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RESEARCH PAPER

Local Co-Delivery of Pancreatic Islets and Liposomal Clodronate Using Injectable Hydrogel to Prevent Acute Immune Reactions in a Type 1 Diabetes

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ABSTRACT

Purpose The purpose of this study was to investigate the effect of locally delivered pancreatic islet with liposomal clodronate (Clodrosome®) as an immunoprotection agent for the treatment of type 1 diabetes.

Method The bio-distribution of liposomal clodronate in matrigel was checked by imaging analyzer. To verify the therapeutic efficacy of locally delivered islet with liposomal clodronate using injectable hydrogel, four groups of islet transplanted mice ($n = 6$ in each group) were prepared: 1) the islet group, 2) the islet-Clodrosome group, 3) the islet-Matrigel group, and 4) the islet-Matrigel-Clodrosome group. Immune cell migration and activation, and pro-inflammatory cytokine secretion was evaluated by immunohistochemistry staining and ELISA assay.

Results Cy5.5 labeled liposomes remained in the matrigel for over 7 days. The median survival time of transplanted islets (Islet-Matrigel-Clodrosome group) was significantly increased (>60 days), compared to other groups. Locally delivered liposomal clodronate in matrigel effectively inhibited the activation of macrophages, immune cell migration and activation, and pro-inflammatory cytokine secretion from macrophages.

Conclusions Locally co-delivered pancreatic islets and liposomal clodronate using injectable hydrogel effectively cured type 1 diabetes. Especially, the inhibition of macrophage attack in the early stage after local delivery of islets was very important for the successful long-term survival of delivered islets.

KEY WORDS pancreatic islets · liposomal clodronate · injectable hydrogel · local delivery · macrophage depletion

ABBREVIATIONS

CD	Cluster of differentiation
ELISA	Enzyme-linked immunosorbent assay
IEQ	Islet equivalent
IL-1β	Interleukin-1 beta
RPMI	Roswell park memorial institute medium
SD	Standard deviation
SEM	Standard error of mean
TNF-α	Tumor necrosis factor-alpha

INTRODUCTION

Islet transplantation holds great promise as a successful method of the treatment for type 1 diabetes. One drawback, however, is the fact that only 50% of the type 1 diabetic patients, who receive islet transplant followed by long-term cocktailed immunosuppressive drug therapy, maintained insulin independence for 5 years (1). The main cause of graft failure is the activation of host's immune system and subsequent release of antigen from the transplantation site. The innate immune system is triggered by the activation of

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macrophages and neutrophils, causing inflammation and immune cell infiltration into the transplanted site (2). The activation of macrophages and neutrophils releases the inflammatory cytokines and reactive oxygen species, thereby activating antigen-presenting cells (APCs), helper T cells (CD4) and cytotoxic T cells (CD8) (3,4). Various secreted cytokines from activated macrophages would damage the transplanted islets (5). Thus, macrophages play a key role in the initiation of immune reactions and inflammatory reactions in the microenvironment of transplanted islets (3,6).

Therefore, depletion of macrophage activation is a crucial strategy for inhibiting islet graft rejection. Bottino *et al.* reported the effect of macrophage depletion on graft survival and microenvironment activation (6). It has been reported that liposomal clodronate improved the survival time of grafted islets, which helped to inhibit the initiation of immune reaction (7–10). Wu *et al.* demonstrated that xenografted porcine islets rejection was delayed in macrophage-depleted mice (11). In addition, Frith *et al.* reported that, free clodronate significantly decreased the total viability of cultured J774 macrophages after 48 h of treatment and the encapsulation of clodronate as liposomal form increased the potency by 300-fold (7). However, systemically delivered liposomal clodronate was not effective for inducing macrophage depletion because of its short retention time in the blood. Also, systemically administered liposomal clodronate could induce several kinds of side effects (8,9). Therefore, it is important to find appropriate methods of sustaining delivery of liposomal clodronate. Several studies have investigated the portal vein infusion as an alternative site of delivery since the current clinical pancreatic islet transplantation is performed by intraportal infusion. Nevertheless, the intraportal delivery of liposomal clodronate might not be the optimal delivery route since the liver is known to have a very strong immune response and inflammation reaction (e.g. instant blood-mediated inflammatory reaction) (10). Currently, subcutaneous delivery has been demonstrated to be the proper route for using injectable hydrogel since it ensure minimal invasiveness and easy access (11–15).

In this study, it was hypothesized that locally delivered liposomal clodronate using injectable matrigel at the site, where immune reaction was induced by macrophages, would maximize the survival period of delivered pancreatic islets. And we demonstrated that pancreatic islets embedded within matrigel containing liposomal clondronate significantly enhance the survival time in type 1 diabetic mice model.

