ORIGINAL

Donor-specific tolerance induced by simultaneous allogeneic islet transplantation with $CD4^+CD25^+$ T-cells into hepatic parenchyma in mice

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Abstract : *Background*. The allogeneic islets transplantation is an ideal therapeutic strategy for patients with diabetes mellitus. However, it has been difficult to induce immunological tolerance against islets grafts. The $CD4^+CD25^+$ regulatory T-cells (Treg) play a role in suppressing T-cell activation. Thus, we evaluated whether Treg can regulate donor-specific T-cell tolerance that received allogeneic islets into the hepatic parenchyma (ITxHP) along with Treg.
***Methods*. C3 H/He mice were used as donors ; and streptozotocin-induced diabetic BALB/c mice were recipients. The protocol included three groups : Group A recipients received only 300 IE islets ; Group B was given 300 IE islets and whole splenocytes ; Group C was given 300 IE islets and Treg purified from peripheral lymph nodes.**
***Results*. For all mice in Groups A and B, the fasting blood sugar exceeded 250mg/dl and graft rejection was observed. GVHD was observed earlier in Group B than in Group A. In contrast graft survival exceeded 30 days for two mice in Group C (50%, mean POD 28.5±24.0, $P<0.05$). Mixed lymphocyte reaction showed that T-cells from tolerant mice had very weak responses against spleen cells from C3H mice.**
***Conclusions*. The simultaneous ITxHP with $CD4^+CD25^+$ T-cells administration prolonged islet graft survivals and induced donor-specific hyporesposiveness. J. Med. Invest. 51 : 178-185, August, 2004**

Keywords **:** *islet transplantation, experimental transplantation, donor-specific tolerance, $CD4^+CD25^+$ T-cells, mouse*

INTRODUCTION

Islet transplantation is used clinically for patients with diabetes mellitus (especially for insulin-dependent Diabetes Mellitus, type 1 DM) as a radical therapy, instead of regular insulin injection (1). One of the major obstacles to utilizing islet transplantation more routinely is its use of allogeneic grafts. This is because the allogeneic graft is sometimes rejected by the recipient's immune system in spite of immunosuppressive drugs and new regimen (1). Thus, a primary goal for improving allogeneic islet transplantation is to make the eternal transplanted islets less invasive in character so that the graft can survive without immunosuppression.

Many studies have shown that the liver has a great potential to induce immunological tolerance. It also showed that the liver represented one of the immunologically privileged site for transplantation (2). Thus, the transportal islet transplantation system has been used frequently, both experimentally and clinically (3, 4). According to Griffith *et al.* (5) islets transplanted from the portal vein migrate into the hepatic parenchyma five or six days after transplantation. As for other sites of islet transplantation, several studies have sug-

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gested direct transplantation of islets into hepatic parenchyma, although the transplantation efficiency is not good (6). However, considering the great potential of liver to induce immunological tolerance by itself (7), the possibility can allow the less invasive technique like percutaneous administration (8), we have tried here to establish direct islet transplantation into the hepatic parenchyma system.

It is well known that immunological tolerance can be acquired by transfer of donor-specific antigens into the recipient beforehand (9, 10). Many experiments have suggested that T-cells play an important role in inducing such a tolerant state (11, 12), and one of them showed that T-cells from untreated mice could prevent acute allograft rejection (13). Hara *et al*. have recently shown that $CD4^+CD45RB^{low}$ and $CD4^+CD25^+$ T-cell subpopulations isolated from animals with long-term surviving allografts can control rejection of skin allografts (14). Those studies suggest that donor MHC or antigen-specific regulatory T cells could be generated before transplantation and could then afford graft protection. However, it is not practical to prime regulatory T-cells before transplantation and use them in clinical islet transplantation because freshly isolated islets are recommended (22). We have to try to minimize the cold ischemia of islet (4). Thus, we examined whether unprimed donor-derived $CD4^+CD25^+$regulatory T-cells (Treg) could control allogeneic islet transplantation. We aimed to show whether donor-derived Treg could induce allograft-specific T-cell tolerance, resulting in long-term survival of allogeneic islets.

