• BASIC RESEARCH •

Immune tolerance in pancreatic islet xenotransplantation

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Abstract

AIM: To observe the effect of tail vein injection with donor hepatocytes and/or splenocytes on the islet xenotransplantation rejection.

METHODS: New-born male pigs and BALB/C mice were selected as donors and recipients respectively. Islet xenotransplantation was performed in recipients just after the third time of tail vein injection with donor hepatocytes and/or splenocytes. Macrophage phagocytosis, NK(natural killing cell) killing activity, T lymphocyte transforming function of spleen cells, antibody forming function of B lymphocytes, and T lymphocyte subsets were taken to monitor transplantation rejection. The effects of this kind of transplantation were indicated as variation of blood glucose and survival days of recipients.

RESULTS: The results showed that streptozotocin (STZ) could induce diabetes mellitus models of mice. The pre-injection of donor hepatocytes, splenocytes or their mixture by tail vein injection was effective in preventing donor islet transplantation from rejection, which was demonstrated by the above-mentioned immunological marks. Each group of transplantation could decrease blood glucose in recipients and increase survival days. Pre-injection of mixture of donor hepatocytes and splenocytes was more effective in preventing rejection as compared with that of donor hepatocyte or splenocyte pre-injection respectively.

CONCLUSION: Pre-injection of donor hepatocytes, splenocytes or their mixture before donor islet transplantation is a good way in preventing rejection.

INTRODUCTION

Insulin dependent diabetes mellitus (IDDM) so far is treated with a constant low dose insulin injection and immunosuppressive agents. But a large quantity of clinical data indicated that long-term insulin injection could induce insulin resistance and was not favorable to prevent some serious complications[1]. Long-term application of immunosuppressive drugs, for example, high-dose cyclosporine and tacrolimus, could bring about toxic effects on islets and normal liver and kidney[2,3]. So in some cases, it is limited to use immunosuppressive drugs for a long period at high dose. However, islet transplantation has the potential to cure diabetes mellitus[4], especially insulin-dependent diabetes mellitus[5]. Islet transplantation could make insulin maintain normal level of blood glucose and prevent serious complications. In general, islet allotransplantation is safe and efficient to reduce hyperglycemia, and leads to insulin independence in patients with insulin dependent diabetes[6,7]. But this kind of islets source is limited, which becomes the chief obstacle for treating patients. So it is necessary to find alternative islet sources, such as xenotransplantation of islets of new-born pigs. However, the rejection for xenotransplantation should be mornitored efficiently. Usually, the way of preventing rejection lies in using high dose of immunosuppressive drugs, which gives rise to serious side effects. On the other hand, pre-injection of a suitable number of the same donor lymphocytes or blood cells could induce immune tolerance in different kinds of organ transplantations[8-14]. Some other laboratories also reported that immunologic isolation, or transplantation of microencapsulated islets, could reduce rejection of islet xenotransplantation[15,16]. In the present study, pig islet xenotransplantation in mice was performed after injection of donor hepatocytes, splenocytes or their mixture.

MATERIALS AND METHODS

Animals and induction of diabetes mellitus model
Eighty-four BALB/C female mice (22-24 g) and 6 new born male pigs were used as recipients and donors respectively. Each BALB/C mouse was injected 0.18-0.2 mL streptozotocin (STZ) (220 mg/kg) via tail vein. When blood glucose was increased to 11.1 mmol/L, the diabetes mellitus model of mice was used in the present experiments.

Isolation of islets, hepatocytes and splenocytes
Under sterile condition, Pig pancreases were washed three times with Hanks' solution. Trypsin digestion method was used to isolate single β cells or islets, referring to the method reported by Heiser[17]. Activity of β cells was evaluated by dithiozon (DTZ) staining and the activity should be equal or over 90%. Under bacteria-free condition, pig livers or spleens were washed three times with serum-free RPMI1640, then ground on a 200# stainless steel net to make single cell suspension, washing two times (2 000 rpm/min, 5 min) with normal saline (N.S). The number of cells was adjusted to 2×10^7/mL. The cell viability was detected by Hoechst/propidium iodine (Ho/PI), both hepatocytes and splenocytes were used as donor cells with viability of more than 95%.