MATERIALS AND METHODS

Animal

Sprague–Dawley (SD) rats (male, 8 weeks old) were used as islet donors and inbred C57BL/6 mice (male, 7–8 weeks old) were used as recipients. They were purchased from Orient Bio Inc. (Seongnam, Republic of Korea) and were housed under a specific pathogen-free condition at our institution. Type 1 diabetes was induced chemically in the recipient C57BL/6 mice by a single intraperitoneal injection of 180 mg/kg of streptozocin (STZ; Sigma, St. Louis, MO). Mice with the blood glucose level of over 350 mg/dl for two consecutive days were selected as diabetic recipients for transplantation. All experimental and surgical procedures were conducted by following the guidelines of the Institute of Laboratory Animal Resources, Seoul National University (IACUC no. SNU-070822-5).

Pancreatic Islet Isolation

Pancreatic islets were isolated from the pancreases of outbred male SD rats. Briefly, SD rats were anaesthetized with intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture, and their pancreases were exposed by laparotomy. The common bile duct was ligated, cannulated with a 25-gauge-needle, and then injected with 10 ml of Hank's balanced salt solution (HBSS; Sigma) containing 0.8 mg/ml Collagenase P (Roche, Indianapolis, IN). Distended pancreases were removed and incubated at 37°C for 20 min. Islets were then purified by centrifuging in the solution of discontinuous Histopaque (Sigma) density gradient at 2,400 rpm for 18 min. Isolated islets were cultured for 3 days in RPMI-1640 (Sigma) containing 10% fetal bovine serum (FBS; Sigma) at 37°C in a humidified atmosphere of 5% CO_2.

Optical Imaging of Liposomal Clodronate

Noninvasive imaging of the locally delivered liposomal clodronate was carried out using Cy5.5 labeled liposome (Encapsula NanoScience, Nashvile, TN) in male Balb/c nude mice (Orient Bio Inc.). Since the C57BL/6 mouse strain possesses immunity, we used athymic Balb/c nude mice to evaluate the release rate for an extended period of time without causing any immune reactions. Cy5.5 labeled liposome (25 μl; same volume of drug containing liposome) was added to the growth factor reduced Matrigel® (BD Biosciences, Franklin Lakes, NJ), which was mixed homogeneously using pre-cooled pipet tips and injected immediately through the subcutaneous part on the scruff region of the pre-anesthetized mice. Next they were laid down in a prone position, fixed on scanning plate and placed inside Optix acquisition system (Optix MX3™, ART Advanced Research Technologies Inc., Montreal, Canada). The localization of Cy5.5 labeled liposome in mice was assessed for 14 days by using OptiScan™ software (ART Advanced Research Technologies Inc.). After 14 days, the locally delivered matrigel containing Cy5.5 labeled liposomes was also retrieved and the

fluorescence intensity was compared with the matrigel excised right after a local injection. The intensity profiles were obtained using OptiView™ software (ART Advanced Research Technologies Inc.) on all images.

Local Delivery of Pancreatic Islets

To evaluate the therapeutic effect of locally delivered islets, four groups of islet delivered mice ($n=6$) were prepared as below; 1) 2000 IEQ islets alone subcutaneously injected (Islet group), 2) 2000 IEQ islets and liposomal clodronate (Clodrosome®, Encapsula NanoSciences, Nashville, TN) both separately injected subcutaneously (Islet-Clodrosome group), 3) 2000 IEQ islets subcutaneously injected in matrigel (Islet-Matrigel group), 4) 2000 IEQ islets subcutaneously injected in matrigel containing liposomal clodronate (Islet-Matrigel-Clodrosome group).

Matrigel was stored in −20°C and thawed overnight at 4°C in ice before use. When matrigel reached its jellylike state, 500 µl of it was added to 20 µl PBS (Life technologies, Grand Island, NY), where 2000 IEQ of islets were suspended with or without 25 µl of liposomal clodronate at 6.25 mg/kg dose. The concoction was mixed by pipetting several times using pre-cooled pipette tips. This process was conducted in ice to prevent matrigel gelation since it rapidly formed gel above 22°C. Diabetic mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). Pre-cooled 1 ml syringe with 26-gauge-needle was used to load the matrigel solution and islets with or without liposomal clodronate. The mixture was subcutaneously injected into the scruff of the mice; the matrigel solution rapidly gelated just after injection at body temperature.

The islet delivery procedure was considered successful if the blood glucose level decreased less than 200 mg/dl within 3 days after injection. Non-fasting blood glucose level was monitored everyday using a glucometer (Super glucocard II, Arkray, Kyoto, Japan) and body weight was also checked as well. The delivered islets were considered graft rejection if the blood glucose concentration was higher than 300 mg/dl for three consecutive days.

Intraperitoneal Glucose Tolerance Test

Intraperitoneal glucose tolerance testing (IPGTT) was performed at 60 days of local delivery to evaluate the glucose responsiveness of the delivered islets. Mice were administered 20% glucose solution (Sigma) at a dose of 2 g/kg into the peritoneal cavity following an overnight fasting. Blood glucose levels were measured at 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min.

