells was used in the experiments and determined whether the IFN- γ production of NKT cells in response to α -GalCer was suppressed by adenosine. As originally reported (20), it is well known that NKT cells produce large amount of IFN- γ in response to α -GalCer. The fluorescence-activated cell sorting analysis of MNC in the liver of mice at 2 hr after the IV injection of α -GalCer (10 μ g/kg/injection/mouse) revealed a marked increase in IFN- γ production of α -GalCer/CD1d-tetramer⁺ CD3⁺NKT cells and Gr-1⁺CD11b⁺ cells (neutrophils) in comparison with control mice treated with vehicle (Fig. 4, I and II). In contrast, the IFN- γ production of NKT cells and Gr-1⁺CD11b⁺ cells in the liver of mice treated with adenosine in conjunction with α -GalCer was suppressed (Fig. 4, III). Thus, adenosine has an inhibitory effect on activation of NKT cell and Gr-1⁺CD11b⁺ cell activation in response to α -GalCer.

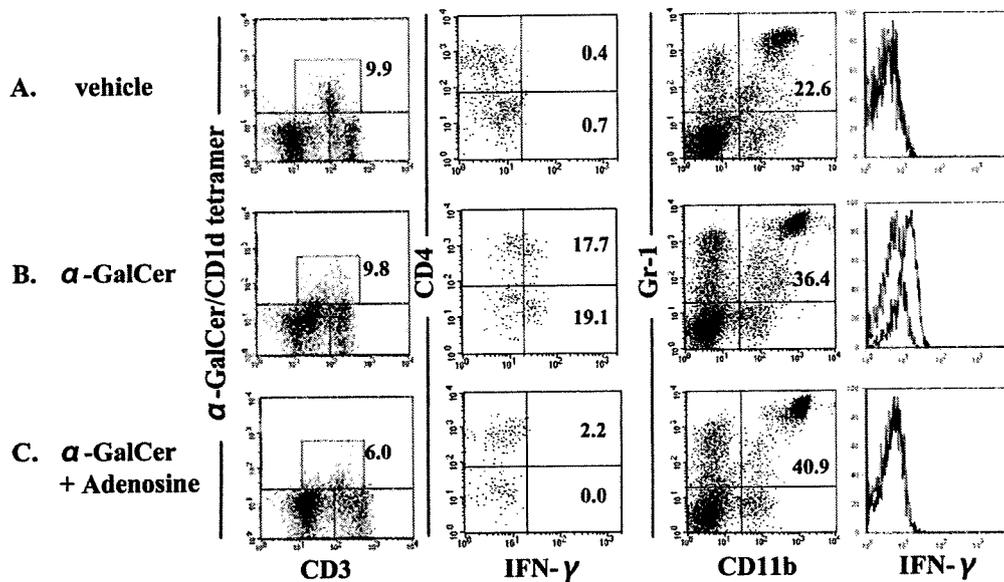


FIGURE 4. Flow cytometry of mononuclear cells in the liver of mice treated with intravenous administration of α -galactosylceramide (α -GalCer). Mononuclear cells in the liver of mice treated with (C) or without adenosine (5 mg/kg) (B) were isolated at 2 hr after the intravenous injection of α -GalCer (10 μ g/kg) and examined by the flow cytometry. Mice treated with vehicle served as the control (A). The α -GalCer-CD1d tetramer⁺CD3⁺ NKT cells were further gated to examine the interferon- γ production of CD4⁺ or CD4⁻ NKT cells (second column). The Gr-1⁺CD11b⁺ cells were further examined with respect to the interferon- γ production (4th column). The numbers in the figures represent the percentage of cells in the corresponding areas. Representative data from the three experiments are shown.

Beneficial Effects of Adenosine Transporter Inhibitors, Dipyridamole, and Nitrobenzylthioinosine on Prevention of Early Loss of Transplanted Islets

Thereafter, we determined whether adenosine transporter inhibitors, such as dipyridamole and NBTI, which increase extracellular levels of adenosine have a similar effect to adenosine with respect to prevention of early loss of transplanted islets. When diabetic mice receiving 200 syngenic islets into the liver were treated with vehicle as controls, all the recipient mice remained hyperglycemic for more than 60 days after the transplantation (Fig. 5, A). In contrast, diabetic mice receiving 200 syngenic islets into the liver and treated with dipyridamole or NBTI became normoglycemic by 60 days after the transplantation (Fig. 5, B and C). Histologically, intact islets with well-granulated or degranulated β cells were identified in the liver of the normoglycemic or hyperglycemic mice, respectively at 60 days after the transplantation (histology not shown).

Adenosine Prevents Early Loss of Transplanted Allogenic Islets in the Liver of Recipient Mice Treated With Anti-CD4 Antibody

Finally, we determined whether the beneficial effect of adenosine on prevention of early loss of transplanted syngenic islets in the liver of mice is similar for islet allotransplantation. When 200 BALB/c islets were grafted into the liver of STZ-diabetic C57BL/6 mice treated with saline, recipient mice did not become normoglycemic and remained hyperglycemic by 60 days after the transplantation (Fig. 6, A, left panel). Histologic examinations revealed that foci of MNCs were seen in the liver of mice receiving islet allografts at 14

days after the transplantation, whereas only a few insulin producing cells were identified (Fig. 6, A, right panels). In contrast, when 200 BALB/c islets were grafted into the liver of STZ-diabetic C57BL/6 mice treated with adenosine once at the time of islet transplantation, hyperglycemia of recipient mice was ameliorated by 3 days and the mice became hyperglycemic again by 7 days after the transplantation (Fig. 6, B, left panel). Histologically, islet grafts infiltrated with MNCs were seen in the liver of the recipient mice at the time of rejection (Fig. 6, B, right panels). When diabetic mice received 200 allogenic islets and were treated with anti-CD4 antibody, the recipient mice remained hyperglycemic by 60 days after transplantation (Fig. 6, C, left panel). Histologically, islets with degranulated β cells were seen in the liver of the recipient mice at 60 days after the transplantation as reported previously (19; data not shown). When diabetic mice received 200 allogenic islets and were treated with adenosine in conjunction with anti-CD4 antibody, the recipient mice became normoglycemic by 5 days and remained in this state for more than 60 days after transplantation (Fig. 6, D, left panel). Histologically, intact islets with well-granulated β cells were seen in the liver of the recipient mice at 60 days after the transplantation (Fig. 6, D, right panels). These findings indicate that adenosine promotes early engraftment while anti-CD4 antibody prevents the alloimmune rejection of transplanted allogenic islets.

DISCUSSION

These findings clearly show that adenosine prevents the early loss of transplanted syngenic islets by suppressing the NKT cell-mediated IFN- γ production of Gr-1⁺CD11b⁺ cells in the liver of mice receiving islets, thus enabling islet trans-

plantation from one donor to one recipient in mice. Moreover, the beneficial effect of adenosine was also found in islet allotransplantation when alloimmune rejection was prevented under an immunosuppressive agent such as anti-CD4 antibody.

Adenosine has long been used as a supplemental component of preservation solution for organ transplantation to minimize ischemic damage of donor organs during preservation (21). Accumulating evidences now show that adenosine is produced in various cells when they are placed under stress such as ischemia and inflammation, and it serve as a suppressive molecule through its receptor on cellular membrane,

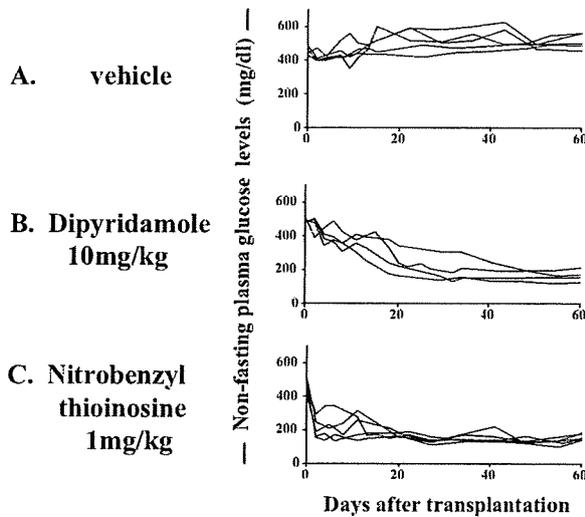
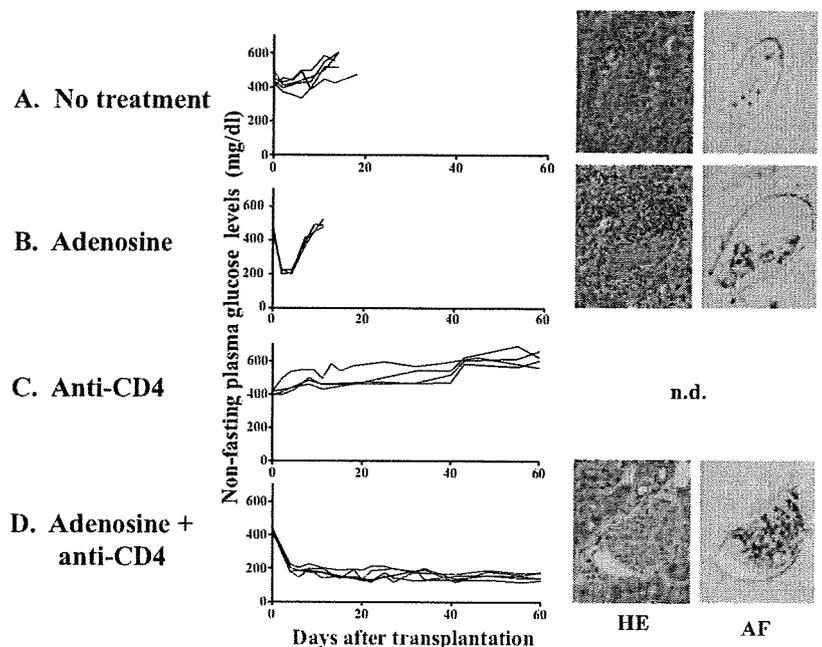