Islet transplantation
For hepatocyte injection group, 0.2 mL liver single cell suspension was injected into diabetic mice via tail vein every 24 h for 3 times. After the last injection of donor hepatocytes, 0.5 mL pig islets (the number of islets was 980) was transplanted into peritoneal of mouse recipient.

For splenocyte injection group, 0.2 mL spleen single cell suspension was injected into diabetes mellitus mice via tail vein every 24 h for 3 times. After the last time of injection, 0.5 mL pig islets (the number of islets was 980) was transplanted into peritoneal mouse recipient.

For mixture injection group, 0.1 mL single hepatocyte cell suspension and 0.1 mL splenocyte single cell suspension were injected into model mice via tail vein every 24 h for 3 times. After the last time of injection, 0.5 mL pig islets (the number of islets was 980) was transplanted into their peritoneal.

For islets group, 0.2 mL serum-free RPMI1640 was injected into model mice via tail vein every 24 h for 3 times. After the last time of injection, 0.5 mL pig islets (the number of islets was 980) was transplanted into recipient peritoneal. Three mice in parallel were measured for each mark and the result was indicated as an average. Twelve mice in each group were observed in terms of their living days and survival rate.

Blood glucose measurement

The level of blood glucose in mice was measured every three days by a Glucose monitoring system before and after islet transplantation. The level of blood glucose in model mice before transplantation should be over 11.1 mmol/L. The survival of transplanted-islets was indicated as at least decreasing 2-fold as compared with that before transplantation, which was indicated as functional surviving. Rejection was monitored by the level of blood glucose in mice after islet transplantation with more than 16.5 mmol/L for a continuation of 2 times.

Macrophage phagocytosis assay

Candida albicans were used as targets, constant method in our laboratory was taken to detect phagocytosis of macrophages in abdominal cavity of mice.

Phagocytosis percent=(phagocytosis of macrophages/total macrophages)×100%,

Phagocytosis index=(number of phagocytotic candida albicans in 100 macrophages /total macrophages)×100%.

Lymphocyte transformation assay

Pig spleens were washed three times with serum-free RPMI1640. The spleens were ground on a 200# stainless steel net. The number of cells was adjusted to 1×10^6/mL with RPMI1640. Each well of the culture plate was added 100 μL cell suspension, 6 wells in parallel, 3 wells were added conA solution 50 μL, the other 3 wells were added RPMI1640 50 μL as control. The cells were incubated in 50 mL/L CO_2, at 37 ℃ for 56 h, then ^{3}H-TdR 1 μCi/well was added and cultured cells for 72 h. Cpm value was detected with a β-liquid scintillation counter. Transformation function was evaluated as average Cpm value.

Assay of NK killing activity

For effector cells, mice spleens were made into single cell suspension as the same as above-mentioned method, and the cell density was adjusted to 1×10^7/mL in 100 mL/L fetal calf serum(FCS) RPMI1640 medium. For target cells, exponentially growing YAC-1 cells were adjusted to 2×10^5/mL cell suspension with 100 mL/L FCS RPMI1640. A 100 μL effector cell suspension and 100 μL target cell suspension were added into each well respectively in a 40-well plate as experimental group, 3 wells in parallel. On the other hand, 100 μL/well effector cells and 100 μL/well RPMI1640 were used as effector group, 100 μL 100 mL/L FCS RPMI1640 and 100 uL target cells as target group. They were cultured them in 50 mL/L CO_2, at 37 ℃ for 24 h. Then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide(MTT) 20 μL/well, continually cultured

for another 4 h, centrifuged at 2 000 rpm/min 10 min, the supernatant was discarded, add 1 000 g/L dimethyl sulfoxide (MDS) 100 μL was added into each well, cells were lysed with vibration. After 10 min, A value was measured at 570 nm with an ELISA reader. NK killing activity=[1-(Experimental A-Effector A)/Target$A$]×100%.

T lymphocytes and subpopulations

The total number of lymphocytes was calculated directly under microscopy. T lymphocytes and its subpopulations were measured by constant strept avidin-biotin complex (SABC) staining. SABC kit was supplied by Wuhan Boshide Company.