Quantification of Insulin and Cytokine Levels in Serum

After anesthetizing the mice by diethyl ether (Sigma), the blood was collected by retro-orbital sinus puncturing. The whole blood was allowed to clot by leaving it at room temperature for 30 min. Clot was removed by centrifugation at 2,000 g for 10 min in a refrigerated centrifuge. The supernatant was immediately transferred into another tube, aliquoted and stored at −70°C. The insulin level was measured using a rat/mouse insulin ELISA kit (Millipore Corp., Billerica, MA), cytokine IL-1ß (R&D Systems, Minneapolis, MN) and TNF-α (BioLegend, Inc., San Diego, CA) concentration were also measured in accordance with the manufacturer's instructions.

Quantification of Insulin and Cytokine Levels in Matrigel

Injected matrigel was retrieved from the injected site, followed by homogenization in hypotonic lysis buffer (1% RIPA buffer, Sigma) and centrifugation at 5,000 g for 5 min. The supernatant was collected, aliquot and stored at −70°C. Insulin concentration was measured by using a rat/mouse insulin ELISA kit (Millipore Corp.). Cytokine IL-1ß (R&D Systems) and TNF-α (BioLegend) concentrations were also measured by ELISA.

Immunohistochemistry

At day 60 of islet delivery, the matrigel was retrieved and fixed in 10% formalin for 2 days at room temperature. The sections were deparaffinized by heating in a dry oven for 1 h and washing vigorously in xylene. The slides were then rehydrated serially in 100%, 90%, 80% and 70% alcohol. The antigens were retrieved by heating the slides in 10 mM citrate buffer (pH 6.0, Sigma) using microwave (5 min, 3 times, 700 W), and then cooling down to room temperature for 20 min. Citric acid was neutralized by immersing the slides in 3% H_2O_2 (Sigma) for 15 min. After washing in PBS, the slides were incubated overnight at 4°C with mouse monoclonal anti-insulin (1:50; Abcam Inc., Cambridge, MA), anti-somatostatin (1:50; Biomeda, Foster City, CA), anti-glucagon (1:200; DAKO, Carpinteria, CA), anti-CD31 (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-CD11b (1:10; eBioscience, San Diego, CA), anti-$CD20^+$ (1:40; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-$CD4^+$ (1:100; Abcam Inc., Cambridge, MA), and anti-$CD8a^+$ (1:25; BioLegend, San Diego, CA) in a humidified chamber, respectively. Next day, before further treatments, the slides were kept at room temperature for 1 h. After washing, the tissue sections were observed with a peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins in Tris–HCl buffer (Envision plus System-HRP labeled polyer; Dako, Glostrup, Denmark), which was incubated for

2 h at room temperature. The slides were washed, and the chromogen was developed for 15 min with liquid 3, 30-diaminbenzidine (Dako). The slides were counterstained with Mayer hematoxylin. Negative controls were treated similarly with the exception of primary antibodies. All slides were gradually dehydrated using 70%, 80%, 90% and 100% alcohol. Finally, tissue slides were fixed using mounting medium (Dako) with a glass coverslip.

Statistical Analysis

The survival time was analyzed as the median ± SEM. *In-vivo* optical intensity, IPGTT of the injected islets, insulin and cytokine concentrations were expressed as the mean ± SD. Statistical analysis was carried out using the unpaired t-test or ANOVA one-way test. The p value of less than 0.05 was considered to be statistically significant.

RESULTS

Releasing Profiles of Cy5.5 Labeled Liposome from the Locally Delivered Matrigel in Nude Mouse

The release profile of encapsulated liposomal clodronate from the matrigel in nude mice was evaluated using the molecular imaging technique for 14 days after local injection. Cy5.5 labeled liposome loaded in the matrigel solution was injected subcutaneously and the fluorescence images were taken at different time points (Fig. 1a). The fluorescence intensity at the injected site was gradually decreased with time, and it was remarkably decreased as low as 95.60±7.33 photons at day 14, compared to the initial intensity (497.33±88.57 photons), indicating that the liposomes were gradually released out from the matrigel (Fig. 1b). Afterwards, the matrigel was retrieved, and the fluorescence intensity was measured. It was also confirmed that 98% of the Cy5.5 labeled liposome was released out from the matrigel after 14 days of local injection (Fig. 1c and d).

