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Prevention of Early Loss of Transplanted Islets in the Liver of Mice by Adenosine

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Background. The low efficiency of islet transplantation necessitating sequential transplantations with the use of 2 to 3 donors for a recipient has been a major obstacle facing clinical islet transplantation. We determined whether adenosine has any beneficial effects on preventing early loss of transplanted islets in the liver, thereby facilitating successful islet transplantation from one donor to one recipient in mice.

Methods. Two hundred islets, the number of islets from a single mouse pancreas, were grafted into the liver of streptozotocin-induced diabetic C57BL/6 mice. Adenosine was administered once at the time of islet transplantation. Mononuclear cells in the liver of mice receiving islets were isolated and examined by flow cytometry.

Results. A single injection of adenosine at the time of transplantation ameliorated hyperglycemia of diabetic mice receiving 200 syngenic islets with suppression of interferon (IFN)-γ production of hepatic NKT cells and neutrophils, while that of control did not. The IFN-γ production of NKT cells and neutrophils in the liver of mice treated with α-galactosylceramide, a synthetic ligand of NKT cells was suppressed by adenosine. The beneficial effect of adenosine was also observed for BALB/c islet allografts when alloimmune rejection was prevented by anti-CD4 antibody.

Conclusions. Adenosine suppresses the NKT cell-mediated IFN-γ production of neutrophils in the liver of mice receiving islets, thus leading to prevention of early loss of transplanted syngenic and allogenic islets. The findings indicate that adenosine may improve efficiency of clinical islet transplantation.

Keywords: Islet transplantation, Adenosine, Early graft loss, NKT cells, Neutrophils.

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Pancreatic islet transplantation has now become a procedure of choice for the treatment of insulin-dependent diabetes mellitus (*1*). Currently, however, pancreatic islet transplantation has limited success in achieving insulin independence of a diabetic patient after transplantation of islets from a single donor (*2*), and therefore sequential transplantations of islets with the use of 2 to 3 donor pancreases are required for the treatment of a single recipient (*1*). Therefore, the inability to produce successful islet transplantation from one donor to one recipient has been a major obstacle facing clinical islet transplantation.

The necessity of sequential transplantations of islets to achieve insulin independence from a single donor after transplantation may imply that transplanted islets are lost after transplantation and that the amount of insulin produced from transplanted islets is not enough to maintain glycemic control without an exogenous insulin treatment. Ryan et al. (*3*) recently reported that an islet graft mass of insulin-independent diabetic patients after having received sequential transplantations of islets is only 36% even though they received a total of 1 million islets, which is the equivalent number of islets in a single human pancreas. Therefore, the islet graft loss after transplantation seems to be a major limiting factor for successful islet transplantation.

There are several major factors responsible for the islet graft loss after transplantation including the toxic effects of calcineurin inhibitors as immunosuppressive agents such as FK506 and cyclosporine A (*4*) and of sustained hyperglycemia (*5*) on transplanted islets and allo- and auto-immune rejection. We have previously shown another novel mechanism that is involved in islet graft loss after transplantation, namely an early loss of transplanted islets within 24 hr after transplantation, in which NKT cell-dependent interferon (IFN)-γ production of Gr-1^+CD11b$^+$ cells (neutrophils) plays an essential role (*6*). These previous findings indicate that NKT cells and Gr-1^+CD11b$^+$ cells may be targets for intervention to improve efficiency of islet transplantation.

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Adenosine is a purine nucleoside and is emerging as a key regulatory molecule which has anti-inflammatory, anti-coagulatory, and pro-angiogenic effects in hypoxic and inflamed tissues (*7*). From an immunological point of view, adenosine has been reported to attenuate neutrophil and macrophage functions (*8, 9*), and it is a potent inhibitor of T cell-mediated immune responses (*10, 11*). Recently, adenosine has been found to be a key molecule that is responsible for the suppressive function of regulatory T cells (*12*). Moreover, adenosine has been reported to have an inhibitory effect that is mediated by NKT cells on the ischemia-induced reperfusion injury of the liver in mice (*13*).

On the basis of these previous reports, we hypothesize that adenosine may have a beneficial effect on preventing early loss of transplanted islets by improving the efficiency of islet transplantation because the NKT cell-mediated IFN-γ production of Gr-1$^+$CD11b$^+$ cells (neutrophil) is an essential component of the early loss of transplanted islets (*6*) and because adenosine is expected to have an inhibitory effect on NKT cells. Importantly, adenosine and an adenosine transporter inhibitor that increases the extracellular levels of adenosine such as dipyridamole have already been used in the clinical practice for the examination and the treatment of heart function and disease, respectively. Therefore, the safety issue related to the clinical use for islet transplantation has already been cleared.

The present study demonstrates that adenosine and an adenosine transporter inhibitor, dipyridamole can suppress the NKT cell-mediated IFN-γ production of Gr-1$^+$CD11b$^+$ cells, enabling islet transplantation from one donor to one recipient in mice, and thus suggesting that adenosine may improve the efficiency of clinical islet transplantation.

MATERIALS AND METHODS

Animals

Male BALB/c (H-2d) and C57BL/6 (H-2b) mice were purchased from Charles River Japan (Kanagawa, Japan) and used for the experiments. Because the severity of diabetes made with streptozotocin (STZ) injection differed depending on the weight of mice, only the mice weighing 23 to 25g were used as recipients. Mice weighing 25 to 30g served as donors. Diabetes was induced in the recipients by the intravenous injection of STZ (180 mg/kg) (Sigma, St. Louis, MO). The plasma glucose levels of the mice exceeded 400 mg/dL at 2 to 3 days after the STZ injection, and the mice remained hyperglycemic at the time of islet transplantation. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of Fukuoka University.