Antibody-forming assay in vitro

Quantitative hemolytic spectrometry (QHS) was used to run antibody-forming assay. Mice spleens were washed three times with normal saline. A 200# stainless steel net was used to grind spleen tissues into single cell suspension. The cells were re-suspended and adjusted to a final concentration of 2×10^7/mL Hanks buffer (pH 7.2). A 1 mL cell suspension and 1 mL 2 mL/L sheep red blood cell (SRBC) (1.5×10^8/mL cells), then 1:10 complement 1 mL was added at 37 ℃ for 1 h, centrifuged at 3 000 rpm/min for 5 min, the A value of supernatant at 413 nm detected by 721 spectrophotometer. A value was indicated as the ability of antibody formation of B lymphocytes in vitro.

Pathological examination

Pathologic changes including inflammatory infiltration of local site of islet transplantation in different experimental groups were examined.

Statistical analysis

The experiments were repeated 3 times and the data were expressed as mean±SE, and significant difference was assessed by Student's t test.

RESULTS

The Variations of blood glucose level after islet transplantation is shown in Figure 1.
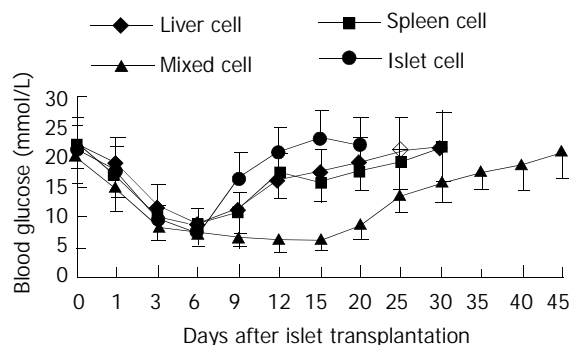

Figure 1 Variations of blood glucose after islet transplantation.

The survival time of hepatocyte injection group, splenocyte injection group, mixture group and islet group was 26.42±5.87 d, 29.56±4.52 d, 43.00±4.55 d and 16.92±2.47 d respectively (survival time of diabetic model mice was 3.86±0.85 d). The survival time of islet transplantation group was shorter than any other experimental group ($P<0.05$). So pre-injection of hepatocytes, splenocytes or their mixture from the same donor was effective in preventing rejection and delaying the time of death.

Macrophage phagocytosis in diabetic mice after pig islet xenotransplantation

Phagocytosis of macrophages in diabetic mice after pig islet xenotransplantation is summarized in Table 1.

Table 1 shows that phagocytosis percentage of islet group was increased, and the phagocytosis index of islet or hepatocyte group was obviously increased ($P<0.05$). There was no significant variation of phagocytosis percentage or index in other transplantation groups that received pre-injection of hepatocytes or mixture of hepatocytes and splenocytes 7 d after islet transplantation ($P<0.05$). After 14 d, phagocytosis percentage in hepatocyte, splenocyte and islet groups was significantly increased ($P<0.05$), phagocytosis index of islet or hepatocyte group was significantly increased ($P<0.05$).

Effect of pig islet xenotransplantation on splenic lymphocyte transforming function in mice

Seven days after transplantation, the cpm value of spleen lymphocytes in recipients of hepatocyte group, splenocyte group, mixture group or islet group was higher after pig islet xenotransplantation than before transplantation ($P<0.05$). After 14 d, the value of hepatocyte group or islet group was still higher than that before transplantation ($P<0.05$). But there was no significant difference between splenocyte group and mixture group ($P>0.05$) (Table 2).

Effect of pig islet transplantation on NK activity of diabetic mice

NK activity of each experimental group, as compared with that before transplantation, was increased 7 d after islet transplantation ($P<0.05$), and their NK activity was continually higher than that before transplantation even 14 d after transplantation ($P<0.05$). As to the mixture group, there was no significant increase of NK activity 7 or 14 d after islet transplantation as compared with that before islet transplantation ($P>0.05$) (Table 3).

Variations of T lymphocytes in diabetic mice after pig islet xenotransplantation(Table 4)

CD_3 and CD_4 counts in hepatocyte, splenocyte or islet group

Table 1 Macrophage phagocytosis in diabetic mice after pig islet xenotransplantation

Group	Pre-treatment		7 d post-treatment		14 d post-treatment	
	Percent (%)	Index	Percent (%)	Index	Percent (%)	Index
Hepatocytes group	59.3±4.5	1.05±0.04	62.8±4.37	1.28±0.13	70.6±3.82	1.25±0.07
Splenocytes group	62.5±3.0	1.04±0.07	63.1±6.45	1.18±0.14	69.5±3.62	1.21±0.09
Mixture group	63.1±3.8	1.07±0.06	61.6±3.54	1.12±0.09	62.8±4.70	1.09±0.08
Islet group	62.0±3.3	1.06±0.08	73.5±5.51	1.41±0.18	72.7±5.71	1.47±0.10