Survival Time of Locally Delivered Islets

In order to evaluate the therapeutic potential of liposomal clodronate as an immunosuppressive drug and also to evaluate the efficacy of matrigel in protecting islets, 2000 IEQ of islets were injected into STZ-induced diabetic C57BL/6 mice. Non-fasting blood glucose levels of the recipients were measured to determine the viability of islets after local delivery. For the Islet group and the Islet-Clodrosome group, none of the mice achieved normoglycemia, as shown in Fig. 2a and b. However, the blood glucose level of mice in the Islet-Matrigel group returned to normoglycemia after islet delivery (MST: 5.50±0.22 days, Fig. 2c). This result illustrates the importance of matrigel as an extracellular matrix to maintain the viability of delivered islets. To improve the survival time of delivered islets in matrigel, we further evaluated the combination and immunosuppressive effects of matrigel along with liposomal clodronate (Islet-Matrigel-Clodrosome group, Fig. 2d) at 6.25 mg/kg dose. For this group, all the recipient mice achieved normoglyemia within 24 h after injection, and 83.33% of the recipient mice maintained normoglycemia for more than 60 days (Fig. 2e). We began evaluating the immunoprotection effect of clodrosome at 50 mg/kg dose, reducing the dose sequentially by half to 50, 25, 12.5, 6.25 and 3.125 mg/kg. When the dose was above 6.25 mg/kg, the survival rates were not so significantly different that the glucose levels in all groups were maintained in the normal range up to 60 days. On the other hand, 3.125 mg/kg dose showed a significantly reduced survival rate of 10.5±1.0 days (data not shown). Therefore, the dose of liposomal clodronate was optimized as 6.25 mg/kg for islet transplantation. Since the clinical dose of clodronate is 1,600 mg/person (26.7 mg/kg) one or two times a day, the optimized dose of clodronate in this study was very low compared to its clinical dose. The combination system of matrigel and liposomal clondronate significantly enhanced the survival time of delivered islets.

At 60th day of the islet delivery of Islet-Matrigel-Clodrosome group, IPGTT was performed to evaluate the glucose responsiveness *in vivo* (Fig. 3). After the administration of a high dose of glucose, non-diabetic mice maintained blood glucose level rapidly to the normal level within 2 h. On the other hand, the blood glucose level of diabetic mice remained over 500 mg/dl after 5 min of the glucose injection without returning to the normal range. Compared to the diabetic group, the blood glucose level profile of Islet-Matrigel-Clodrosome group was well maintained, indicating that islet delivered recipient had normal glucose sensitivity up to 60 days.

Inhibition Effects of Liposomal Clodronate and Matrigel on Inflammation and Immune Cell Activation

To further investigate the functionality of locally delivered islets and immune cell invasion to the graft, histological analysis as well as insulin and cytokine concentrations for IL-1β and TNF-α were carried out for two groups, the Islet-Matrigel group and the Islet-Matrigel-Clodrosome group. Both immunohistological analysis and ELISA for checking insulin and cytokine concentrations were conducted for the Islet-Matrigel-Clodrosome group on the 60th day of local delivery and for the Islet-Matrigel group right after rejection at different time points ranging from day 4 to day 10. Hematoxylin and eosin (H&E) staining demonstrated that islet morphology was disrupted when islets were delivered in matrigel, but when liposomal clodronate was loaded in the matrigel, the structure of delivered islets was remained intact (Fig. 4a). Insulin,