FIGURE 5. Beneficial effects of dipyridamole and nitrobenzylethioinosine on prevention of early loss of transplanted islets. Individual line represents the nonfasting plasma glucose levels of each diabetic mouse receiving 200 syngenic islets and treated with vehicle (A), dipyridamole (10 mg/kg) (B), or nitrobenzylethioinosine (1 mg/kg) (C).

such as adenosine A2A receptor (22). Thus, the presence of adenosine outside of the cells seems to be essential for the inhibitory effect of adenosine. Adenosine is present in equal amounts between inside and outside the cells and its transport is controlled by the equilibrium nucleoside transporter (ENT) on the cellular membrane (23). Adenosine is produced from adenosine triphosphate/adenosine diphosphate and adenosine monophosphate mediated by CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5-nucleotidase), respectively, and from *s*-adenosylhomosistein by adenosine hydrolase (24). Adenosine is degraded by adenosine deaminase (25). Therefore, there are several ways to increase the extracellular concentration of adenosine including the administration of adenosine itself, the blockade of ENT to prevent transportation of adenosine from outside to inside the cells, the prevention of adenosine degradation and the promotion of adenosine production. In this study, we focused on the former two interventions because adenosine and the ENT inhibitor, dipyridamole have already been used in the clinic and are ready to be applied to clinical islet transplantation. First, we determined whether adenosine has any beneficial effect on prevention of the early loss of syngenic transplanted islets and found that this was the case as shown in Figure 1. The flowcytometry of MNC in the liver of mice receiving islets and treated with adenosine revealed that the IFN- γ production of NKT cells and neutrophils infiltrated into the liver of mice after islet transplantation was suppressed. The finding suggests that the beneficial effect of adenosine was mediated through the inhibition of NKT cells and not through the activation of neutrophils, because the IFN- γ production of neutrophils in the liver of mice receiving islets, which is an essential component of the early loss of transplanted islets, was dependent on NKT cells (6). To examine further whether NKT cells were responsible for the inhibitory effect of adenosine, we determined whether IFN- γ production of NKT cells in response to α -GalCer, a synthetic ligand of NKT cells (20), was inhibited by adenosine. The flow

FIGURE 6. Early loss of transplanted allogenic islets was prevented by adenosine. The plasma glucose levels of diabetic mice receiving 200 allogenic islets and treated with saline or adenosine (5 mg/kg, administered intravenously) are shown in groups A or B, respectively. In groups D or C, recipient mice receiving 200 allogenic islets were treated with anti-CD4 antibody combined with and without the treatment by adenosine, respectively. The livers of mice receiving islet allografts were examined morphologically at 14 (A and B) or 60 days (D) after the transplantation (right columns). nd, not done; HE, hematoxylin-eosin stain; AF, aldehyde and fuchsin stain. Original magnification $\times 100$.



cytometry of MNC in the liver of mice treated with the IV injection of α -GalCer showed that the IFN- γ production of NKT cells in response to α -GalCer was suppressed by the treatment with adenosine and, consequently, the IFN- γ production of neutrophils that infiltrated the liver was also suppressed. Recently, Lappas et al. (13) reported that hepatic reperfusion injury was initiated by the activation of NKT cells that was inhibited by the adenosine A_{2A} receptor agonist. Thus, these findings strongly suggest that the inhibitory effect of adenosine on prevention of early loss of transplanted islets is primarily mediated by NKT cells, although it remains uncertain whether the site of inhibitory action by adenosine includes the upstream pathway(s) of NKT cell activation, in which dendritic cells and macrophages (kupffer cells) might be involved.

Another important issue to consider is whether adenosine has protective effects directly on transplanted islets and whether islet cells express adenosine receptor(s). Recently, an adenosine receptor knockout mouse was developed (26), and it was reported that the tissue damage in Con A hepatitis was exacerbated in adenosine receptor 2A^{-/-} mice compared with wild-type mice (27). However, no information is available regarding whether islet cells themselves express adenosine receptors and whether adenosine has a direct protective effect on islet cells under stresses including hypoxia and inflammation. More importantly, the molecular mechanisms of the inhibitory effects by adenosine in the individual cells after binding adenosine receptor remain unclear. These issues are matters of interests for future investigations.

Because the low efficiency of islet transplantation remains a major obstacle to overcome in clinical islet transplantation, it is important and interesting to understand the extent and the efficiency of islet transplantation improvement by adenosine. The IPGTT at 60 days after transplantation disclosed that the glucose tolerance of STZ-induced diabetic mice receiving 200 islets from a single donor and treated with adenosine was superior in comparison with diabetic mice receiving 400 islets from two donors, thus indicating that adenosine facilitates a greater than 2-fold improvement in the efficiency of islet transplantation.

We found that the inhibitory effect of adenosine on the early loss of transplanted syngenic islets was also similar for islet allotransplantation, in which STZ-diabetic mice receiving 200 allogenic islets from a single donor and treated with adenosine became normoglycemic after the transplantation. However, the normoglycemic recipient mice became hyperglycemic again by 7 days after the transplantation, thus indicating that a single injection of adenosine has an inhibitory effect on the early loss of transplanted islets but not on the alloimmune rejection. Previously, we have shown that NKT cells play an essential role in alloimmune rejection of islet allografts in the liver of mice by using V α 14 NKT cell- and CD1d-deficient mice, in which the survival of islet allografts is prolonged without any immunosuppression (28). These findings suggest that adenosine may have an inhibitory effect on alloimmune rejection when administered appropriately with respect to its dosage and its duration, and with respect to the timing of the treatment. To clarify these, further studies are required. Importantly, a beneficial effect of adenosine on preventing the early loss of transplanted allogenic islets was found to be maintained when alloimmune rejection was pre-

vented by anti-CD4 antibody. It is important to determine whether immunosuppressive agents such as antithymocyte globulin, which has been recently introduced into clinical islet transplantation, have any beneficial effect on the engraftment of transplanted islets. Furthermore, it is important to clarify whether the adenosine receptor agonist such as ATL-146e (29), which is currently being developed for clinical application, has any beneficial effect and how adenosine itself is effective in the improvement of the engraftment of transplanted islets in comparison to the other strategies including the procedures targeting proinflammatory cytokines (19) and instant blood-mediated inflammatory reactions (30, 31).

In summary, this study demonstrates that adenosine produces beneficial effects for the prevention of early loss of transplanted islets, enabling islet transplantation from one donor to one recipient in mice. Because adenosine and the adenosine transporter inhibitor, dipyridamole have already been used in the clinic, the safety issues with respect to the clinical use for islet transplantation has been cleared. Thus, adenosine may improve the efficiency of clinical islet transplantation provided that the beneficial effect of adenosine demonstrated in this study holds true in humans, although the effective dosage and the duration of the treatment still need to be clarified in a clinical setting.

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High-mobility group box 1 is involved in the initial events of early loss of transplanted islets in mice

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Islet transplantation for the treatment of type 1 diabetes mellitus is limited in its clinical application mainly due to early loss of the transplanted islets, resulting in low transplantation efficiency. NKT cell-dependent IFN- γ production by Gr-1⁺CD11b⁺ cells is essential for this loss, but the upstream events in the process remain undetermined. Here, we have demonstrated that high-mobility group box 1 (HMGB1) plays a crucial role in the initial events of early loss of transplanted islets in a mouse model of diabetes. Pancreatic islets contained abundant HMGB1, which was released into the circulation soon after islet transplantation into the liver. Treatment with an HMGB1-specific antibody prevented the early islet graft loss and inhibited IFN- γ production by NKT cells and Gr-1⁺CD11b⁺ cells. Moreover, mice lacking either of the known HMGB1 receptors TLR2 or receptor for advanced glycation end products (RAGE), but not the known HMGB1 receptor TLR4, failed to exhibit early islet graft loss. Mechanistically, HMGB1 stimulated hepatic mononuclear cells (MNCs) *in vivo* and *in vitro*; in particular, it upregulated CD40 expression and enhanced IL-12 production by DCs, leading to NKT cell activation and subsequent NKT cell-dependent augmented IFN- γ production by Gr-1⁺CD11b⁺ cells. Thus, treatment with either IL-12- or CD40L-specific antibody prevented the early islet graft loss. These findings indicate that the HMGB1-mediated pathway eliciting early islet loss is a potential target for intervention to improve the efficiency of islet transplantation.

Introduction

Pancreatic islet transplantation, although an attractive procedure for the treatment of type 1 diabetes mellitus, usually fails to achieve insulin independence of a diabetic recipient from a single donor due to early loss of transplanted islets and therefore requires sequential transplantations of islets with the use of 2–3 donors (1). Thus, the low efficiency of islet transplantation has been a major obstacle facing islet transplantation and hampers its clinical application.