Islet Isolation and Transplantation

Islets were isolated by the static digestion method using collagenase (*14*) and then separated by centrifugation using Ficoll-Conray gradients (*15*). Islets of 150 to 250 μm in diameter were hand-selected using Pasteur pipette with the aid of a dissecting microscope, because it was critical to minimize the size variation of individual islets to compare the effects of the difference in the number of donor islets. The size of individual islets in each islet isolation procedure was confirmed by using a phase-contrast microscope equipped with a scale in the eyepiece. Hand-picked islets were transplanted into the liver through the recipient's portal vein (*16*) at 3 days after the induction of diabetes with STZ injection.

Monitoring Plasma Glucose and Body Weight

The nonfasting plasma glucose levels and body weight were monitored three times a week in all the recipients for 60 days after islet transplantation. The plasma glucose was measured using a Beckman glucose analyzer (Beckman Japan, Tokyo, Japan). Normoglycemia after transplantation was defined as two consecutive plasma glucose levels with a reading below 200 mg/dL.

Administration of Adenosine, α-Galactosylceramide, Dipyridamole, Nitrobenzylthioinosine, and Anti-CD4 Antibody

Adenosine (Daiichi Pharmaceutical Co., Tokyo, Japan) and dipyridamole (Boehringer Ingelheim, Ingelheim, Germany) were dissolved in saline and were administered intravenously; nitrobenzylthioinosine (NBTI) (Sigma Aldrich, St. Louis, MO) was dissolved in 10%DMSO and administered intraperitoneally (IP) into appropriate groups of diabetic recipient mice, once at the time of islet transplantation.

α-Galactosylceramide (α-GalCer) was a generous gift from Dr. Masaru Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and was administered intravenously to naïve mice.

Anti-CD4 antibody (200 μg/injection/mouse, YTS177, rat IgG1; R&D, Minneapolis, MN) was administered IP to appropriate groups of diabetic recipient mice receiving allogenic islets into the liver.

Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance test (IPGTT) was performed in recipient mice at 60 days after the islet transplantation. The mice were fasted for 8 hr before the start of the examination. Blood samples were obtained from the orbital sinuses of recipient mice at 0, 30, and 120 min after the IP injection of glucose (1 g/kg body weight), and the plasma glucose was measured as previously described.

Morphological Study

The livers bearing islet grafts and pancreas were examined morphologically at 60 days after transplantation in appropriate groups of mice, and the pancreases of recipient mice were also examined simultaneously. The liver and pancreas were fixed with Bouin's solution, processed, and then were embedded in paraffin. The sections were prepared for light microscopy and stained with hematoxylin-eosin, and aldehyde and fuchsin.

Preparation of Hepatic Mononuclear Cells

Hepatic mononuclear cells (MNCs) were prepared as described previously (*17*). In brief, an excised liver was pressed through a stainless steel mesh, and the resulting dissociated liver tissues were suspended in Dulbecco's modified Eagle medium (D-MEM/F-12, Life Technologies, Tokyo, Japan) and washed twice. The mixture was resuspended in an

isotonic 33% Percoll solution containing heparin (67 U/mL) and centrifuged at 2000*g* 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 FcRγIII/II mAb, 2.4G2), fluorescein isothiocyanate-conjugated anti-CD3ε (clone 145–2C11, American Hamster IgG1κ), 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-FcRγIII/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

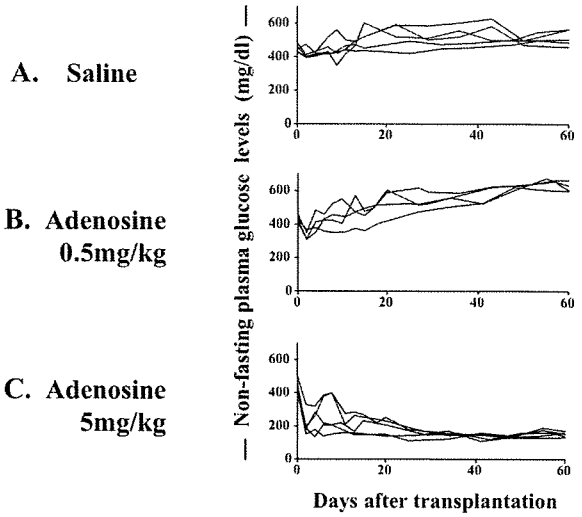

FIGURE 1. Plasma glucose levels of streptozotosin-induced diabetic mice receiving 200 syngenic islets into the liver. Diabetic mice receiving 200 syngenic islets were treated with saline (A), 0.5 mg/kg (B), or 5 mg/kg adenosine (C) once at the time of islet transplantation. Individual line represents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

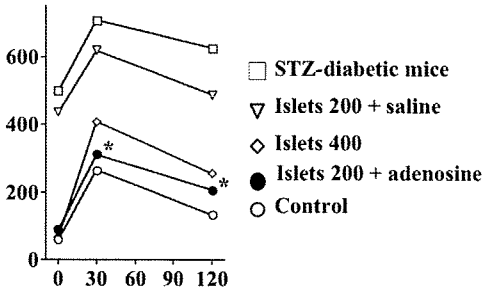

FIGURE 2. Intraperitoneal glucose tolerance test of diabetic mice receiving syngenic islets at 60 days after transplantation. Recipient mice were fasted for 8 hr before the examination, and plasma glucose levels were determined at 0, 30, and 120 min after the glucose injection (intraperitoneally, 1 g/kg). Experimental groups included diabetic mice without islet transplantation (□, n=4), those receiving 400 islets without treatment (◇, n=5), 200 islets treated with adenosine (5 mg/kg) (●, n=5), or saline (▽, n=4). Untreated mice served as control (○, 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