Table 2 Effect of transplantation with pig islets on splenic lymphocyte transforming function in mice

Group	Pre-transplantation (cpm)	7 d post-transplantation (cpm)	14 d post-transplantation (cpm)
Hepatocytes group	11 437.56±1 137.55	23 453.74±4 157.28	20 178.56±4 159.74
Splenocytes group	11 437.56±1 137.55	25 834.66±3 763.37	15 336.25±3 782.38
Mixture group	11 437.56±1 137.55	21 920.05±600.08	14 345.53±2 653.28
Islets group	11 437.56±1 137.55	32 339.13±5 071.67	22 182.16±750.28

Table 3 Effect of pig islet xenotransplantation on NK activity (%) in diabetic mice

Group	Pre-treatment	7 d post-treatment	14 d post-treatment
Hepatocytes group	31.56±4.23	63.17±3.47	60.26±7.65
Splenocytes group	31.76±3.48	62.50±4.86	56.19±8.13
Mixture group	31.37±8.09	48.00±12.0	34.33±14.57
Islet group	35.67±3.51	76.23±12.50	71.00±3.0

Table 4 Variations of T lymphocytes in diabetic mice after pig islet xenotransplantation

Group	Pretreatment(%)			7 d post-treatment (%)			14 d post-treatment (%)		
	CD3	CD4	CD8	CD3	CD4	CD8	CD3	CD4	CD8
Hepatocyte group	61.6±4.5	42.0±4.6	24.3±1.5	72.7±5.5	51.6±4.5	28.0±3.6	70.0±2.0	52.7±4.9	26.7±4.2
Splenocyte group	66.0±3.6	46.3±4.0	24.6±2.5	73.0±4.4	53.3±4.5	31.3±4.1	68.7±5.7	45.3±4.1	33.0±3.6
Mixture group	64.7±2.5	44.3±6.7	25.3±4.9	65.0±5.3	47.6±3.8	28.3±2.5	61.6±3.5	47.0±4.4	27.7±2.1
Islet group	65.0±7.0	43.3±3.0	24.3±3.2	80.0±2.0	60.6±3.2	32.3±3.1	72.3±6.7	58.7±9.1	32.0±4.0

Table 5 B lymphocyte antibody-forming function (*A* value) in mice after transplantation (*A*)

Group	Pretransplantation	7 d post-transplantation	14 d post-transplantation
Hepatocyte group	0.34±0.06	0.44±0.02	0.63±0.07
Splenocyte group	0.34±0.06	0.55±0.02	0.63±0.08
Mixture group	0.34±0.06	0.55±0.01	0.50±0.06
Islet group	0.34±0.06	0.74±0.11	0.81±0.13

were up-regulated 7 days after islet transplantation. CD_8 counts in splenocyte or islet group was higher than that before islet transplantation ($P<0.05$). Fourteen days after transplantation, CD_3 and CD_4 in hepatocyte and islet groups increased ($P<0.05$), and CD_8 in splenocyte and islet groups became higher than that before transplantation ($P<0.05$).

Antibody forming assay of B lymphocytes in mice after transplantation (Table 5)

Table 5 shows that antibody forming function of all four kinds of islet transplantation group was significantly increased as compared with that before islets transplantation 7 or 14 d after transplantation ($P<0.05$).

Pathological examination

In islet transplantation group, there were a lot of lymphocyte infiltration, disruption of islets structure, or denaturation and necrosis of islet cells. Hepatocyte group with moderate lymphocyte infiltration. The mixture group had light lymphocytes infiltration.