We have previously shown in mice that loss of transplanted islets soon after transplantation is caused by NKT cell-dependent IFN- γ production by Gr-1⁺CD11b⁺ cells and is successfully prevented by treatment of NKT cells with repeated stimulation with their synthetic ligand, α -galactosylceramide (α -GalCer), to downregulate IFN- γ production of NKT cells, or by depletion of Gr-1⁺CD11b⁺ cells with anti-Gr-1 antibody (2). However, precisely how it is involved in the upstream events in the activation of NKT cells and Gr-1⁺CD11b⁺ cells in the early loss of transplanted islets remains to be solved.

High-mobility group box 1 (HMGB1) protein was initially found to be a DNA-binding protein present in almost all eukaryotic cells, where it stabilizes nucleosome formation and acts as a nuclear factor that enhances transcription (3, 4). Recently,

HMGB1 has been demonstrated to play crucial roles in response to tissue damage, indicating that HMGB1 is a prototype of the emerging damage-associated molecular pattern molecule (4, 5). HMGB1 is also known to be secreted by activated immune cells, including macrophages (6, 7), DCs (8), and NK cells (9) in response to infection and inflammatory stimuli. Once secreted, HMGB1 induces inflammatory responses by transduction of cellular signals through its receptors, such as TLR2, TLR4 (10–12), and receptor for advanced glycation end products (RAGE) (8, 13, 14). Moreover, HMGB1 levels are markedly increased during severe sepsis in humans and animals, and administration of neutralizing HMGB1-specific antibodies prevents lethality from sepsis (6). Recent accumulating evidence now suggests that HMGB1 acquires or augments proinflammatory activity by binding to proinflammatory mediators such as LPS, IL-1 (14), and DNA (15–17). These observations indicate that HMGB1 is an essential mediator of organ damage; however, its precise role and mechanism remain unknown. Here, we investigate the mechanisms of action of HMGB1 in the early loss of transplanted islets.

Results

Involvement of HMGB1 in early loss of transplanted islets. It has previously been shown that hyperglycemia of streptozotocin-induced (STZ-induced) diabetic recipient mice was ameliorated after transplantation of 400 syngenic islets in the liver but not of 200 islets (Figure 1A, no treatment), the number of islets isolated from a

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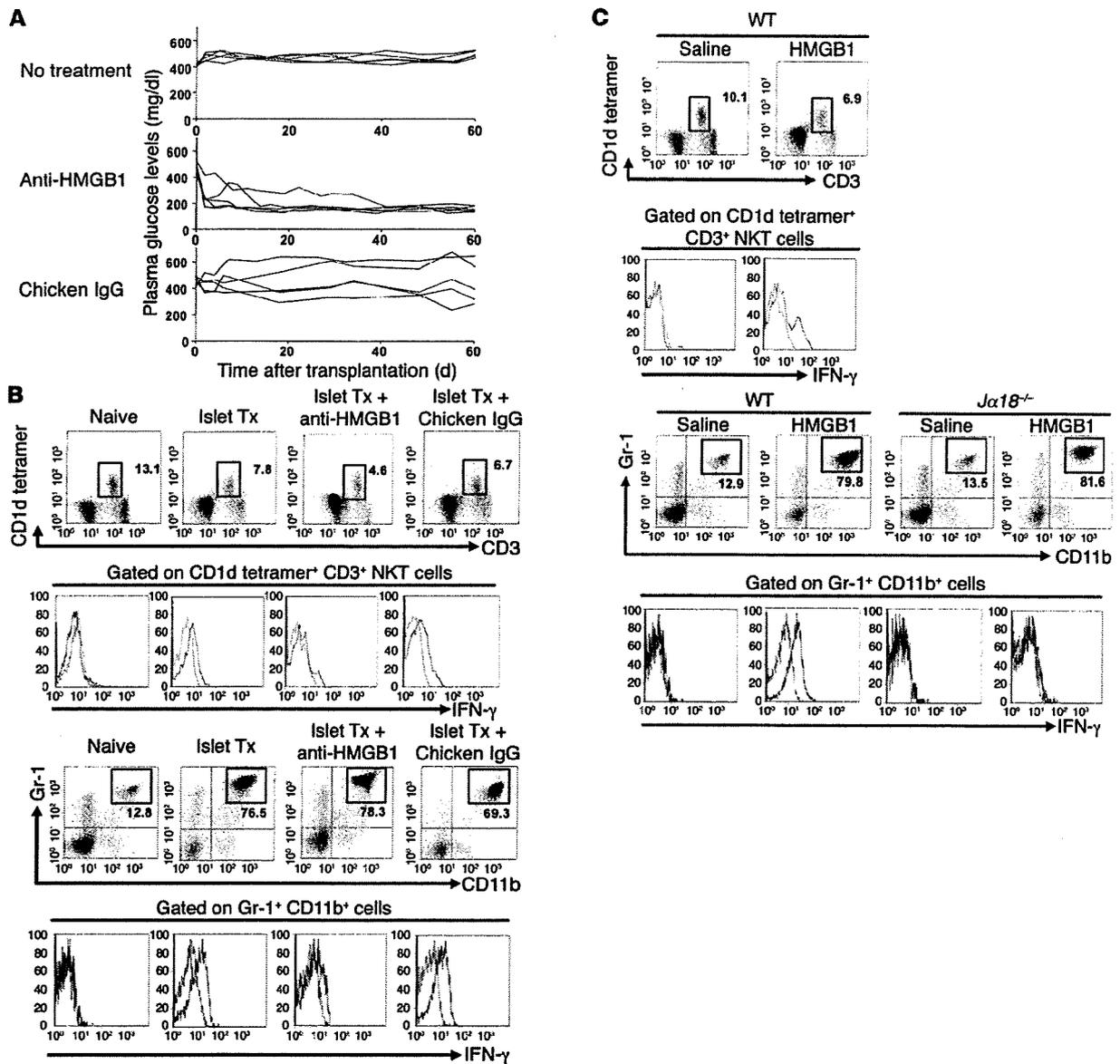


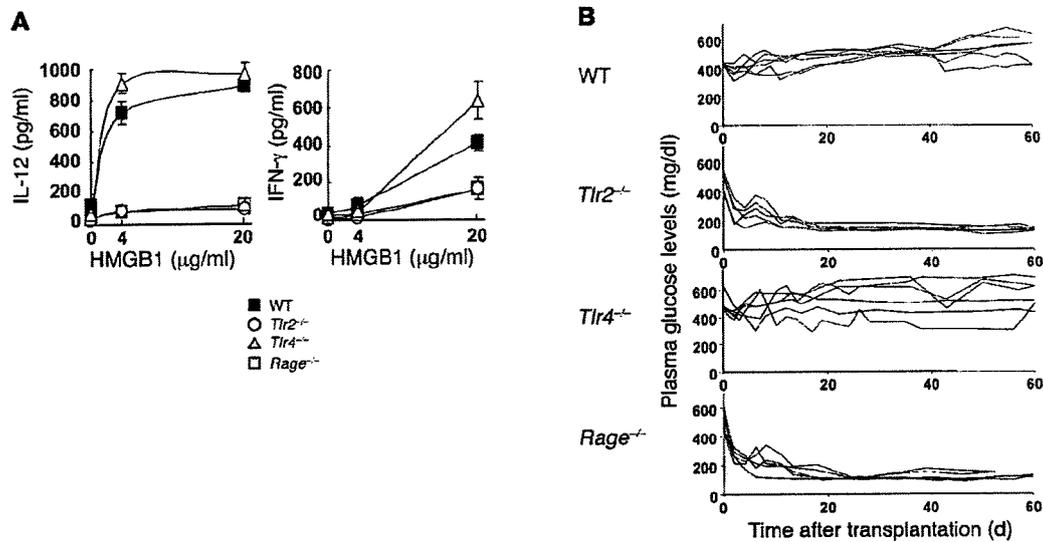
Figure 1

Essential roles of HMGB1 in early loss of transplanted islets. **(A)** Nonfasting plasma glucose levels in STZ-induced diabetic mice received 200 syngeneic islets (top panel) and those treated with chicken anti-HMGB1 antibody or control chicken IgG. Individual lines represent glucose levels of each animal. **(B)** FACS profiles of liver MNCs from naive mice, STZ-induced diabetic mice that received 200 syngeneic islets (Islet Tx), and islet transplanted mice treated with anti-HMGB1 antibody or with chicken IgG. NKT cells (top 2 rows) and Gr-1⁺CD11b⁺ cells (bottom 2 rows) were analyzed for IFN- γ (second and fourth rows). The numbers in the figures represent the percentage of cells in the corresponding square areas. Representative data from 4 experiments are shown. **(C)** FACS profiles of NKT cells and Gr-1⁺CD11b⁺ cells after HMGB1 treatment. Liver MNCs from wild-type or *Jα18^{-/-}* mice treated with i.v. injection of saline or HMGB1 (100 μ g/mouse) were isolated 2 hours after the injection and examined by flow cytometry for IFN- γ production by NKT cells and Gr-1⁺CD11b⁺ cells. The numbers in the figures represent the percentage of cells in the corresponding square areas. Representative data from 4 experiments are shown.

single mouse pancreas (2). By using the diabetes model mice, we first investigated the effects of anti-HMGB1 antibody to examine whether HMGB1 is directly involved in early loss of transplanted islets. STZ-induced diabetic mice that received 200 islets together with anti-HMGB1 antibody once at the time of islet transplantation became normoglycemic, in contrast to mice treated with control chicken IgG (Figure 1A). The results demonstrated that

the anti-HMGB1 antibody ameliorates hyperglycemia of diabetic mice, indicating that the early loss of transplanted islets is prevented by anti-HMGB1. Thus, HMGB1 plays a crucial role in early loss of transplanted islets.