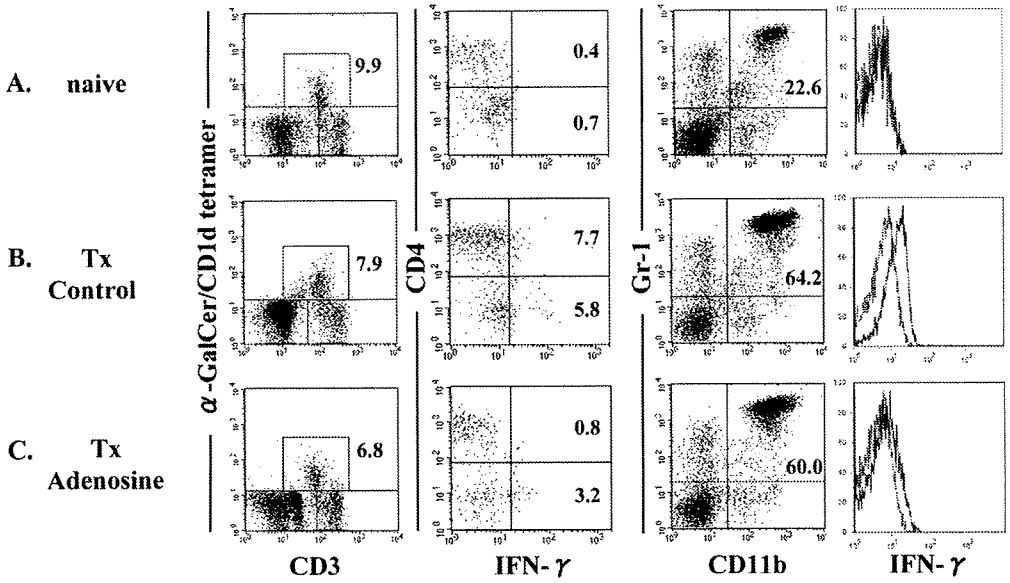

FIGURE 3. Flowcytometry of mononuclear cells in the liver of mice. Mononuclear cells in the liver of diabetic mice receiving 200 syngenic islets and treated with saline (B) or adenosine (5 mg/kg) (C) were isolated at 6 hr after islet transplantation and examined by the flow cytometry. NKT cells defined 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 suppressed 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 μg/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.

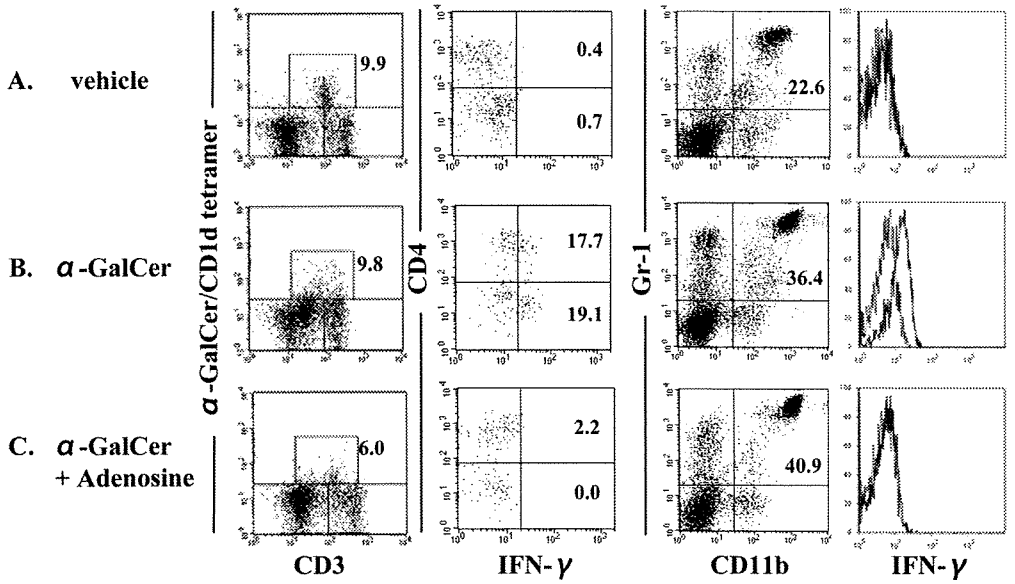

FIGURE 4. Flow cytometry of mononuclear cells in the liver of mice treated with intravenous administration of α-galactosylceramide (α-GalCer). Mononuclear cells in the liver of mice treated with (C) or without adenosine (5 mg/kg) (B) were isolated at 2 hr after the intravenous injection of α-GalCer (10 μg/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, 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 though the activation of neutrophils, because the IFN-γ production of neutrohils 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

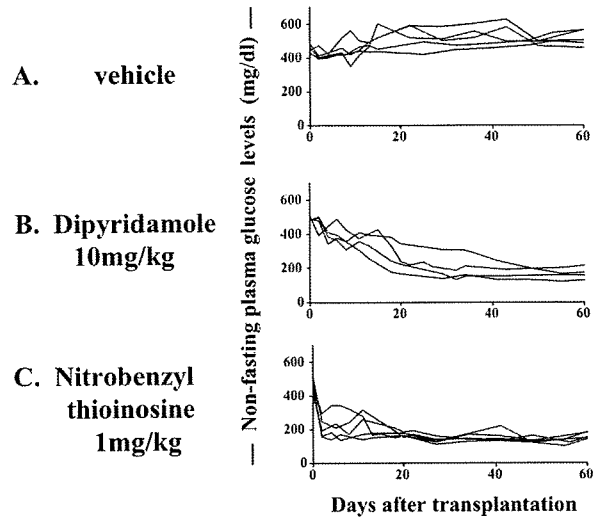

FIGURE 5. Beneficial effects of dipyridamole and nitrobenzylethioinosine on prevention of early loss of transplanted islets. Individual line represents the nonfasting plasma glucose levels of each diabetic mouse receiving 200 syngenic islets and treated with vehicle (A), dipyridamole (10 mg/kg) (B),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