MATERIALS AND METHODS

Animals

C3H/He male (8-10 weeks old) and BALB/c male (6-7 weeks old) were purchased from Japan CLER Co., Tokyo. Three C3H/He mice were used as donors. BALB/c mice were injected intraperitoneally with 200 mg/kg Streptozotocin (STZ, WAKO pure chemical industries, Ltd, Tokyo, Japan.). Only mice whose fasting blood sugar level (FBS level) exceeded 350 mg/dl with polyuria and polyposia after the 6th day of the medication were used as recipients. These mice were usually fed with CE-2 solid food (Japan CLER Co ; Tokyo, Japan) and drinking water was given freely. All experiments were conducted in accordance with the animal experimental guidelines of The University of Tokushima.

Preparation of islets

Islet isolation was carried out using the collagenase technique (15) and centrifugation on a Ficoll gradient (16). Briefly, an upper middle incision was made in the abdomen of the donor mouse under general anesthesia, and the distal end of the common bile duct (CBD) was clamped. The CBD was punctured carefully by a 27-gauge needle, and pancreas tissue was flowed back to retrograde with 2.5ml of Hanks Balanced Salt Solution (HBSS, SIGMA CHEMICAL CO, St Louise, U.S.A.) that contained 1 mg/ml collagenase S-1(SIGMA CHEMICAL CO, St Louis, U.S.A.), in order to inhibit the pancreas tissue mechanically. The donors were sacrificed after extracting the pancreas. These procedures were undergone under a light microscope. After extraction of the pancreatic tissue, the tissue was incubated for 35 minutes at 37 degrees in a shaker water bath. After washing three times (1500rpm, 4°C, 5min.), impurities were removed by a sterile stainless steel mesh and the islets were separated by a Ficoll gradient (WAKO pure chemical industries, Ltd, Tokyo, Japan. Specific gravity1.023, 1.033, 1.043, and 1.050 ; 2200 rpm, 4°C, 10 min.). The antibiotics Penicillin and Streptomycin (ICN Biomedicals Inc., Ohio, U.S.A.) were added to RPMI 1640 (SIGMA CHEMICAL CO, St Louise, U.S.A.) in a total volume of 50 μl, along with 300 IE (islet equivalent) islets, after removal of the exocrine pancreatic tissue by the handpicking method. All procedures involving cell adjustments were performed on a clean bench at room temperature.

Transplantation

Transplantation was performed under general anesthesia. All recipient mice received an upper midline lapatomy. The hepatic parenchyma of the right lobe was punctured carefully with a 30-gauge needle to 1mm depth, and islets were transplanted from a 1 ml syringe. The total injection volume was adjusted to less than 0.1 ml. To prevent leakage of islets out of the hepatic parenchyma, we injected the fluid over 90 seconds. After removal of the needle, gentle pressure was applied for three minutes with a sterile swab for hepatic hemostasis at the puncture point. The abdomen was closed using two layers (peritoneum and fascia, 3-0 vicryl ; skin, 3-0 nylon). The operation was performed in a sterile environment. All of the postoperative mice were wormed for10 minutes, and then they were returned to the cage, where drinking water and solid feed were given freely.

Preparation of lymphocytes

Three C3H/He mice 8-10 weeks of age were eutha-

nized. The spleen and the mesenteric and inguinal lymph nodes were extracted from each. After carrying out homogenization with 3ml RPMI 1640 that contained 10% heat-inactivated fetal bovine serum (FBS), hemolyzation of the spleen and the lymph nodes was performed using 7% NH_4Cl, and the lymphocytes were washed and spun down (1500rpm, 4°C, 5min. twice). The viable lymphocytes were counted under a light microscope after dyeing dead cells in Turk solution (WAKO pure chemical industries, Ltd, Tokyo, Japan), and splenocytes were adjusted to 1.0×10^8(10μl). Afterwards, $CD4^+CD25^+$ T-cells were purified from peripheral lymph nodes by positive selection using Magnetic Cell Separation (MACS) procedure with an anti-CD25 antibody (e-bioscience, U.S.A) and Goat anti-rat IgG microbeads (Militienyi Biotec, Germany). $CD4^+$ $CD\ 25^+$ T-cells were adjusted to 1.0×10^5(10μl). These splenocytes and purified lymphocytes were used immediately for transplantation with islets. All procedures were completed within two hours. All pre-and post-purified lymphocytes were investigated for contents of lymphocyte populations by FACSCalibur (BD Biosciences, U.S.A.).