IFN- γ production of NKT cells and Gr-1⁺CD11b⁺ cells in the liver receiving islets is inhibited by anti-HMGB1 antibody. Next, we determined whether anti-HMGB1 antibody treatment has any effect on IFN- γ

**Figure 2**

HMGB1 receptors involved in early loss of transplanted islets. **(A)** In vitro cytokine production by liver MNCs. Liver MNCs (2×10^6 /well) isolated from wild-type, *Tlr2*^{-/-}, *Tlr4*^{-/-}, or *Rage*^{-/-} mice were cultured with indicated doses of HMGB1 in vitro for 48 hours, and IL-12 or IFN- γ levels in the culture supernatant were measured. Representative data from 2 experiments are shown. **(B)** Nonfasting plasma glucose levels of STZ-induced diabetic wild-type, *Tlr2*^{-/-}, *Tlr4*^{-/-}, or *Rage*^{-/-} mice that received 200 syngeneic islets. Individual lines represent the glucose level of each animal.

production by NKT cells and Gr-1⁺CD11b⁺ cells in the liver of mice receiving islets, which are essential components of early loss of transplanted islets, as shown previously (2). For those purposes, mononuclear cells (MNCs) in the liver of recipient mice were isolated at 6 hours after islet transplantation of 200 syngeneic islets into the liver and examined by FACS as to IFN- γ production by NKT cells and Gr-1⁺CD11b⁺ cells in the liver. The results are in agreement with the previous findings (2) that, within 6 hours after transplantation of syngeneic islets into the liver, NKT cells and Gr-1⁺CD11b⁺ cells accumulated into the liver with upregulated production of IFN- γ (Figure 1B). This upregulated production of IFN- γ after islet transplantation was inhibited by anti-HMGB1. Since the treatment with anti-HMGB1 antibody did not affect the number of infiltrated Gr-1⁺CD11b⁺ cells (Figure 1B), the recruitment of Gr-1⁺CD11b⁺ cells was due not to HMGB1, but rather probably to the events of transplantation itself. These findings suggest that HMGB1 is essentially involved in the activation of NKT cells and/or Gr-1⁺CD11b⁺ cells in the liver after islet transplantation.

NKT cell-dependent IFN- γ production by Gr-1⁺CD11b⁺ cells upon stimulation with HMGB1. In order to confirm HMGB1-dependent IFN- γ production, we investigated whether HMGB1 has any stimulatory effects in vivo on NKT cells and/or Gr-1⁺CD11b⁺ cells in the liver of mice (Figure 1C). For those purposes, HMGB1 was administered i.v. into naive wild-type and NKT cell-deficient *J α 18*^{-/-} mice, and their hepatic MNCs were isolated at 2 hours after the injection and examined by flow cytometry. It was found that IFN- γ production was upregulated in NKT cells and Gr-1⁺CD11b⁺ cells in the liver of wild-type mice treated with HMGB1 (Figure 1C). Importantly, the IFN- γ production by Gr-1⁺CD11b⁺ cells in the liver of *J α 18*^{-/-} mice treated with HMGB1 was not upregulated, although accumulation of Gr-1⁺CD11b⁺ cells was similar to that in wild-type mice (Figure 1C). These findings indicate that IFN- γ production by Gr-1⁺CD11b⁺ cells in the liver of mice treated with HMGB1 is dependent on NKT cells.

Involvement of TLR2 and RAGE but not TLR4 in HMGB1-dependent early loss of transplanted islets. We further investigated whether HMGB1-dependent early loss of transplanted islets is dependent on TLR2, TLR4, and/or RAGE, which is known to be a potential receptor of HMGB1 (10–14). Isolated liver MNCs from wild-type mice induced augmented production of IL-12 and IFN- γ in response to HMGB1 in vitro (Figure 2A), which were greatly reduced in *Tlr2*^{-/-} and *Rage*^{-/-} liver MNCs but not in *Tlr4*^{-/-} liver MNCs, whose cytokine production levels were equivalent to those of wild-type mice in response to HMGB1.

To elucidate which receptor(s) for HMGB1 are actually involved in early loss of transplanted islets, STZ-induced diabetic *Tlr2*^{-/-}, *Tlr4*^{-/-}, or *Rage*^{-/-} mice that received 200 syngeneic islets were investigated for glucose levels in the serum. Interestingly, all of *Tlr2*^{-/-} or *Rage*^{-/-} mice (5 of 5) became normoglycemic, while *Tlr4*^{-/-} mice remained hyperglycemic after transplantation, indicating that TLR2 and RAGE, but not TLR4, play an essential role in the early loss of transplanted islets (Figure 2B).

Pancreatic islet cells are a major source of HMGB1, which mediates IFN- γ production by NKT cells and Gr-1⁺CD11b⁺ cells. To validate the involvement of HMGB1 in early loss of transplanted islets, we carried out histological examination on islets before and after transplantation. HMGB1 was detected at a high level in cytoplasm as well as nucleus of transplanted islets as early as 3 hours after transplantation, while HMGB1 was stained only in the nucleus of islets in the naive pancreas and of isolated islets (Figure 3A). The results suggest that HMGB1 is localized in the nucleus of pancreatic islets, shuttled to cytoplasm, and possibly secreted into the circulation soon after transplantation.

Next, we examined the amounts of HMGB1 in isolated islets in comparison with those in other organs, including the thymus, lung, spleen, liver, and pancreas, as well as of FACS-sorted liver MNCs, including NK, NKT, T, B, Gr-1⁺CD11b⁺, and Gr-1⁺CD11b⁻ cells. Currently, there are no data available with respect to the HMGB1 content in the different cell types, although tissue dis-