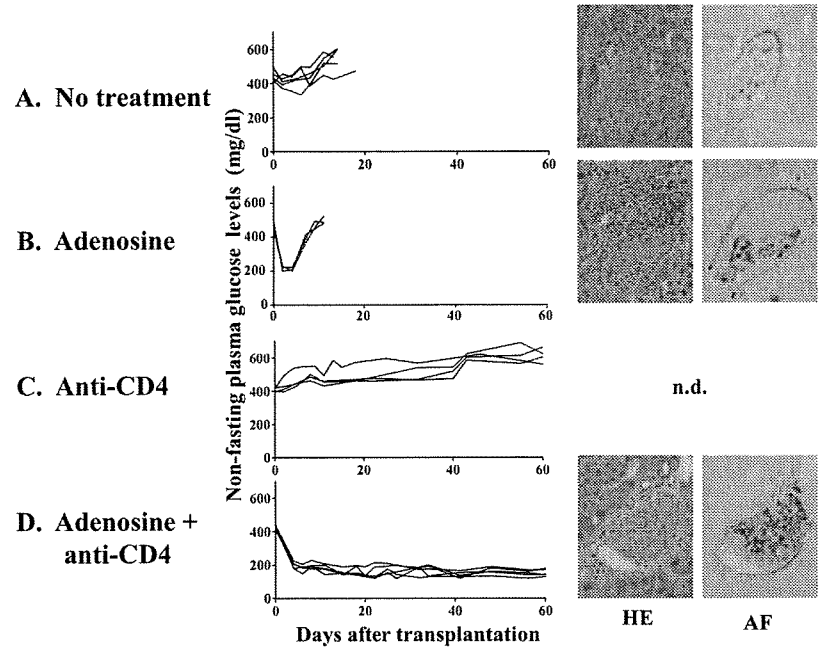

FIGURE 6. Early loss of transplanted allogenic islets was prevented by adenosine. The plasma glucose levels of diabetic mice receiving 200 allogenic islets and treated with saline or adenosine (5 mg/kg, administered intravenously) are shown in groups A or B, respectively. In groups D or C, recipient 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 ×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 A2A 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 Vα14 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 prevented 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

Authorship note: Nobuhide Matsuoka, Takeshi Itoh, and Hiroshi Watarai contributed equally to this work.

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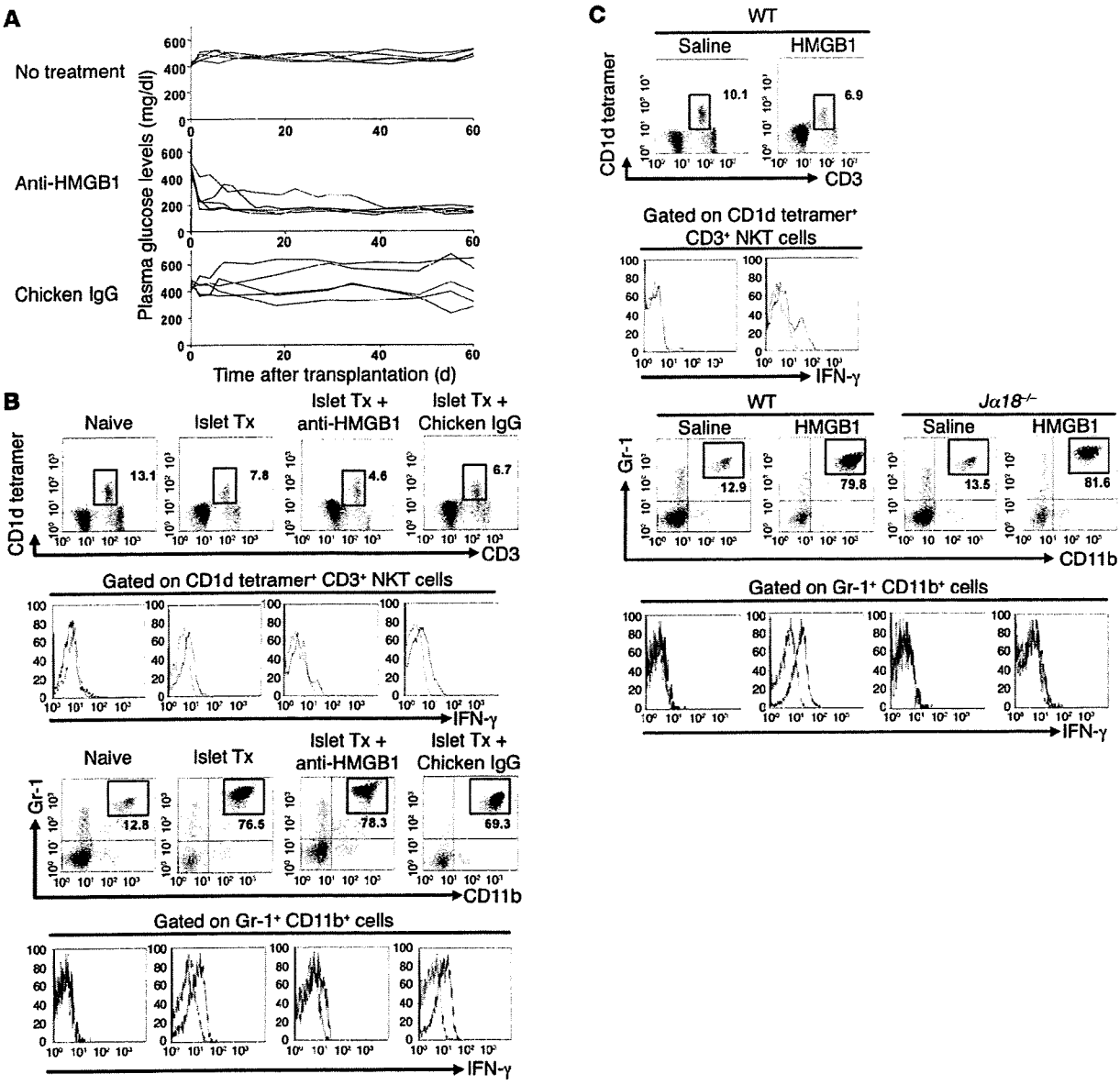