Criteria for rejection

FBS levels of all recipients were measured two or three times per week, starting from the day of transplantation, by the tail cut method (using Precision Q.I.E, Medisense, Coleshill, U.K). Two consecutive days of a high blood glucose level (over 250mg/ml) were judged to indicate rejection of the transplanted islets.

Experimental design

The protocol included three treatment groups : Group A (n=5), recipient mice receiving only 300 IE islets as a control ; Group B (n=5), recipients given 300 IE islets and whole splenocytes (1.0×10^8), as already described ; Group C (n=4), recipients given 300 IE islets and $CD4^+CD25^+$ T-cells (1.0×10^5) purified from inguinal lymph nodes and mesenteric lymph nodes. For all recipients within each group, islets and lymphocytes were transplanted into the hepatic parenchyma (ITxHP) simultaneously. Immunosuppressive agents were not given.

Mixed Lymphocyte Reaction (MLR)

In order to investigate whether or not donor-specific tolerance was induced, a mixed lymphocyte reaction (MLR) was performed. Stimulator splenocytes (from C3H/He, BALB/cJClr and C57/BL6 as 3rd party) were treated with 50μg/ml Mitomycin C (MMC, Kyowa Hakko Co ; Tokyo, Japan) at 37°C for 45min. Using the method of Goossens *et al.* (17), we separated the free lymphocytes from the livers of the recipients whose grafts survived over 30 days. These lymphocytes were designated responders. Each set of stimulator and responder cells (5.0×10^5) was added to 0.2mL RPMI 1640 medium with 100U/mL penicillin, 0.1mg/ml streptomycin and 10% heat-inactivated FBS. These cells were add to round-bottom 96-well microplates and incubated at 37°C in 5% CO_2. After 66 hours, 0.5μCi of [^{3}H] methylthymidine was added to each well, and the MLR response was determined by measuring incorporation of [^{3}H] methylthymidine by direct β counting (Scintillation Counter, Aloka Co ; U.S.A.). The results were reported as the mean counts per minute (cpm) of triplet measurements. The stimulation indices (S.I.s) were calculated by the formula : S.I.=cpm (mean experimental culture)/cpm (naïve BALB/c culture). The Median test was used for the statistical analysis.

Histological examination

Several of the recipients were sacrificed, their livers bearing transplanted islets were extirpated without perfusion, and histological examination was performed. One part of the sections was stained by Hematoxylin-Eosin, and others were stained using an anti-insulin antibody, in order to evaluate islet function (using DAKO LSAB2 kit/HRP, DAKO Cytomation Co ; U.S.A.).

Frozen sections

Isolated islets were also stained by 5 (and 6)-carboxy fluorescein diacetate succinimidyl ester (CFSE ; purchased from Molecular Probes, Eugene, OR) in order to estimate location and function of the transplanted islets (30). Isogeneic transplantation (BALB/c to BALB/c) was used for this model. Isolated islets were allowed to float in 4 ml Phosphate Buffered Saline (PBS) to which was added 4 mM CFSE. The islets were left floating for 10 min. at room temperature. After washing with RPMI 1640 medium three times (1500 rpm, 4°C, one min.), these islets were transplanted as described earlier. After 14 POD, livers bearing transplanted islets were extirpated without perfusion, mounted in O.C.T. compound (Miles Laboratories, Inc. Elkhart, Indiana), and frozen in liquid nitrogen. Frozen sections of the liver were made in the Cryostat at -24°C. All sections were observed under a fluorescence microscope, and sections that contained islets were stained by an anti-insulin antibody and by Hematoxylin.

Table 1. All transplantations (with or without lymphocytes) were performed directly into the hepatic parenchyma. Group A, rejection was occurred at 20±2.1 POD. On the contrary, in Group B, rejection was accelerated, and fatal GVHD was observed. In two mice from Group C (IT×HP with CD 4^+CD 25^+ T-cells purified from peripheral lymph nodes) grafts survival exceeded 30 post-operative days. However, as in Group B,fatal GVHD was observed in two mice from Group C.

Group	Donor lymphocyte	n	Graft survival (days)	Mean±SD	
A	0	5	18, 18, 20, 21, 23	20.0±2.1	
B	1.0×10^7	5	7+, 7, 8+, 15, 16	10.6±4.5	¶ Δ
C	1.0×10^5	4	10+, 12+, 30*, 62	28.5±24.0	

* : sacrificed for immunological and histological examinations. $^+$: death of an individual mouse (fatal GVHD) occurred. There were significant differences between total graft survival days between Groups A and C and Group B and C, according to Dunn's test. ¶ : $P<0.001$, Δ : $P<0.05$.