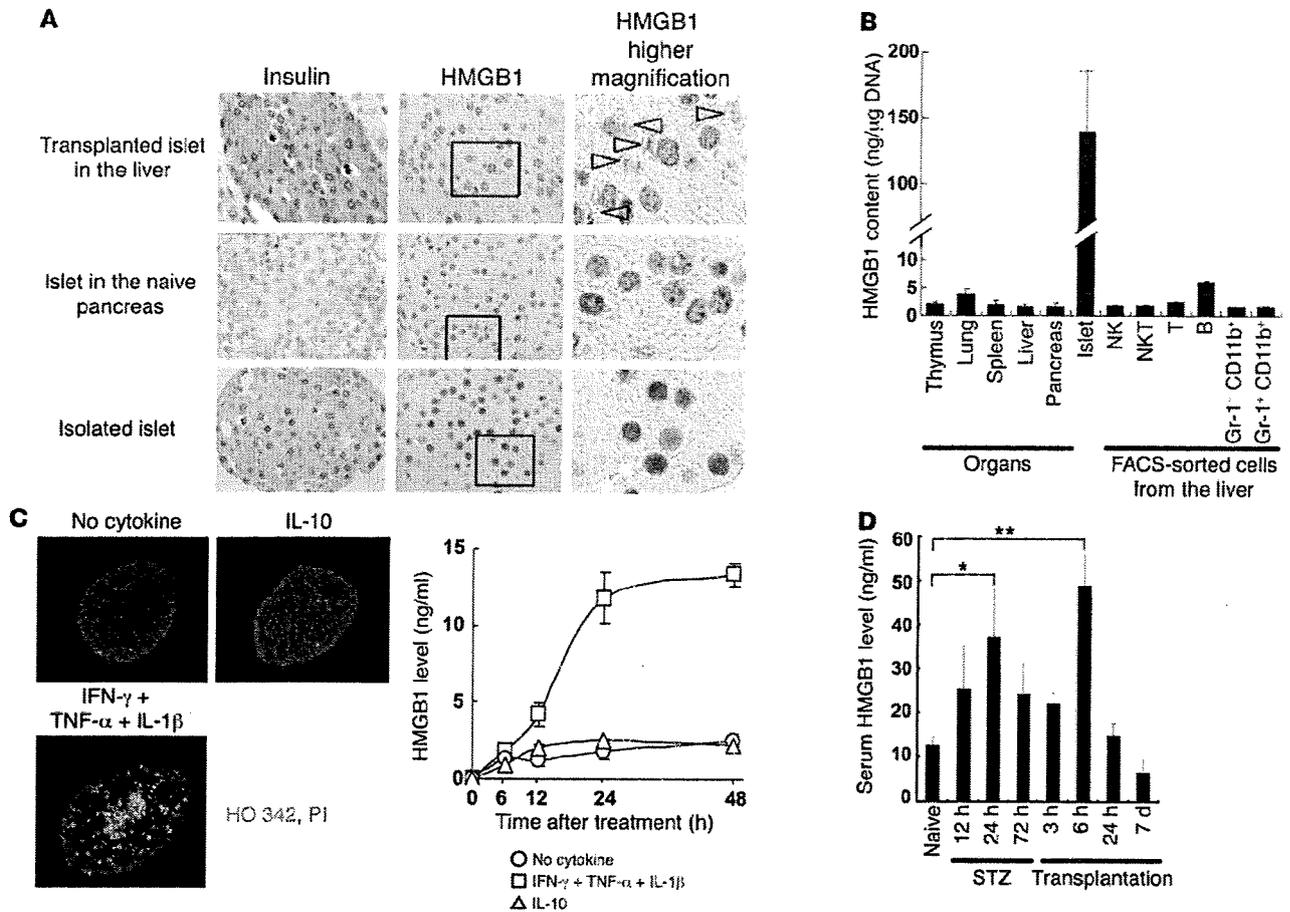


Figure 3

HMGB1 production in tissues and cell types. (A) Photomicrographs of islets. Islet cells 3 hours after transplantation in the liver (top row) and those of naive pancreas or isolated islets were examined. Sections stained with anti-insulin or anti-HMGB1 followed by staining with hematoxylin are shown. In general, HMGB1 was detected in the nucleus (brown), while some was detected in cytoplasm, as indicated by arrowheads. Original magnification: $\times 100$ (first and second columns) and $\times 800$ (third column). Boxed regions in the second column were enlarged. (B) HMGB1 contents (ng/ μ g DNA) of individual organs ($n = 5$), isolated islets, or FACS-sorted liver MNCs ($n = 3$). (C) Left panels: Fluorescence photomicrographs of isolated islets (original magnification, $\times 200$) stained with HO 342 (blue) and PI (red) at 24 hours after *in vitro* culture with or without IFN- γ , TNF- α , and IL-1 β (20 ng/ml each) or IL-10 (20 ng/ml). Right panels: HMGB1 levels in the culture (200 cells/dish) were also measured at the indicated time points in the absence of cytokine or the presence of cytokine mixtures or of control cytokine (IL-10). The values are expressed as the mean \pm SD in each group ($n = 5$). (D) Serum HMGB1 levels were measured after STZ injection and also after transplantation of 400 syngeneic islets, which had been performed 72 hours after STZ injection ($n = 5-6$). The values are expressed as the mean \pm SD. * $P < 0.05$; ** $P < 0.01$.

tribution of HMGB1 has been reported previously (18). To our surprise, isolated islets contained high levels of HMGB1, which were 20 times more greater than in other organs or FACS-sorted cells tested (Figure 3B). The physiological roles of high concentrations of HMGB1 in islet cells as well as their etiology are a matter of interest and need to be clarified in future studies.

To investigate a direct relationship between HMGB1 and islet cell damage, we cultured isolated mouse islets in the absence or presence of cytotoxic proinflammatory cytokines, including IFN- γ , TNF- α , and IL-1 β , which are known to induce islet cell death *in vitro* (19) with elevated concentrations of HMGB1 in the culture medium (20). IL-10 was used as a control. Islet cell death was assessed by fluorescence microscopy with the use of the DNA-binding dye propidium iodide (PI) and Hoechst 33342 (HO 342) (19). PI, a highly polar dye that is impermeable to cells with preserved membranes, stains DNA

red when membranes are damaged. HO 342 freely passes the plasma membrane, readily enters cells with intact membranes, and stains DNA blue. Thus, the nuclei of dead cells stained red by PI, while those of intact cells stained blue without fragmentation and condensation by HO 342. PI-positive islet cells were increased in number with time in the presence of cytotoxic cytokines, while those in the absence of cytotoxic cytokines and in the presence of the control cytokine remained low in number (Figure 3C). In parallel, HMGB1 concentration in the islet cell culture medium increased with time in the presence of cytotoxic cytokines, while, in contrast, that in the absence of cytotoxic cytokines as well as in the presence of the control cytokine remained low (Figure 3C).

The above findings suggest that HMGB1 of transplanted islets may be released into the circulation of recipient mice in association with their damage soon after transplantation. In fact, the serum

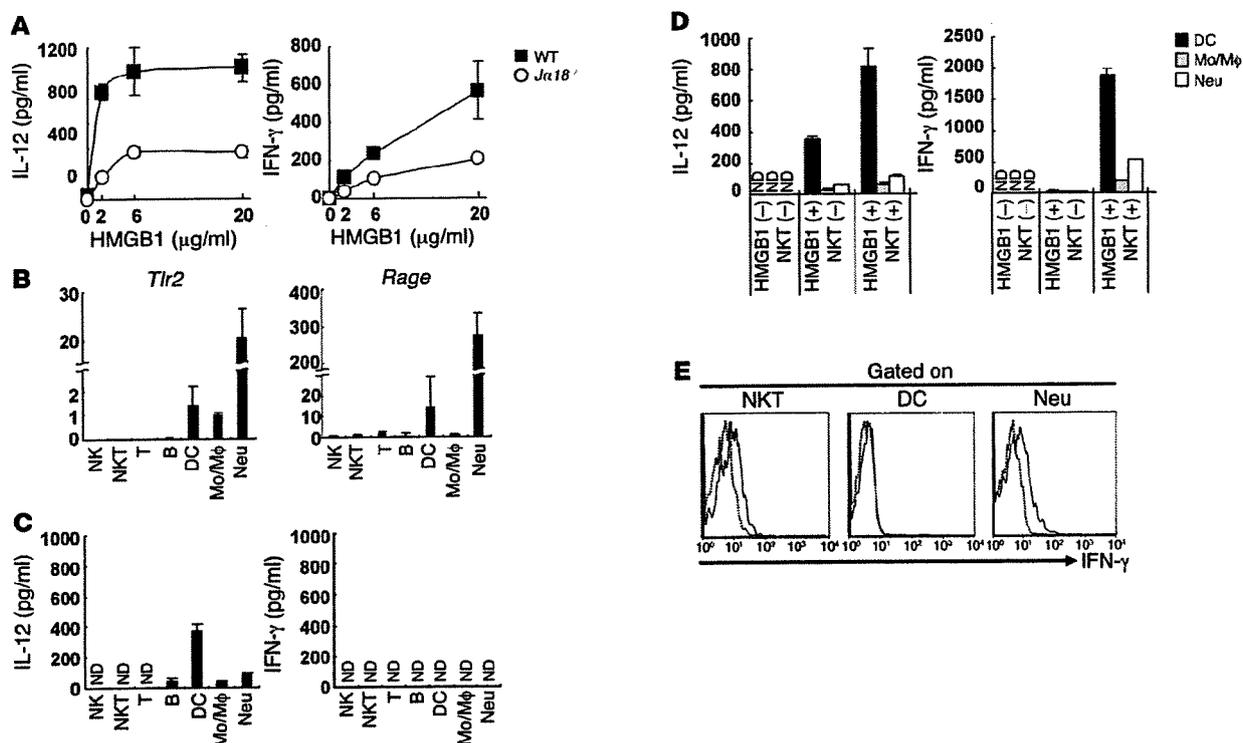


Figure 4

NKT cell-dependent IL-12 and IFN- γ production by liver MNCs in response to HMGB1. (A) Liver MNCs (2×10^6 /well) isolated from wild-type or *Jα18*^{-/-} mice were cultured with the indicated doses of HMGB1 in vitro for 48 hours and measured for IL-12 and IFN- γ . Representative data from 2 experiments are shown. (B) PCR analysis on HMGB1 receptors. FACS-sorted liver MNCs (2×10^3 for *Tlr2*, *Rage*, or *Hprt*) were analyzed for mRNA levels by quantitative real-time PCR. Data were analyzed by the $\Delta\Delta C_t$ method using the expression level in Mo/M ϕ as normalized control. (C) Cytokine production in FACS-sorted liver MNCs upon stimulation with HMGB1. FACS-sorted cells were cultured in vitro (1×10^5 cells/well) for 48 hours in the presence of HMGB1 (20 μ g/ml). The amounts of IL-12 and IFN- γ were measured by CBA ($n = 3$). (D) Cytokine production by DCs, Mo/M ϕ , or Neu in the presence of NKT cells. FACS-sorted Gr-1⁺CD11b⁺CD11c⁻ DCs, Gr-1⁺CD11b⁺CD11c⁻ Mo/M ϕ , and Gr-1⁺CD11b⁺CD11c⁻ Neu (4×10^4) were cocultured in vitro with NKT cells (2×10^5) in the presence of HMGB1 (20 μ g/ml) for 48 hours. The amounts of IL-12 and IFN- γ were measured by CBA ($n = 3$). (E) Intracellular cytokine staining of liver MNCs after HMGB1 treatment. Liver MNCs (2×10^6) were cultured with HMGB1 (20 μ g/ml) for 24 hours, and the indicated cells were gated and analyzed for their production of IFN- γ by intracellular cytokine staining.

HMGB1 levels in the STZ-induced diabetic mice were elevated, with a peak at 24 hours, and returned to the preinjection levels by 72 hours after i.v. injection of STZ, while, after islet transplantation, HMGB1 peaked at 6 hours and returned to pretransplant levels by 7 days (Figure 3D). The findings suggest that the first peak of the serum HMGB1 elevation is due to islet cell damage caused by STZ injection, which is a toxic agent to β cells of islets, while the second HMGB1 peak is due to the early loss of transplanted islets.

Cell types responsible for HMGB1-mediated cytokine production. We investigated the mechanisms of action of HMGB1 by measuring in vitro production of IFN- γ and IL-12 in the culture of isolated liver MNCs from wild-type and *Jα18*^{-/-} mice in response to HMGB1, since IFN- γ is critical in the early islet graft loss (2) and also because IL-12 is essential for IFN- γ production by NKT cells (21). Isolated liver MNCs from wild-type mice induced augmented production of IL-12 and IFN- γ in response to HMGB1 in vitro (Figure 4A). Importantly, the amount of IL-12 and IFN- γ produced by liver MNCs in NKT cell-deficient *Jα18*^{-/-} mice treated with HMGB1 was greatly reduced (Figure 4A), indicating that NKT cells augment HMGB1-dependent IL-12 and IFN- γ production.