Figure 1

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 syngenic 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 μg/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

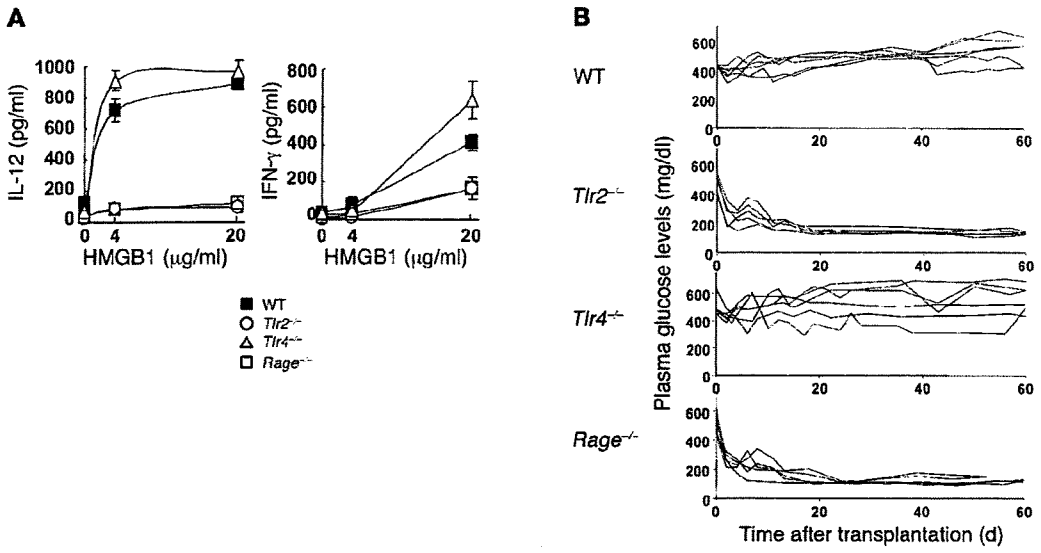

Figure 2

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 syngenic 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 Jα18$^{-/-}$ 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 Jα18$^{-/-}$ 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 syngenic 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-