RESULTS

Prolonged graft survival achieved by ITxHP with Treg

We first examined whether direct injection of islet cells into hepatic parenchyma induces stable engraftment of functional islets. The islets from BALB/c mice were transplanted into the hepatic parenchyma of BALB/c mice that had been treated by STZ, and fasting blood sugar (FBS) levels in peripheral blood were monitored. The FBS of mice that had been treated by STZ began to increase 4-6 days after treatment, and never decreased to less than 250mg/ml (data not shown). In contrast, the FBS of STZ-treated mice that received 300 IE islets into the hepatic parenchyma showed reduced hyperglycemia, and achieved normoglycemia within seven days of transplantation. Their FBS never increased above 250mg/ml to at least 50 days following transplantation (data not shown). These results show that direct injection of islets into the hepatic parenchyma successfully induces stable engraftment of functional islets. Using this transplantation system, we examined whether donor-derived CD4$^+$CD25$^+$ T-cells control allogeneic islet transplantation into hepatic parenchyma.

After islet transplantation into the hepatic parenchyma (ITxHP), all recipients were monitored for FBS levels and were examined for rejection or the appearance of clinical GVHD two or three times a week (Table 1, Figure1). For all mice in the control group (Group A : The 300 IE islets from C3H mice were transplanted into BALB/c mice), the FBS level descended to less than 250 mg/dl within seven postoperative days (PODs), and FBS levels began to increase around the 20 th postoperative day (Figure 1). All of these grafts were rejected on POD 20.0±2.1. For all mice in Group B (who received simultaneous ITxHP with 1.0×10^8 whole splenocytes), increased FBS levels were observed within 10 PODs (Table 1), and clinical observations included depilation and profuse diarrhea. The graft was rejected on POD 10.6±4.5 (Table 1). These

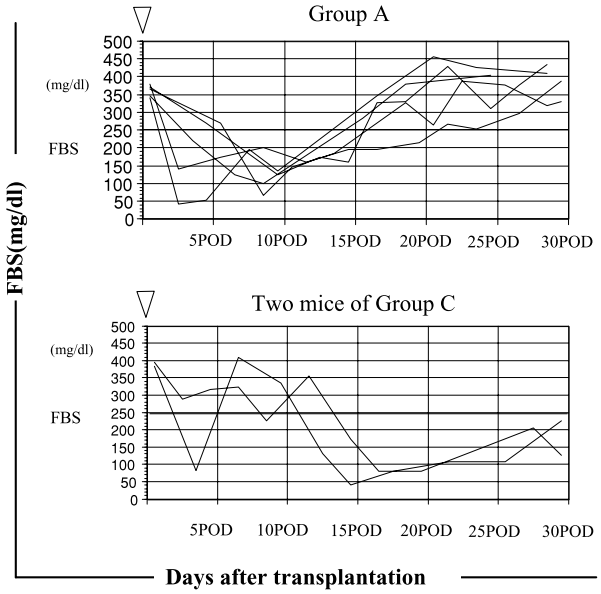

Figure 1. Graft survivals of transplanted islets. FBS measurements of all cases of Group A (control group) and two mice in Group C (excepted two mice because of fatal GVHD) are shown. FBS measurements for the control group (Group A) had never decreased below 250mg/dl after rejection. Double line in both graphs is 250 mg/dl, i.e. the rejection criteria as described. POD, Post Operative days. FBS, fasting blood sugar. The triangle on the graphs shows the day of the transplantation.

results showed that co-transfusion of total splenocytes without immunosuppression aggravates GVHD. This was consistent with previous reports of co-injection of total splenocytes with allo-transplantation through the portal vein without immunosupression from agents such as FK506 (18). In the case of Group C (mice that received simultaneous ITxHP with 1.0×10^5 Treg purified from peripheral lymph nodes), two of the four mice achieved normoglycemia after the 30 th postoperative day (Figure 2). Graft survival was significantly prolonged (mean POD 28.5±24.0, $P<0.05$) compared to the control group (Group A) (Table 1). The modes of FBS levels after transplantation were completely different between Groups A and C (Figure 1). Briefly, the FBS level of Group A had never decreased below

250 mg/dl after graft rejection around POD 20. On the other hand, the FBS levels of two mice in Group C (long-term survivors) approached normoglycemia gradually around POD11 and never rose above 250 mg/dl during the observation period (up to POD 30).