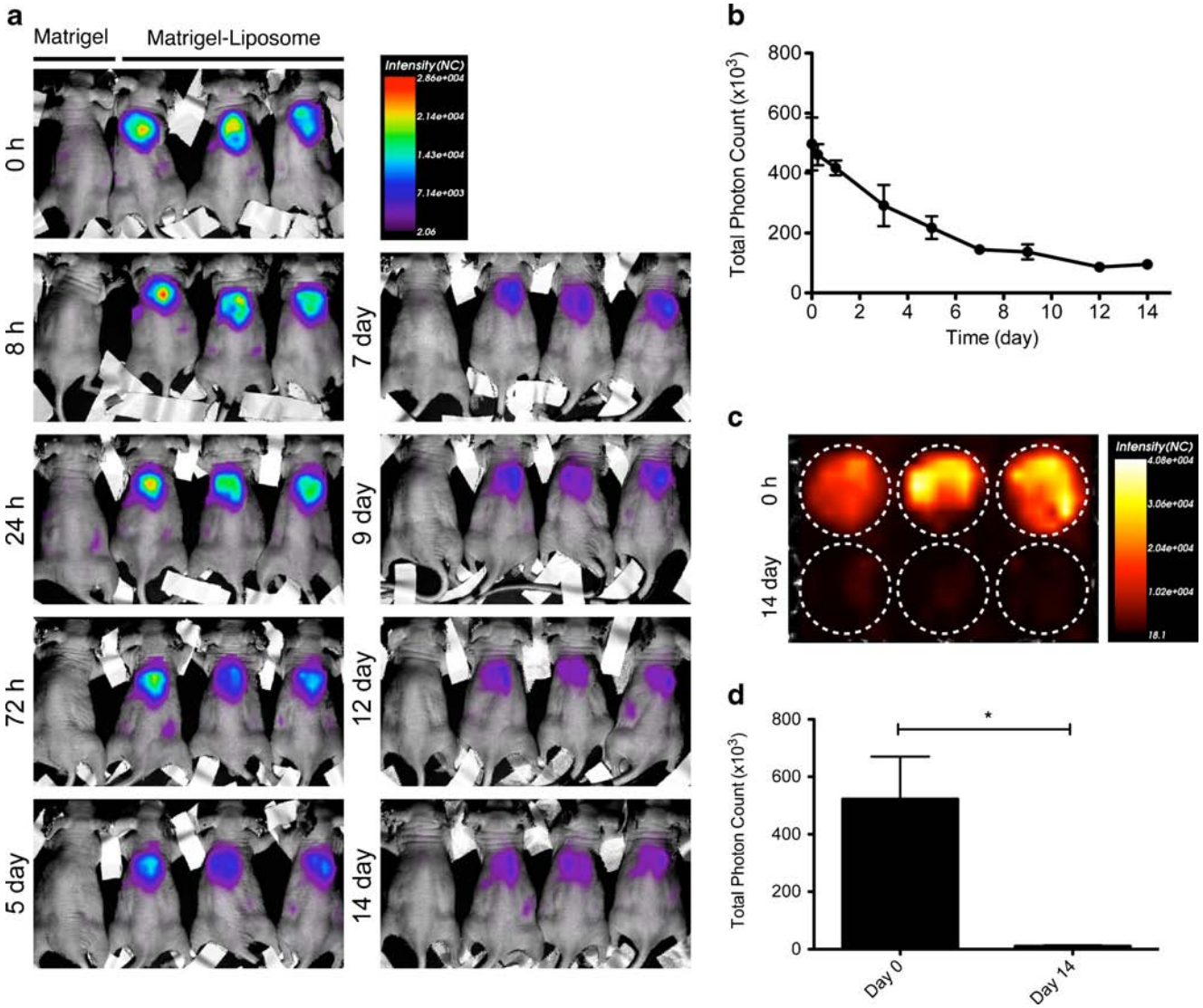

Fig. 1 *In vivo* bio-distribution of Cy5.5 labeled liposome. (**a**) Balb/c nude mice were subcutaneously injected matrigel with Cy5.5 labeled liposome. (**b**) Photon counts of injected sites in comparison with untreated group were quantitated at 14 day by *in vivo* imaging system (**c**) *Ex vivo* bio-distribution image of matrigel containing Cy5.5 labeled liposome extracted from Balb/c nude mice after 14 days. (**d**) Photon counts of extracted matrigel containing Cy5.5 labeled liposome. Data were expressed as mean ± SD ($n = 3$). *Significantly lower ($p < 0.001$) compared to Day 0 group

glucagon and somatostatin were released from well-structured islets in the Islet-Matrigel-Clodrosome group, on the other hand, those hormones in Islet-Matrigel group were stained irregularly (Fig. 4a).

One possible reason for the destruction of islet structure in the Islet-Matrigel group might be due to the infiltration of lymphatic cells to the transplanted graft since a significantly great number of $CD4^+$, $CD8^+$, $CD20^+$ and $CD11b^+$ positive cells were detected in this group. However, much less infiltration of $CD4^+$, $CD8^+$, $CD20^+$ and $CD11b^+$ positive cells into the matrigel were found in the Islet-Matrigel-Clodrosome group (Fig. 4b and c). Furthermore, we measured insulin and pro-inflammatory cytokine concentration quantitatively both in serum and matrigel in order to confirm the functionality of delivered islets and immune reactions. The insulin concentration of Islet-Matrigel-Clodrosome group was higher in both matrigel and serum (14,350±11,190 pg/ml and 1,240±560 pg/ml) than those of Islet-Matrigel group (460±340 pg/ml and 410±330 pg/ml), which were statistically significant ($p < 0.05$) (Fig. 5a).

To assess whether macrophage-depleting agent could reduce the macrophage activation and the related cytokine expression in the delivered site, the concentrations of IL-1β and TNF-α had been determined. As shown in Fig. 5b, the IL-1β concentration in the matrigel was significantly decreased as low as 0.5% by the liposomal clodronate in the matrigel; that is, the IL-1β concentrations in the matrigels of Islet-Matrigel group and Islet-Matrigel-Clodrosome group