We then investigated expression of *Tlr2* and *Rage* by quantitative real-time PCR in each FACS-sorted cellular population from the liver, including NK1.1⁺CD3⁻ NK, α -GalCer/CD1d dimer⁺ NKT, CD3⁺ T, and CD19⁺ B cells; Gr-1⁺CD11b⁺CD11c⁻ neutrophils (Neu); and Gr-1⁺CD11b⁺ cells, which were further divided into CD11c⁺F4/80⁻ DCs and CD11c⁺F4/80⁺ monocytes/macrophages (Mo/M ϕ) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI41360DS1). *Tlr2* and *Rage* were detected at high levels on Neu (Figure 4B). DCs also expressed modest levels of both *Tlr2* and *Rage*. However, Mo/M ϕ expressed modest levels of *Tlr2* but low levels of *Rage*, while expression of either *Tlr2* or *Rage* was barely detected in other cell populations (NK, NKT, T, and B cells) (Figure 4B).

In order to dissect further the mechanisms of action of HMGB1, we investigated in vitro IL-12 and IFN- γ production in the culture of FACS-sorted individual cellular populations from liver MNCs in response to HMGB1. IL-12 was mainly produced from DCs rather than Neu or Mo/M ϕ (Figure 4C). However, IFN- γ production was not detected in any individual cell population among all liver MNC subpopulations tested (Figure 4C).

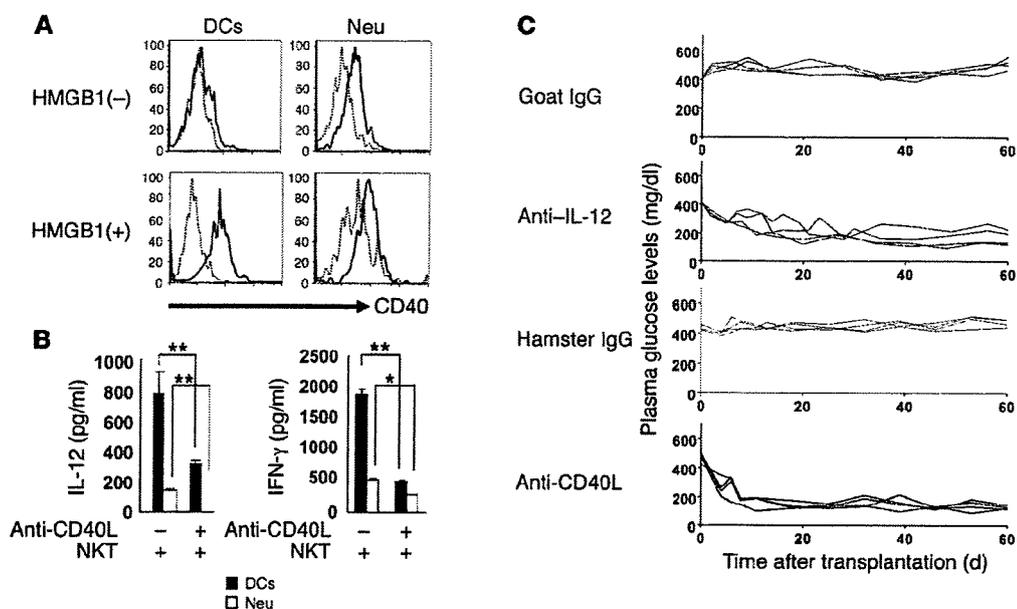


Figure 5
 Involvement of CD40-CD40L interaction in production of IL-12 and IFN- γ in early loss of transplanted islets. (A) CD40 expression in DCs and Neu before or after treatment with HMGB1. Liver MNCs (2×10^6) were treated without or with HMGB1 (20 μ g/ml) for 24 hours and analyzed for CD40 expression ($n = 3$). (B) Requirement of CD40-CD40L interaction in the production of IL-12 and IFN- γ in the presence of NKT cells. DCs or Neu (4×10^4) were cocultured in vitro with NKT cells (2×10^5) in the presence of HMGB1 (20 μ g/ml) for 48 hours with or without addition of anti-CD40L antibody. IL-12 and IFN- γ levels were measured by CBA ($n = 3$). The values are expressed as the mean \pm SD. * $P < 0.05$; ** $P < 0.01$. (C) Nonfasting plasma glucose levels of STZ-induced diabetic mice that had received 200 syngeneic islets and were treated with control goat IgG or goat anti-mouse IL-12 antibody and those with control hamster IgG or hamster anti-mouse CD40L antibody with 200 μ g intraperitoneal injection per mouse at the time of transplantation. Individual lines represent the nonfasting plasma glucose levels of each diabetic mouse after islet transplantation.

Since IL-12 was produced in vitro from DCs in response to HMGB1 (Figure 4C) and since NKT cell-dependent IFN- γ production by Neu is an essential component of early loss of transplanted islets as shown previously (2), IL-12 and IFN- γ production of FACS-sorted DCs, Mo/M ϕ , or Neu cocultured in the presence of NKT cells with addition of HMGB1 was examined. The production of IL-12 was greatly augmented in response to HMGB1, especially when DCs were cocultured with NKT cells (Figure 4D). The production of IFN- γ became evident in the culture medium of DCs cocultured with NKT cells in the presence of HMGB1 (Figure 4D). The cell types responsible for the production of IFN- γ in response to HMGB1 in Figure 4D were NKT cells but not DCs, because intracellular cytokine staining revealed that NKT cells, but not DCs, produced IFN- γ (Figure 4E). It was also shown that Neu production of IFN- γ was augmented in the presence of NKT cells (Figure 4D).

It is known that IFN- γ production by NKT cells is largely dependent on the interaction between CD40L expression on activated NKT cells and CD40 expression on DCs (22). Thus, we measured CD40 expression on DCs and Neu stimulated with HMGB1. CD40 surface expression was detected in both cell types in resting conditions, while upregulation of CD40 was observed in DCs rather than Neu in HMGB1-treated conditions (Figure 5A). Furthermore, production of both IL-12 and IFN- γ mounted in vitro by HMGB1 stimulation was blocked by anti-CD40L antibody (Figure 5B), indicating that augmented IL-12 production from DCs and Neu and also IFN- γ production by NKT cells and Neu are triggered by CD40/CD40L interaction.

To confirm the data shown in Figure 5, A and B, we determined in vivo requirement of IL-12 and CD40-CD40L interaction in early loss of transplanted islets. Hyperglycemia of STZ diabetic mice receiving 200 syngeneic islets in the liver was ameliorated by treatment with either anti-IL-12 or anti-CD40L antibody once at the time of islet transplantation, while that of mice treated with control antibody was not (Figure 5C). Together with the previous studies showing that the anti-IFN- γ treatment normalizes hyperglycemia (22), the results indicate that IL-12 and CD40-CD40L interaction together with IFN- γ actually play a crucial role in vivo in early loss of transplanted islets.

Discussion

Among the most important findings of the present study is that pancreatic islets contain abundant HMGB1 compared with other organs and individual cell populations in the liver, the site of islet transplantation. Immunohistochemical staining of the pancreas revealed that HMGB1 is mainly stained in the nucleus of islet cells but not in other cell types, while HMGB1 is detected in the circulation after islet cell damage. In fact, the plasma concentration of HMGB1 in wild-type mice was elevated and peaked at 24 hours after i.v. injection of STZ and returned to the preinjection level 72 hours after STZ injection. The plasma levels of HMGB1 in diabetic recipient mice were elevated after islet transplantation with a peak at 6 hours and returned to pretransplant levels by 24 hours. These findings suggest that the first peak of the elevated HMGB1 levels is caused by destruction of islet cells by a toxic agent of STZ specific to β cells of islets and that the second peak in recipient



mice after islet transplantation is related to the damage of islet grafts soon after transplantation. Thus, combined with *in vitro* findings of elevated concentrations of HMGB1 in the culture medium of isolated islets in the presence of cytotoxic cytokines, the plasma levels of HMGB1 may reflect the degree of islet damage in the liver after transplantation. Furthermore, the treatment with anti-HMGB1 antibody delayed the onset of diabetes in NOD mice, suggesting that HMGB1 plays a significant role in disease progression (23).

The above findings prompted us to determine whether HMGB1 is involved in the early loss of transplanted islets, which occurs within 6 hours after islet transplantation and is an event caused by inflammatory cytokines, as we previously reported (2, 23). In fact, the hyperglycemia of islet-transplanted diabetic mice was ameliorated by treatment with anti-HMGB1 antibody, indicating that HMGB1 is essentially involved in the early loss of transplanted islets.

Concerning the mechanisms of HMGB1-mediated early islet graft loss, 3 cell types, NKT cells, Gr-1⁺CD11b⁺CD11c⁺F4/80⁻ DCs, and Gr-1⁺CD11b⁺ Neu, were found to be involved in the initial phase of early loss of transplanted islets. Among these cell types, the primary cellular targets of HMGB1 does not seem to be NKT cells, since the receptors for HMGB1 (10–14) TLR2 and RAGE but not TLR4 (Figure 2B) are expressed on DCs, Mo/M ϕ , and Neu, but not on NKT cells (Figure 4B). IL-12, which is essential for NKT cell-dependent production of IFN- γ , was mainly produced by DCs after HMGB1 stimulation (Figure 4C). Thus, it is likely that the first target for HMGB1 is DCs, which in turn activate NKT cells. Then, activated NKT cells themselves produce IFN- γ and also stimulate Neu to produce IFN- γ (Figure 4D), which is an essential component of HMGB1-mediated early loss of transplanted islets.