The highly purified Treg prolonged graft survival

In Group C, Treg were transferred with islets into hepatic parenchyma but the rate of graft survival was significantly different. The major difference was the purity of the $CD4^+CD25^+$ T-cells. The absolute numbers of $CD4^+CD25^+$ T-cells in Group C were set to be the same, but the purity of each sample was different (Figure 2). In all of the Group C samples, the purity of $CD4^+CD25^+$ T-cells was more than 70% after the magnetic cell sorting (MACS) procedure. However, the purity of cell populations other than $CD4^+CD25^+$ T-cells was also different in Group C. The percentage of $CD4^+CD25^-$ T-cells in each Group C sample ranged from 4.2% to 14.4%, and the recipients receiving Treg that were highly contaminated with $CD4^+CD25^-$ T-cells did not acquire long-term graft survivals (Figure 2). These findings showed that highly pure $CD4^+CD25^+$ T-cells that contain little contamination by $CD4^+CD25^-$ T-cells are required to prolong islet graft survival.

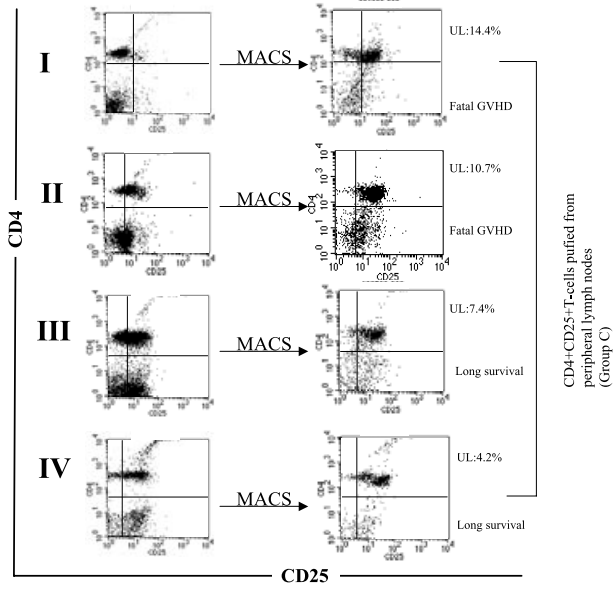

Figure 2. I-IV. All cases of $CD4^+CD25^+$T-cells purification from peripheral lymphnodes (Group C) was showed. $CD4^+CD25^+$T-cells ratio of purification by MACS was over 70%. However, extra $CD4^+CD25^+$ T-cells, especially $CD4^+CD25^-$ T-cells, were not stable (UL, upper left area of the graphs). The graphs show that over 70% of $CD4^+CD25^+$T-cells purification with less than 10% contamination by $CD4^+CD25^-$ T-cells was required to achieve long-term survival of islet cells. MACS, Magnetic cell separation.

ITxHP with highly purified Treg induced donor-specific T-cell tolerance

We examined whether transplanted Treg induced donor-specific T-cell tolerance using a mixed lymphocyte reaction (MLR). Free liver lymphocytes from long-term graft survival recipients were used as responders and splenocytes from BALB/c, C3H/He, and C57BL/6 mice were used as stimulators (Figure 3). The reaction of T-cells from BALB/c mice against spleen cells from C3H/He (Donor strain) or C57/BL6 (3rd party) mice was shown by S.I. values of 10.3 and 12.2, respectively (S.I., formula was already described) (Figure 3, left pair). In contrast, the reaction of T-cells from the liver of a recipient in Group C (simultaneous ITxHP with Treg purified from peripheral lymph nodes) to C3H/He (Donor strain) or C57/BL6 (3rd party) mice was indicated by S.I. values of 2.06 and 6.12, respectively (Figure 3, right pair. S.I., significant difference. $P<0.05$). These results show that donor-specific tolerance (hyporesponsiveness) was induced, at least in recipient liver, on POD 30 for Group C (simultaneous ITxHP with Treg purified from peripheral lymph nodes).