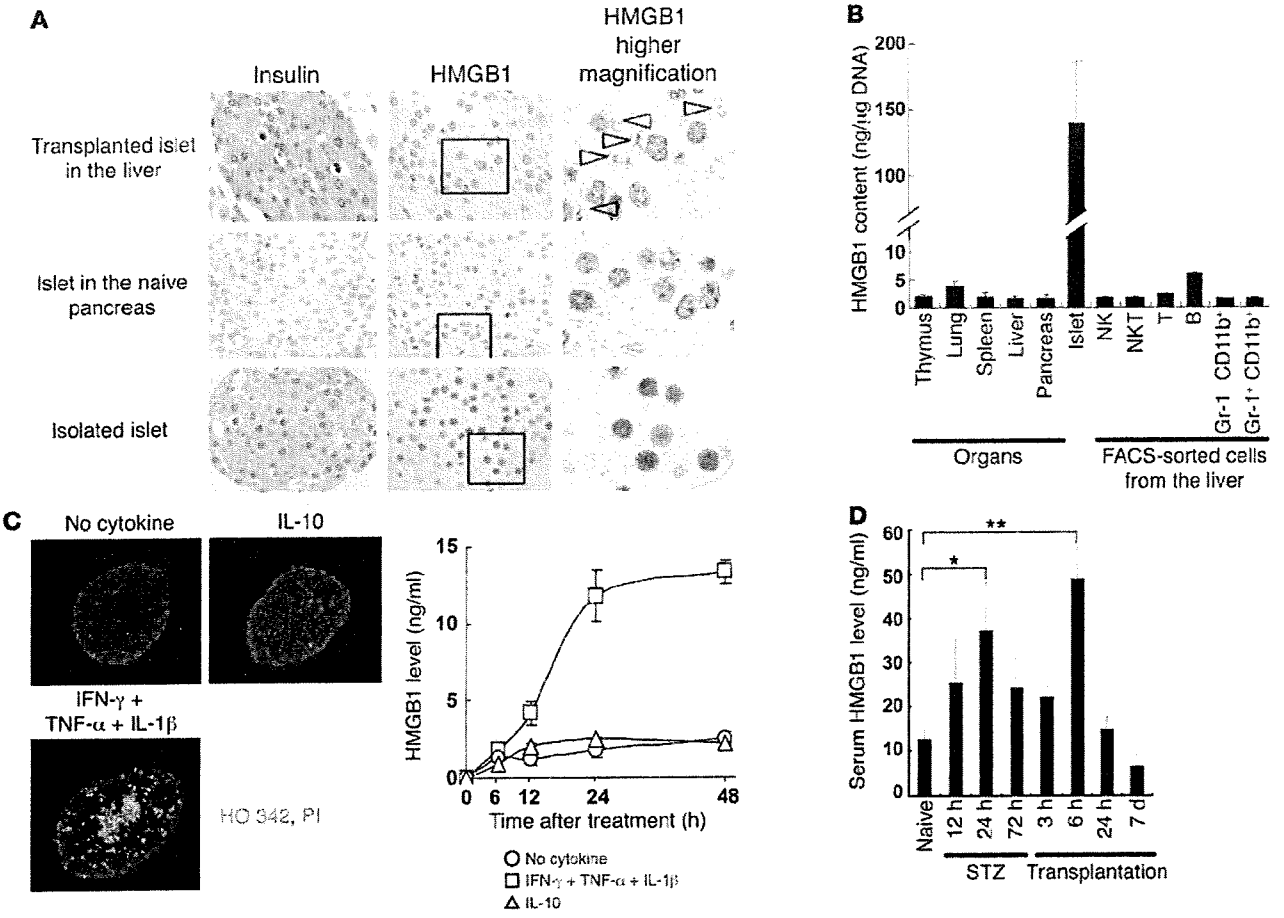

Figure 3

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: ×100 (first and second columns) and ×800 (third column). Boxed regions in the second column were enlarged. (**B**) HMGB1 contents (ng/μg 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, ×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-1β (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 ± 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 ± 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-1β, 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

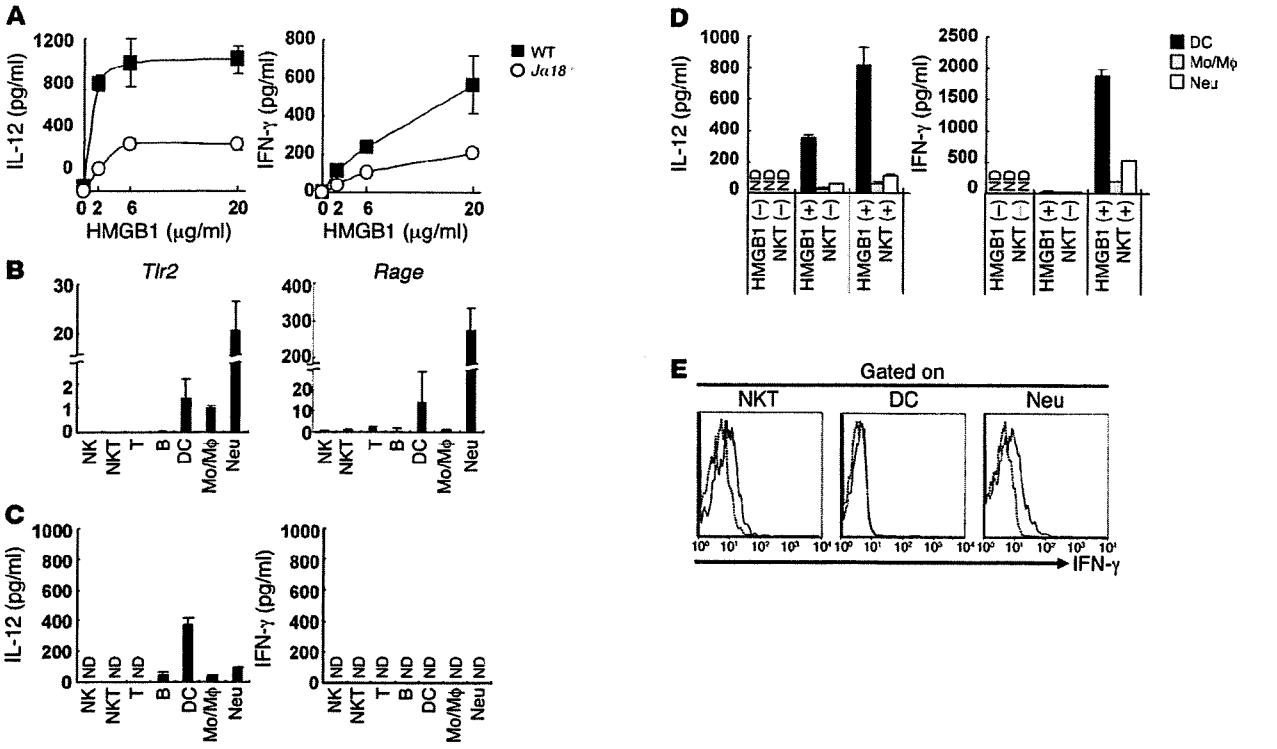

Figure 4

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 ΔΔCt method using the expression level in Mo/Mϕ 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 μg/ml). The amounts of IL-12 and IFN-γ were measured by CBA ($n = 3$). (**D**) Cytokine production by DCs, Mo/Mϕ, or Neu in the presence of NKT cells. FACS-sorted Gr-1$^-$CD11b$^+$CD11c$^+$ DCs, Gr-1$^-$CD11b$^+$CD11c$^-$ Mo/Mϕ, 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 μg/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 μg/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/Mϕ) (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/Mϕ 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/Mϕ (Figure 4C). However, IFN-γ production was not detected in any individual cell population among all liver MNC subpopulations tested (Figure 4C).