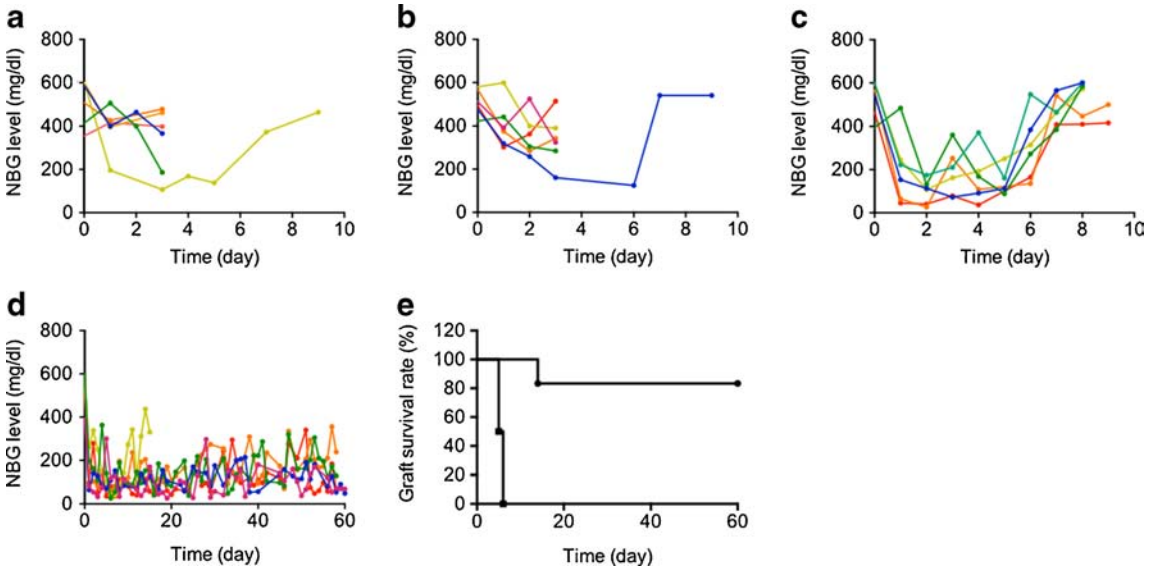

Fig. 2 Non-fasting blood glucose level after islet transplantation subcutaneously into diabetic mice (**a**) Islet group ($n=6$), (**b**) Islet-Clodromsome group (Subcutaneously; 6.25 mg/kg) ($n=6$), (**c**) Islet-Matrigel group ($n=6$), (**d**) Islet-Matrigel-Clodrosome group ($n=6$), (**e**) Graft survival rate of matrigel encapsulated islet recipients with (*black circle*) or without (*black square*) liposomal clodronate

were 582.75±269.49 pg/ml and 3.25±6.50 pg/ml, respectively. In addition, the TNF-α concentration in the injected matrigel was also significantly decreased by the liposomal clodronate such that the TNF-α concentrations in matrigels of Islet-Matrigel group and Islet-Matrigel-Clodrosome group were 72.33±10.40 pg/ml and 6.80±4.73 pg/ml ($p<0.001$), respectively. Likewise, the serum concentrations of IL-1β and TNF-α were also decreased by liposomal clodronate in the matrigel from 202.33±73.08 pg/ml to 138.67±49.51 pg/ml and 20.54±11.42 pg/ml to 15.59±12.14 pg/ml, respectively (Fig. 5c). These results indicated that the macrophage-depleting agent significantly reduce the secretion of IL-1β and TNF-α from macrophage and inflammatory reaction. In addition, the concentration of these pro-inflammatory cytokines at the site of injected matrigel was higher than in the serum, which justifies the co-delivery of liposomal clodronate along with islets implanted within matrigel instead of systemic delivery of liposomal clodronate.

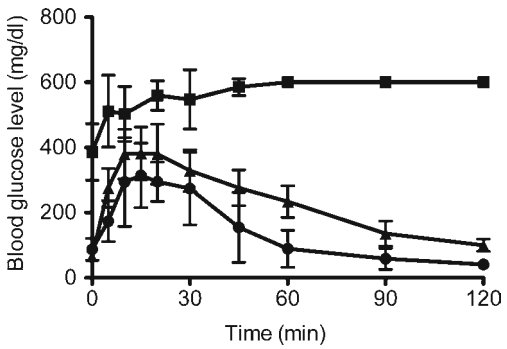

Fig. 3 The IPGTT of normal mouse (*black triangle*; $n=7$), diabetic mouse (*black square*; $n=3$) and Islet-Matrigel-Clodrosome group (*black circle*; $n=5$, at 60 day of transplantation), Data were expressed as mean ± SD

DISCUSSION

In this study, we have developed a new strategy toward improving anti-inflammation and immunoprotection against the locally delivered islets for the treatment of type 1 diabetes. This strategy can be used to maintain functionality and viability of locally delivered islets by co-embedding with macrophage depleting agent, liposomal clodronate, in an injectable hydrogel. The early immune reactions are normally initiated by macrophage and neutrophil activation. Macrophages cause inflammatory reaction in the microenvironment of locally delivered islets and induce immune cell migration into the islet delivered area. Macrophages secrete inflammatory cytokines and reactive oxygen species that can damage the transplanted islets and induce the activation of antigen presenting cells. These activated antigen-presenting cells trigger the adaptive immune reactions. The most potent period of macrophage action against delivered islets are during the first 2 weeks of islet injection (3). Thus, it is important to inhibit the activation of macrophage in the early stage and the progression to adaptive immune reaction.