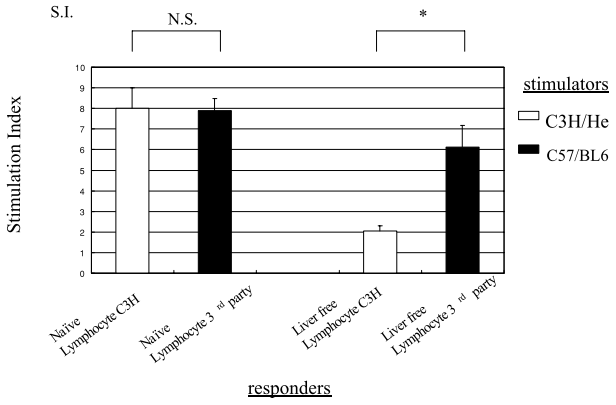

Figure 3. MLR in lymphocytes from long-surviving mice in group C. MLR was performed as described in Materials and Methods. The responses of lymphocytes from naïve BALB/c mice to lymphocytes of C3H/He (donors) and C57/BL6 were not significant. On the other hand, responses of lymphocytes from long-surviving mice (Group C) to donor cells were significantly suppressed, even though the responses to third party cells were maintained (*; significant difference. $P<0.05$).

Functioning transplanted islets surrounded by hepatocytes

Finally, we examined the site where transplanted islets were present in the hepatic parenchyma. Histological examinations were performed for the BALB/c to BALB/c isograft transplantation model on POD14, and for mice receiving simultaneous allogeneic ITxHP with Treg purified from lymph nodes on POD32. The recipient's livers were stained with Hematoxylin-Eosin

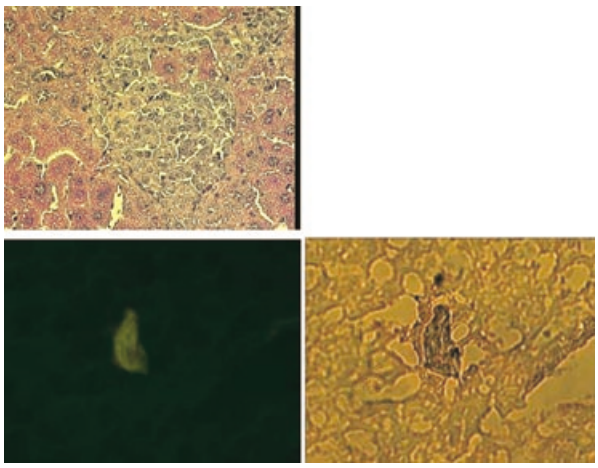

Fig.4a
Fig.4b | Fig. 4c

Figure 4. Figure 4a. Post-transplanted islets existed in a small crevasse of hepatic parenchyma. hematoxylin and eosin,original magnification×400.
Figure 4 b. Transplanted islets stained with CFSE. The islets were found in the hepatic parenchyma. Frozen section, original magnification×100.
Figure 4 c. Same section (Figure 4b) stained with insulin antibody. The islet was shown by an arrow. Staining for insulin shows that the islet cells were functioning well. Hematoxillin, original magnification×100.

and examined under a light microscope. The liver had small crevasse on its surface, and cell clusters that are not normally found in liver were present in the crevasses (Figure 4 a). In order to estimate if the cell clusters were transplanted islets, we stained BALB/c islets with CFSE and transplanted them into STZ-induced BALB/c hepatic parenchyma. Then we examined the frozen sections of recipient livers on POD 14 under a fluorescence microscope and subsequently stained them with both anti-insulin antibody and Hematoxylin. The CFSE labeled islets were localized in the same area of cell clusters found in the crevasses (Figure 4 b). Furthermore, these cell clusters were positive for insulin, which demonstrated that the transplanted islets were localized in the small crevasses of hepatic parenchyma and were producing insulin (Figure 4c).