Thus, the present study unveils a role of DCs in HMGB1-dependent IFN- γ production by NKT cells. DCs stimulated with HMGB1 *in vitro* upregulate their CD40 expression and produce IL-12, which is markedly augmented in the presence of NKT cells, facilitating IFN- γ production by NKT cells and subsequently that of Neu. The requirement of CD40-CD40L interaction and IL-12 is confirmed by the fact that anti-CD40L and anti-IL-12 antibodies prevented early loss of transplanted islets, leading to amelioration of hyperglycemia of islet-transplanted diabetic recipient mice, while the corresponding control antibody did not. Thus, the uncovered pathways involved in the early loss of transplanted islets in the present study afford further new targets for intervention to improve the efficiency of islet transplantation.

TLR2, TLR4, and RAGE as potential receptors for HMGB1 (10–14) are expressed mainly on DCs, Mo/M ϕ , and Neu (Figure 4B). However, *in vitro* and *in vivo* experiments revealed that TLR2 and RAGE but not TLR4 are involved in the early loss of transplanted islets (Figure 2B). It has been reported that HMGB1-mediated biological effects and usage of their receptors are different in the experimental models. For example, TLR4, but not TLR2 or RAGE, has been shown to be an HMGB1 receptor in hepatic reperfusion injury (24). Similarly, HMGB1 signaling through TLR2 and TLR4 but not RAGE contributes to LPS-induced inflammation (11). In the case of SLE, HMGB1 present in DNA-containing immune complexes triggers activation of autoreactive B cells and plasmacytoid DCs through RAGE (17). These differences in HMGB1-mediated effects might be due to the presence, in different systems, of cell types with distinctly different HMGB1 receptor expression profiles, and also due to the formation of complexes of HMGB1 with different molecules under varying disease conditions.

Concerning the form of HMGB1, HMGB1 acquires and/or augments inflammatory effects when it binds to other inflammatory molecules, such as IL-1 β , the TLR4 ligand LPS, the TLR9 ligand CpG-ODN, or the TLR1-TLR2 ligand Pam3CSK (14–17). Recent studies on HMGB1-deficient mice also showed that HMGB proteins function as universal sentinels for nucleic acids (25). However, in the present studies, it still remains unsolved what types of molecules interact with HMGB1 protein to mediate its function.

Chen et al. (26) have reported that the direct effects of RAGE on conventional T cell functions resulted in the prolongation of syngeneic and allogeneic islet graft transplanted in the subcapsular space of kidney, in that anti-CD3/CD28-induced T cell proliferation, mixed lymphocyte reaction, and T cell production of IL-10, IL-5, and TNF- α but not IFN- γ were inhibited in RAGE-deficient mice and mice receiving RAGE inhibitor. Since no conventional T cells were involved in the early loss of islet transplanted in the liver, and also because IFN- γ , but not IL-10 nor IL-5, is a major player in the early islet loss, the mechanisms observed in the present studies are different from those described by Chen et al. (26).

Concerning the potential sites for islet transplantation — including the liver, renal subcapsular space, omental pouch, abdominal cavity, intramuscular site, subcutaneous — the liver is currently the only site where insulin independence in patients with type 1 diabetes mellitus can be achieved with clinical islet transplantation, as reported by Shapiro et al. (1). Although we do not have any data on islet transplantation at non-NKT cell-dense sites, the NKT cell-mediated early loss of islets can occur at any tissue, as it has been demonstrated in the allogeneic heart transplantation model that NKT cells migrate immediately into non-NKT cell-dense transplantation sites, where CXCL16, the ligand for chemokine receptor CXCR6 selectively expressed on NKT cells, is expressed (27).

Taken collectively, the findings in the present study shed light on the mechanisms involved in the early loss of transplanted islets as follows. First, islet cells themselves are a major source of HMGB1, which is released from transplanted islets. Since the plasma levels of HMGB1 reflect the degree of islet damage, HMGB1 could be a marker to predict rejection of transplanted islets. Second, HMGB1 stimulates production of inflammatory cytokines including IL-12 and IFN- γ in concert with DCs, NKT cells, and Neu in the liver receiving islets. Third, these inflammatory cytokines accelerated the injuries of transplanted islets. Thus, a vicious cycle harmful to transplanted islets is now unveiled. Therefore, each pathway involved in the early loss of transplanted islets revealed by the present study is a potential target for intervention to improve efficiency of islet transplantation.

Methods

Mice. C57BL/6 mice were purchased from Charles River Japan Inc. or CLEA Japan Inc. α 18-deficient mice were generated previously (28) and backcrossed more than 10 times to C57BL/6 mice. *Rage*^{-/-} mice (29) were described previously. *Tlr2*^{-/-} and *Tlr4*^{-/-} mice were provided by Shizuo Akira (Osaka University, Osaka, Japan). Mice were kept under specific pathogen-free conditions and used at 8–16 weeks of age. All experiments were in accordance with protocols approved by the Animal Care and Use Committee of Fukuoka University and RIKEN.

Islet isolation and transplantation. Islets were isolated (30, 31) and transplanted into the liver via the portal vein (32) of STZ-induced (180 mg/kg; Sigma-Aldrich) diabetic mice at 3 days after the injection of STZ. The non-fasting plasma glucose levels of mice were measured using a Beckman glucose analyzer (Beckman Japan).



Immunohistochemistry. The pancreas of naive mice, isolated islets, and the liver of transplant recipients were fixed in 10% formaldehyde solution, processed, and embedded in paraffin. The sections were stained immunohistochemically with anti-mouse insulin antibody (Novocastra) and rabbit anti-bovine HMGB1 antibody (Shino-Test Co.) by a streptavidin-biotin-peroxidase complex method (33).

HMGB1 and cytokine measurement. HMGB1 levels in mouse serum and in the culture medium of isolated islets was measured with an ELISA kit (Shino-Test Co.) (34). IFN- γ concentrations in the culture supernatant of liver MNCs were determined by FACS with cytometric beads assay (CBA) (BD Biosciences). IL-12 concentration in the medium was measured by ELISA (Endogen).

For measurement of tissue concentration of HMGB1, individual tissues (1–2 mg wet weight/organ), isolated islets (200 total), and FACS-sorted cells (2×10^5 to 6×10^5) of each population in the liver of mice were sonicated in PBS. Then, the resulting tissues were treated as reported by Sanders (35) in which perchloric acid (HClO₄) was added to the homogenates with a concentration of 0.75 M. The content of HMGB1 in the solution was measured with ELISA after the adjustment of pH to 7.0 as well as the appropriate dilution with PBS containing 1% bovine calf serum. The sonicated tissues were also used for measuring DNA content with a Wako assay kit.

Reagents. Bovine HMGB1 was purchased from Shino-Test Co. Bovine HMGB1 was extracted from the bovine thymus and further purified by CM-Sephadex C25 ion column chromatography according to the method described by Sanders (35). The biological activity of purified HMGB1 was reported elsewhere (36). Anti-HMGB1 antibody was purchased from Shino-Test Co. This is a polyclonal antibody made by immunizing chicken with purified bovine HMGB1, and the neutralizing effect of the anti-HMGB1 antibody was reported previously (37, 38). Goat anti-mouse IL-12 antibodies and rabbit anti-mouse CD40L antibody were purchased from BD Biosciences and Sigma-Aldrich, respectively. Recombinant mouse IFN- γ , TNF- α , IL-1 β , and IL-10 were purchased from Sigma-Aldrich.

Flow cytometry. Antibodies used for flow cytometric analysis were as follows: anti-mouse Fc γ II/III (2.4G2), FITC- or Pacific blue-conjugated anti-CD3 ϵ (145-2C11), FITC- or PerCP-Cy5.5-conjugated anti-CD11b (M1/70), allophycocyanin-conjugated (APC-conjugated) anti-IFN- γ (XMG1.2), peridinin-chlorophyll protein- (PerCP-) or FITC-conjugated anti-Gr-1 (RB6-8C5), PE-conjugated NK1.1 (PK136), PE-Cy7-conjugated CD19 (1D3), biotinylated anti-CD11c (N418), APC-Cy7-conjugated avidin, APC-conjugated anti-F4/80 (BM8) (BD Biosciences or eBioscience), PE- or APC-labeled α -GalCer-loaded CD1d dimer was prepared as described (39). Intracellular cytokine staining was performed as previously described (39). Flow cytometry was performed using a FACSCalibur and FACSaria (BD) with FlowJo software (Tree Star). The purity of sorted cells was usually greater than 99%.

Cell preparation and culture. Liver MNCs were prepared as described previously (40). For in vitro culture, liver MNCs and those of FACS-sorted cells were cultured in RPMI medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (Biosource) and 100 μ g/ml kanamycin (Meiji Seika) and isolated islets in DMEM medium (Nissui) supplemented with 2% BSA (Sigma-Aldrich) and 100 μ g/ml kanamycin in a CO₂ incubator (95% air plus 5% CO₂) at 24°C or 37°C.