In this study, locally co-delivered liposomal clodronate using matrigel effectively inhibited the activation of macrophage after islet injection. The islet survival time of Islet-Matrigel-Clodrosome group was significantly increased compared to that of Islet-Matrigel group because co-encapsulated liposomal clodronate was remained in the matrigel over 7 days, thereby consistently depleting macrophages. The optical imaging analysis showed that liposomal clodronate was present in the matrigel over 7 days. The consistent retaining of liposomal clodronate in the matrigel effectively inhibited the activation of macrophage in the microenvironment of the

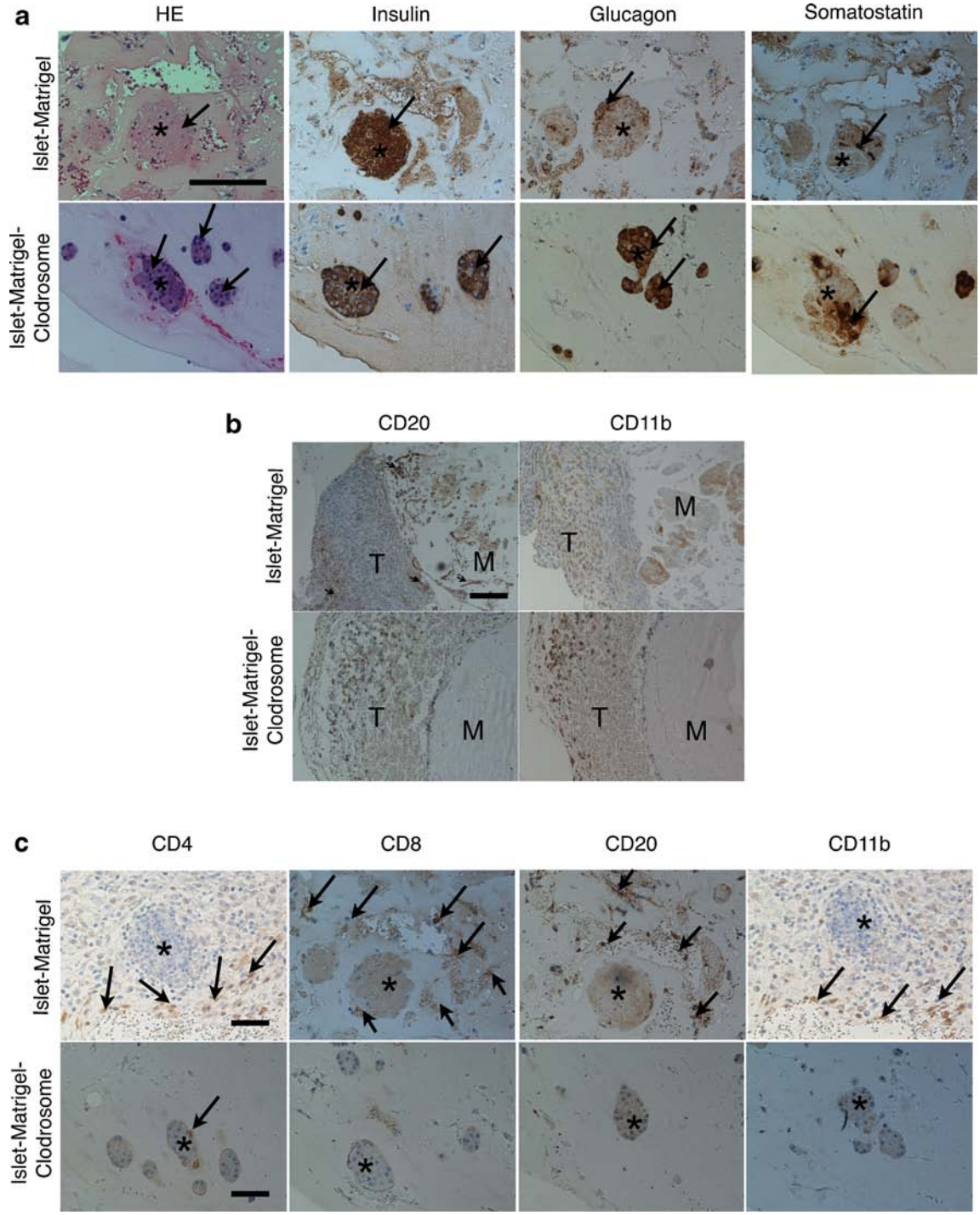

Fig. 4 Immunohistochemistry analysis after local delivery of islets (Islet-Matrigel-Clodrosome group; at 60 day, Islet-Matrigel group; at 10 day). (**a**) HE, insulin, glucagon and somatostatin staining (**b**) Evaluation of immune cell infiltration into injected matrigel. (CD20 and CD11b), (T; Tissue, M; matrigel) (**c**) CD4, CD8, CD20 and CD11b positive cell staining. *Asterisk*: transplanted islets, *Arrows*: pointing at the corresponding staining. Scale bars = 100 μm

islet-loaded matrigel. There are several probable reasons for which transplanted islet graft survival time might increase up to 60 days even though the liposomal clodronate retained time in the transplanted site is only over 7 days. First, the released cytokines from the activated macrophages directly damaged islets. In this way, inhibiting macrophages might preserve a huge mass of transplanted islets for the early period following transplantation. Second, macrophage inhibition slows down the immune recognition process by antigen presentation and by pro-inflammatory cytokines released by macrophages