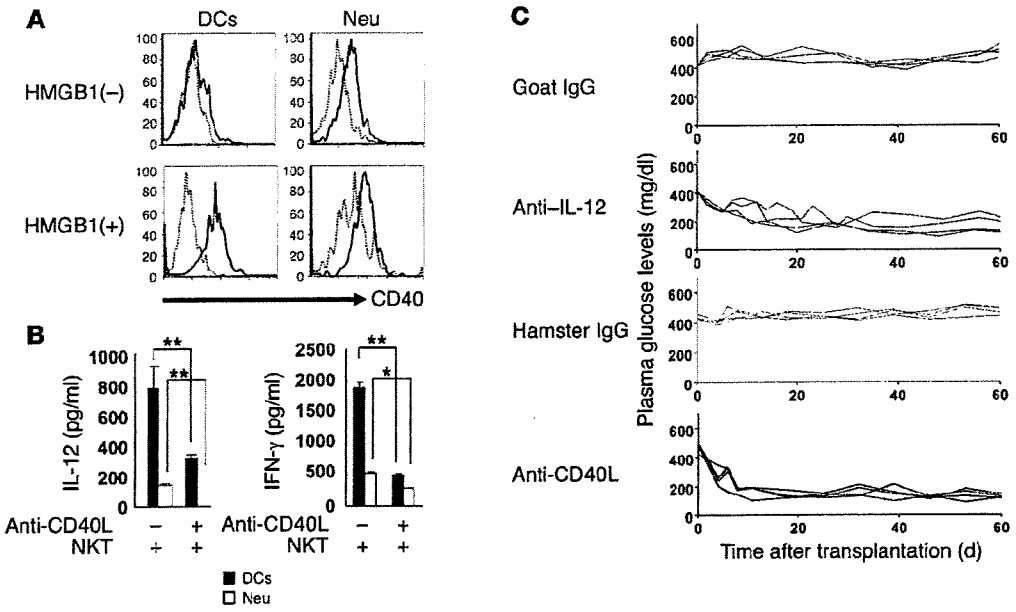

Figure 5

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 μg/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 μg/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 ± 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 μg 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/Mϕ, 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 syngenic 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/Mϕ, 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/Mϕ, 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-1β, 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, subcutaneum — 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. Jα18-deficient mice were generated previously (28) and backcrossed more than10 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 nonfasting 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 ($HClO_4$) 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-1β, and IL-10 were purchased from Sigma-Aldrich.

Flow cytometry. Antibodies used for flow cytometric analysis were as follows: anti-mouse FcRγII/III (2.4G2), FITC- or Pacific blue–conjugated anti-CD3ε (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 μg/ml kanamycin (Meiji Seika) and isolated islets in DMEM medium (Nissui) supplemented with 2% BSA (Sigma-Aldrich) and 100 μg/ml kanamysin in a CO_2 incubator (95% air plus 5% CO_2) 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 SuperMix (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 Hprt. Gene-specific primer sequences were as follows: Tlr2-fw, GGGGCTTCACTTCTCTGCTT; Tlr2-rv, AGCATCCTCTGAGATTTGACG; Rage-fw, 5′-GTGTCGGGCAACTAACAGG-3′; Rage-rv, 5′-CTGGCTTCCCAGGAATCTG-3′; Hprt-fw, 5′-TCCTCCTCAGACCGCTTTT-3′; Hprt-rv, 5′-CCTGGTTCATCATCGCTAATC-3′. Quantitative analysis was performed by the ΔΔCt method by using Hprt as an internal control.

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

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膵島移植拒絶反応とNKT細胞

Novel roles of NKT cells in rejection of pancreatic islet transplantation

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◎著者らは膵島移植の研究を通して，NKT細胞の異種，同種ならびに自然免疫拒絶反応における役割を明らかにしている．いずれも臨床膵島移植の課題を解決し，成績向上をめざすトランスレーショナルリサーチの成果である．とくにNKT細胞が関与する自然免疫拒絶反応の研究は，現在の臨床膵島移植が直面する課題，移植膵島障害の新規制御法開発に直結し，臨床膵島移植のブレイクスルーとなる可能性がある．今後の方向性として，膵島移植に関連してNKT細胞の内因性リガンド発見を含めたNKT細胞活性化機序の解明をめざし，研究を進めている．

Key word：ナチュラルキラーT(NKT)細胞，インスリン依存糖尿病，膵島移植，拒絶反応

インスリン依存糖尿病(insulin-dependent diabetes mellitus：IDDM)の新規治療法として，膵島移植の臨床応用が開始されている[1]．膵島移植は細胞移植であり，侵襲の少ない治療法として今後の発展が期待されているが，解決すべき課題は多い．現在の臨床膵島移植でもっとも重要な課題は細胞移植特有の拒絶反応による移植膵島障害の制御であり，そのメカニズム解析に基づく治療法開発が求められている．著者らは，膵島移植部位(肝)の免疫担当細胞の解析より，NKT細胞を介したあらたな拒絶反応機序，ならびにその制御法を見出した．本稿では，膵島移植拒絶反応におけるNKT細胞の役割と今後の方向性について言及する．

インスリン依存糖尿病と移植医療

IDDMは，生体内で唯一の血糖降下ホルモンであるインスリンを産生する細胞である膵島β細胞の欠落によって発症する疾患であり，インスリンを注射で投与する治療法が行われている．しかし，インスリン治療では十分な血糖管理ができず，治療に難渋する例がある．とくに低血糖が問題で，発汗，手指振戦，頭痛などの低血糖症状を自覚できない病態，自律神経障害による突然の低血糖意識障害により，生命予後に重大な影響を及ぼすことがある．さらには血糖管理が不十分な状態が長期に持続すると，合併症(網膜症，腎症，神経症)を発症する．