DISCUSSION

Allogeneic islet transplantation has been considered an ideal therapeutic strategy for patients with diabetes mellitus, compared with insulin supplementation (1). However, the major obstacle to islet transplantation is the induction or maintenance of immunological tolerance to allogeneic islet grafts. Although this problem is partially addressed by the use of a strong immunosuppression agent such as FK 506, or by effective immunosuppressive methods such as the Edmonton protocol (4), transplanted islets are sometimes rejected. Various experimental approaches have been used for the induction of immunological tolerance, including infusion of donor specific antigen via the portal vein (19), anti-IL 2 receptor antibody (20) or regulating the CTLA-4 pathway (21). We have demonstrated in an allogeneic rat small intestine transplantation model that the infusion of total splenocytes via the portal vein before transplantation allows allogeneic grafts to survive in the recipients under immunosuppression (18). Other groups have shown that the transfer of T-cells from rodents that received allogeneic spleen cells regulated T-cell tolerance against allogeneic grafts. However, it is not practical to transfuse allogeneic cells before clinical transplantation. Generally surgeons try to minimize the period of cold ischemia for organs. As effective islet transplantation, freshly isolated islets are recommended (so-called Edmonton protocol (22)). In order to overcome this problem, we attempted here to use donor-derived naïve lymphocytes, especially Treg to inhibit the host immune system response against islet grafts. The co-injection of Treg with allogeneic islets into the hepatic parenchyma could induce graft-specific T-cell tolerance, and could also allows grafts to survive in the parenchyma. It is hoped that this procedure of islet transplantation will provide a basis for establishing a new clinical protocol for allogeneic islet transplantation.

Treg are known to inhibit autoreactive T-cells as well as T-cell responses against several infectious organisms (23). Several recent papers have shown that Treg from mice that have received allogeneic cells have a strong ability to inhibit T-cell responses against a subsequent allogeneic graft (24). Also, one paper has shown that donor-derived Treg are able to inhibit responses to allogeneic skin grafts (11). In the present study, we used donor-derived Treg in order to control allogeneic islet grafts, and the Treg were capable of inducing donor-cell-specific T-cell tolerance. The purity of $CD4^+$ $CD25^+$ T-cells ranged from 70-80% when peripheral lymph node cells were used as a source of Treg. When the Treg fraction was contaminated by a significant number of T-cells other than Treg, the contaminating cells induced rejection of grafts instead of tolerance. For example, we observed GVHD in two mice in Group C and these mice died. Although Treg purified from lymph nodes were adjusted to include the same number of cells for all Group C samples, the number of cells other than Treg given to the GVHD group was higher than in other samples. These results suggest that allogeneic conventional T-cells were activated by recognizing MHC expressed on transplanted islets in the

GVHD group. This in turn suggests that the removal of conventional T-cells is an important step for avoiding fatal GVHD in this model system.

The T-cells in livers from mice that received allogeneic islets and Treg have very weak responses against spleen cells from donor C3H mice, indicating that T-cells in these recipients acquired tolerance against islet grafts. Whether or not this tolerance is maintained by the persistent presence of donor-derived Treg will be clarified by future experiments. The T-cells responses from ITxHP mice against spleen cells from C57BL/6 mice were low. The majority of T-cells in the liver express the T-cell receptor at intermediate levels (17). Thus, the magnitude of their response would be relatively low compared with conventional T-cells.

We report here transplantation of islets directly into the hepatic parenchyma. Several previous papers have reported attempts to use the hepatic parenchyma (directly and intraportally) as a site of isogeneic islet transplantation (4, 6). Rogers *et al*, described a 'strange' phenomenon of intraportal transplanted islets moving into the hepatic parenchyma a few days after transplantation (5). Moreover, some stated that direct intrahepatic transplantation had advantages with respect to insulin release (25), and was a safe and easy technique (26). Moreover, this could allow for less invasive technique like percutaneous administration. Indeed, in clinical transplantation, intraportal transplantation may be accompanied by serious complications due to portal hypertension (portal embolism) and intrahepatic bleeding, as various rate of complication and measures are reported (27-29). With this in mind, we chose direct hepatic transplantation in order to avoid portal embolism caused by injection of a relatively large volume of cells (T-cells and islets). We found that islets cell clusters secreting insulin were located among the hepatocytes, and this probably occurred through direct inoculation rather than through the circulation. Islets transplanted through portal veins are known to migrate into hepatic parenchyma (5). Thus, the final site where islets are localized should be the same in islet transplantation through portal veins as by direct transplantation to the hepatic parenchyma. Considering that direct injection into hepatic parenchyma is very easy and is less invasive, compared with transportal transplantation, this surgical procedure may be easily applied to clinical islet transplantation.

In summary, the procedure of simultaneous ITxHP with Treg can induce donor-specific tolerance, and it allows islets to survive in hepatic parenchyma without the use of immunosuppressive drugs. This experimental system forms a basis for future work to establish a new clinical protocol for allogeneic islet transplantation.

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