Quantitative real-time PCR. Total RNA was isolated from FACS-purified cell populations using TRIzol reagent (Invitrogen). cDNA was prepared by Superscript III RNase H⁻ Reverse Transcriptase with random hexamers (Invitrogen). Quantitative real-time PCR was performed with SYBR GreenER qPCR Super Mix (Invitrogen) for ABI PRISM 7900HT (Applied Biosystems). Total mRNA from cells (2×10^5) was used as templates to analyze expression levels of Tlr2, Rage, or Hpirt. Gene-specific primer sequences were as follows: Tlr2-fw, GGGGCTTCACTTCTCTGCTT, Tlr2-rv, AGCATCCTCTGAGATTTGACG; Rage-fw, 5'-GTGTCGGGCAAC-TAACAGG-3', Rage-rv, 5'-CTGGCTTCCCAGGAATCTG-3'; Hpirt-fw, 5'-TCCTCCTCAGACCGCTTTT-3', Hpirt-rv, 5'-CCTGGTTCATCATCGCTA-ATC-3'. Quantitative analysis was performed by the $\Delta\Delta$ Ct method by using Hpirt as an internal control.

Statistics. The statistical significance of differences was determined by 1-tailed Student's *t* test. Values were expressed as mean \pm SD from independent experiments. Any difference with a *P* value less than 0.05 was considered significant.

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

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ABSTRACT

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

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

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the demand. Xenotransplantation using pig islets could potentially resolve the shortage of donor islets. Pigs are the favored donor species because of (1) their similar physiology to human beings; (2) unlimited availability owing to a short generation interval because of the high number of pregnancies; and (3) somatic cloning is possible, and, thus, production of transgenic animals can be substantially enhanced.² In particular, pig islets have precise glyco-biologic advantages because they lack α -gal epitopes, which induce complement activation, hyperacute rejection, and acute vascular rejection of xenografts. Therefore, successful short-term xenograft survival of these islets is the result of prevention of hyperacute rejection. However, after initial survival of pig islets, infiltrating cells into pig islet xenografts, including natural killer cells, macrophages, and CD8+ CTLs, are directly cytotoxic to the islets.³⁻⁶ In previous studies, we reported that direct cytotoxicity of human CD8+ CTLs to pig islets is mediated in major part by the Fas/FasL apoptotic pathway.⁴⁻⁶ Cellular FLICE inhibitory protein (c-FLIP) was originally identified as an inhibitor of death-receptor signaling through binding competition with caspase-8 for recruitment to the Fas-associated via death domain.⁷⁻⁹ Two major c-FLIP variants result from alternative messenger RNA splicing: a short 26-KDa protein (c-FLIP_S) and a long 55-KDa form (c-FLIP_L).⁷⁻⁹ We have demonstrated that the overexpression of *c-FLIP_{S/L}* genes markedly suppresses human CD8+ CTL-mediated xenocytotoxicity. In addition, the cytoprotective effects of c-FLIP_L seem to be significantly stronger than those of c-FLIP_S.⁶ The cytoprotective effect of c-FLIP_L in pig islet cells remains controversial. Accordingly, in the present study, we evaluated the cytoprotective activities of the c-FLIP_L molecule by adenovirus-mediated gene expression in pig islets.

MATERIALS AND METHODS

Pig Islet Isolation

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

Construction of Adenovirus Vector

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

Transduction of Pig Islets by Adenovirus Vector

Freshly obtained adult pig islets represented in 500 μL of serum-free medium 199 were exposed to adenovirus encoding human

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

Generation of Human CD8+ CTLs

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

In Vitro Cytotoxicity Assay

The cytotoxic activity of human CD8+ CTLs against pig islets was assessed using a chromium 51 (⁵¹Cr) release assay.¹³ Either parental pig islets, mock islets, or transfected pig islets were incubated with $\text{Na}_2^{51}\text{CrO}_4$ for 24 hours (1 μCi of ⁵¹Cr per 100 islets). Subsequently, ⁵¹Cr-labeled pig islets were plated in 96-well plates as target cells for admixture with human CTLs isolated using magnetic beads at various effector-to-target ratios. The ⁵¹Cr released from dead islet cells was measured in the supernatants.

Transplant Studies and Immunohistochemical Analysis

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

Statistical Analysis

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

RESULTS

Protein Expression of Human c-FLIP_L in Pig Islets

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

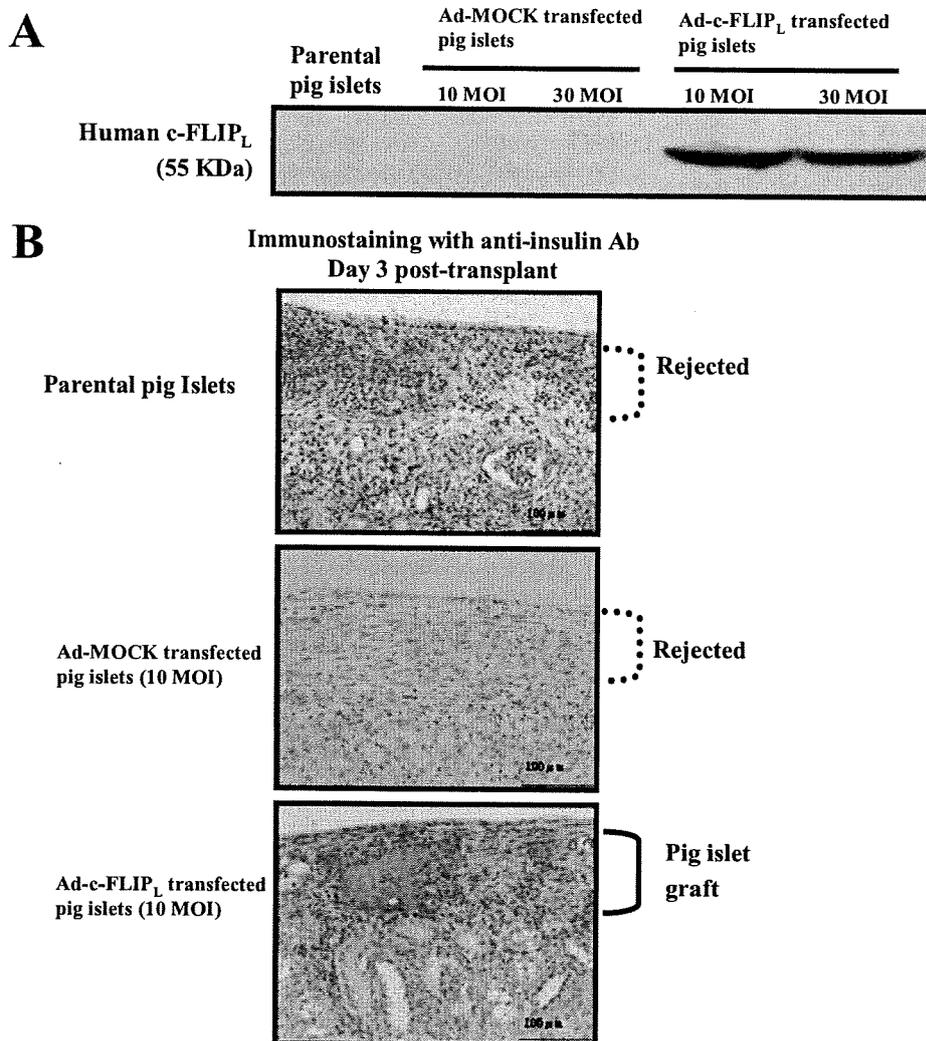


Fig 1. (A) The intracellular expression of c-FLIP_L protein. Adenovirus-mediated gene expression of human c-FLIP_L protein was assessed by Western blot analysis. **(B)** Immunohistological findings of rat kidney tissue of pig islets transplanted rats. Immunostaining with anti-pig insulin Ab for transplanted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

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

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

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

Prolonged Xenograft Survival Was Elicited From c-FLIP_L-Transfected Pig Islet Cells

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

Table 1. ⁵¹Cr Release in Pig Islets

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

Abbreviations: ⁵¹Cr, chromium 51; E/T, effector-target; cFLIP_L, cellular FLICE-like inhibitory protein, long form; MOI, multiplicity of infection.

Amelioration of human CD8+ cytotoxic T lymphocyte-mediated cytotoxicity by transduced pig islets was assessed by ⁵¹Cr release assay. Control parental and mock pig islets were estimated at the E/T Ratio of either 25:1 or 50:1. Values are given as the mean (SD) from five independent experiments.

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

DISCUSSION

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

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

In this pig islet transplant model, large infiltrations of both CD8+ T cells and macrophages were detected. A large number of macrophages infiltrating pig islet xenografts secrete inflammatory cytokines including IL-1 β , tumor necrosis factor- α , and interferon- γ , which may induce β -cell damage through activation of several intracellular stress-signaling pathways.¹⁶ Our preliminary data suggest

that pig islet cells expressing c-FLIP_L induce resistance against cytokine exposure containing 100 U/mL of IL-1 β , 1000 U/mL of tumor necrosis factor- α , and 1000 U/mL of interferon- γ , as assessed using both the tetramethylrhodamine ethyl ester assay and the colorimetric methyl tetrazolium salt Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega Corp, Madison, Wisconsin). Future experiments will be required to further confirm the role of c-FLIP_L expression in pig islet cells.

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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 rapamycin 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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