このようなIDDMを対象としてインスリン産生細胞の移植が行われる(「サイドメモ」参照)．膵島β細胞には血糖に対するセンサーがあり，血糖を生理的範囲に調節している．したがって，インスリン産生細胞を移植すると移植膵島が血糖調節を行い，レシピエントの血糖は移植後ただちに正常となり，低血糖発作は消失，さらには合併症の防止，改善が期待できる．

膵島移植の実際と課題

膵島移植は欧米では脳死ドナー，わが国では心停止ドナーより提供された膵臓より膵島を単離し，移植に用いられる．移植の実際は，局所麻酔下，超音波ガイド下に肝臓内血管(門脈)を穿刺，カテーテルを留置し，点滴の要領でドナー膵島を

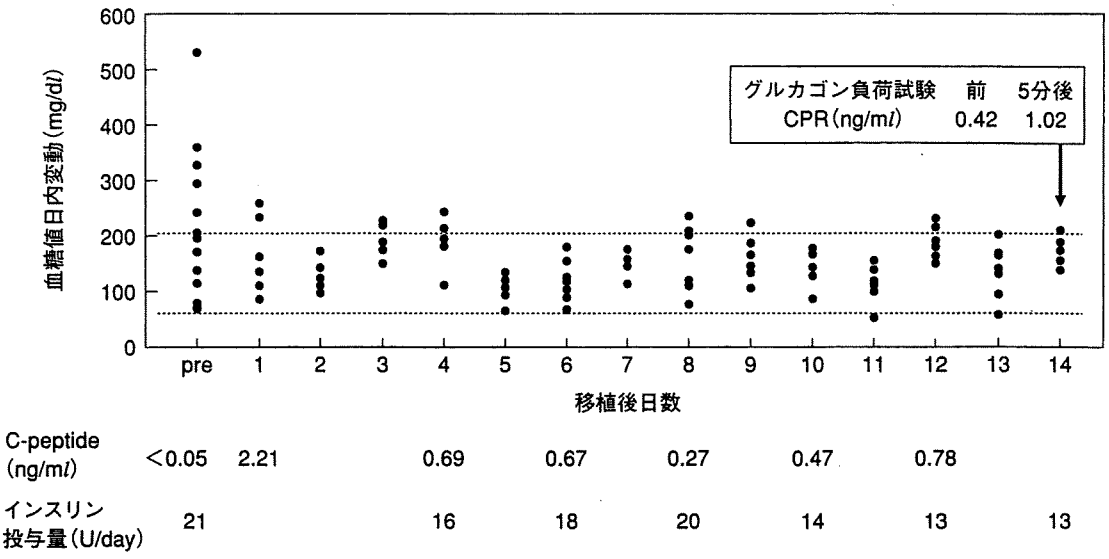

図 1　福岡大学症例の膵島移植後臨床経過

移植前には測定感度以下であったレシピエント血中 C-ペプチドが膵島移植直後より認められ，血糖値はほぼ正常範囲となっている．また，負荷試験による C-ペプチド上昇は移植膵島が機能していることを示している．

注入(移植)する．移植膵島は，門脈末端に塞栓後生着，機能する．その結果，レシピエントの血糖は移植直後より正常範囲となり安定する[1](図 1)．しかし，インスリン治療より離脱するには，引き続き 2〜3 回の移植，すなわち 2〜3 人のドナーが必要である[1]．さらには移植膵島機能は残存しているものの，時間の経過とともに再度インスリン治療が必要になる[2]．

膵島移植レシピエントの解析より，健常人と同等数の膵島移植を受けたにもかかわらず，移植膵島の 36%しか生着していないことが判明し，何らかの原因で早期より移植膵島が障害され，喪失していることが示された．このように臨床膵島移植の現在のもっとも重要な課題は，移植膵島障害の機序を解明し，あらたな治療法を見出すことにあるといっても過言ではない．

サイドメモ

膵移植と膵島移植

インスリン産生細胞移植には膵臓器移植と膵島細胞移植がある．ヒトの膵臓は重さ約 100 g で 100 万個の膵島が存在する．1 個の膵島は大きさ平均(径)150〜250 μM で約 2,000〜3,000 個の細胞からなり，その 60〜70%がインスリン産生(β)細胞である．重要な点は，膵島は膵臓の約 1〜2%(容積)を占めるにすぎず，98%以上は外分泌細胞である．このように，膵臓器移植では 1〜2%の膵島を移植するために膵臓全体を移植している．一方，目的とする膵島のみを取り出し移植に用いるのが膵島細胞移植である．膵臓器移植は全世界で 2 万例以上行われ，すでに確立した医療となっている．膵島移植は 1973 年，Lacy らにより糖尿病が膵島移植により根治できることがはじめて実験的に明らかにされ，臨床応用への研究が開始された．著者は 1980 年に Lacy 研に加わり，臨床膵島移植を目的とした大動物膵島単離法の開発を進めた．1990 年に同グループにより第一例目の膵島移植が実施され，2000 年になり，カナダのグループにより膵島移植の成功例が報告された．わが国では現在まで 18 例の膵島移植が行われ，2004 年に京大グループが第一例目を，福岡大学では 2006 年に実施した．

移植膵島障害とNKT細胞

移植膵島障害の成因としては，同種拒絶反応，自己免疫拒絶反応，免疫抑制剤であるカルシニューリンインヒビター(FK506，cyclosporine A)による膵島(β細胞)障害などがよく知られているが，著者らは移植早期の自然免疫による膵島障害に着目し，あらたな移植膵島障害の機序，ならびに制御法を見出した[3]．

膵島移植は細胞移植であるがゆえに，種々の部位に移植可能である．臨床膵島移植ではインスリ