DISCUSSION

Insulin dependent diabetes mellitus was considered as an autoimmune disease against beta cells[18], which is usually treated with a low dose insulin injection and immunosuppressive agents. But a large quantity of clinical data indicated that long-term insulin injection could induce insulin resistance and could not prevent some serious complications[1]. Recent studies focused on islet transplantation as a primary therapy for insulin dependent mellitus diabetes, accompanied by induction of immune tolerance to immunosuppressive agents[19,20]. Islet allo-transplantation could correct glucose imbalance and related complications, because successful islet transplantation could not only supplies the natural insulins but also other necessary biological factors. But most patients with insulin dependent diabetes mellitus could not be treated by this kind of islet transplantation because the source of allo-islets was limited. However, xenotransplantation, for example pig islet, could resolve the above-mentioned problems. Although xenotransplantation could renew the balance of glucose, rejection usually occurs, thus reducing the effect of transplantation. Islet transplantation combined with other organs or cells from the same donor could reduce rejection. Some laboratories have tried pancreas allotransplantation in combination with the same donor spleen transplantation. The results showed that the survival time of pancreas was significantly prolonged, which was attributed to the induced immunotolerance. It has been reported that bone marrow transplantation could induce the formation of hematopoiesis chimera, which could prevent acute or chronic rejection[13-15,21,22]. So it is possible to use hematopoiesis chimera to treat insulin dependent diabetes mellitus.

T lymphocyte vaccine (TCV) could induce immunotolerance for transplantation[23]. For example, TCV with recipient spleen cells pre-sensitized by donor antigen was used in rat heart transplantation, to observe the survival time of rat heart graft. The results showed that TCV could prolong the survival time of rat heart graft[24]. As to the immune response, T lymphocyte proliferation reaction increased, B lymphocyte proliferation reaction was not affected, but mixed lymphocyte reaction (MLR) declined. The analysis of phenotype showed that CD_8 subpopulation increased, however there was no obvious change of antibody-dependent cell-mediated cytotoxity (ADCC) reaction. Many other studies demonstrated that liver cells (or hepatocytes) and/or spleen cells (or splenocytes) could induce specific immune tolerance in different organ transplantations, such as kidney, marrow, heart, skin[25-28]. In the aspect of islet transplantation, some scholars administrated pre-injection of

donor hepatocytes and/or splenocytes to induce the immune tolerance and got the positive result[29-31].

In the present study, islet xenotransplantation was performed after pre-injection of donor hepatocytes, splenocytes, or their mixture. The results showed that the survival time of hepatocyte group, splenocyte group, mixture group was longer than islet group ($P<0.05$). Pre-injection of hepatocytes, splenocytes or their mixture from the same donor was effective in preventing rejection and prolonging survival time. In the aspect of macrophage function, macrophage phagocytosis percent in islet transplantation group was increased, and the phagocytosis index in islet or hepatocyte group was increased ($P<0.05$). There was no significant variation of phagocytosis percent or index in other transplantation groups 7 d after transplantation. After 14 d, phagocytosis in hepatocyte, splenocyte and islet group was significantly increased, phagocytosis index in islet or hepatocyte group was increased. The antibody-forming function of spleen B lymphocytes of recipients in hepatocyte group, splenocyte group, mixture group or islet group was increased 7 d after pig islet xenotransplantation. After 14 d, the B lymphocyte function in hepatocyte group or islet group was still higher than that before transplantation ($P<0.05$). As to NK cells, NK killing activity of each experimental group, as compared with before transplantation, was increased 7 d after islet transplantation, and their NK activity maintained higher than that before transplantation 14 d after transplantation. As to the mixture group, there was no significant increase in NK activity 7 or 14 d after islet transplantation as compared with that before islet transplantation ($P>0.05$). T lymphocyte subpopulation was also analyzed, the results showed that CD_3 and CD_4 percent of hepatocyte, splenocyte or islet group increased 7 d after islet transplantation. CD_8 percent of splenocyte or islet group was higher than that before islet transplantation. Fourteen days after transplantation, CD_3 and CD_4 percent of hepatocyte and islet group was still higher, and CD_8 percent of splenocyte and islet groups was higher than that before transplantation. To further examine immune tolerance in different experimental groups, pathological examination was performed. In islet transplantation group, lymphocyte infiltration was extensive, disruption of islet structure and necrosis of islet cells were also obvious. Hepatocyte group had moderate lymphocyte infiltration. The mixture group has slight lymphocyte infiltration. The results indicate that pre-injection of hepatocytes, splenocytes or mixture of them could reduce rejection by inducing immunotolerance. Although the concrete mechanism is not completely clear, pre-injection of hepatocytes and splenocytes from the same donor could induce immunotolerance of islet xenotransplantation, and prolong the survival time of islets.

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