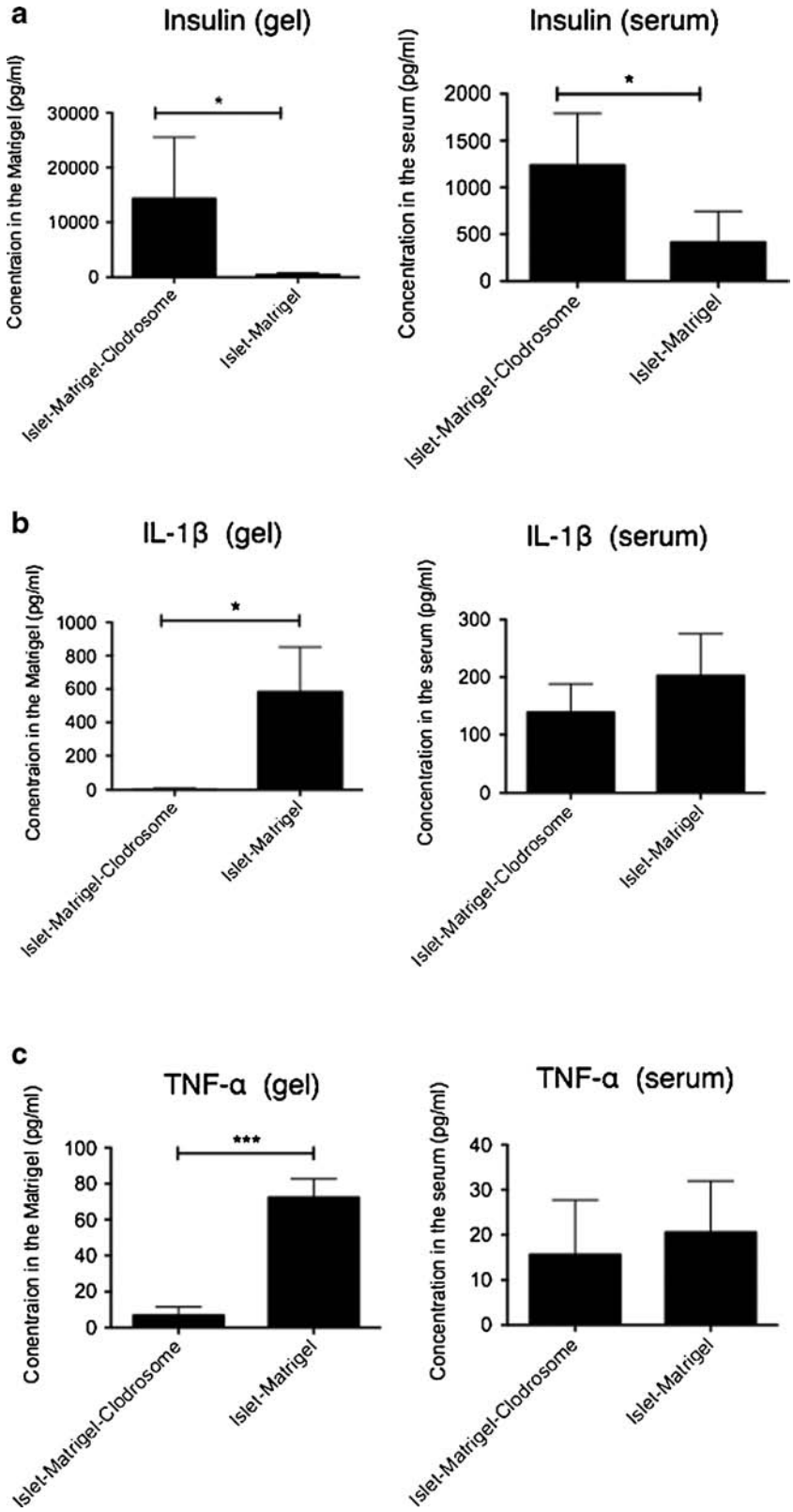

Fig. 5 Insulin and pro-inflammatory cytokine levels in recipient's serum and injected matrigel (Islet-Matrigel-Clodrosome group; at 60 day, Islet-Matrigel group; at 10 day). (**a**) insulin ($n = 5$) (**b**) IL-1β ($n = 3$) (**c**) TNF-α ($n = 3$), Data were expressed as mean ± SD. (*$P < 0.05$ and ***$P < 0.001$)

(16,17). The prolonged acceptance of the transplanted graft by locally delivered clodronate liposome has been argued to be dependent upon tolerance grown in the graft (18). Therefore, the inhibition effects on the early stage immune reaction would induce the adaptation of immune reactions.

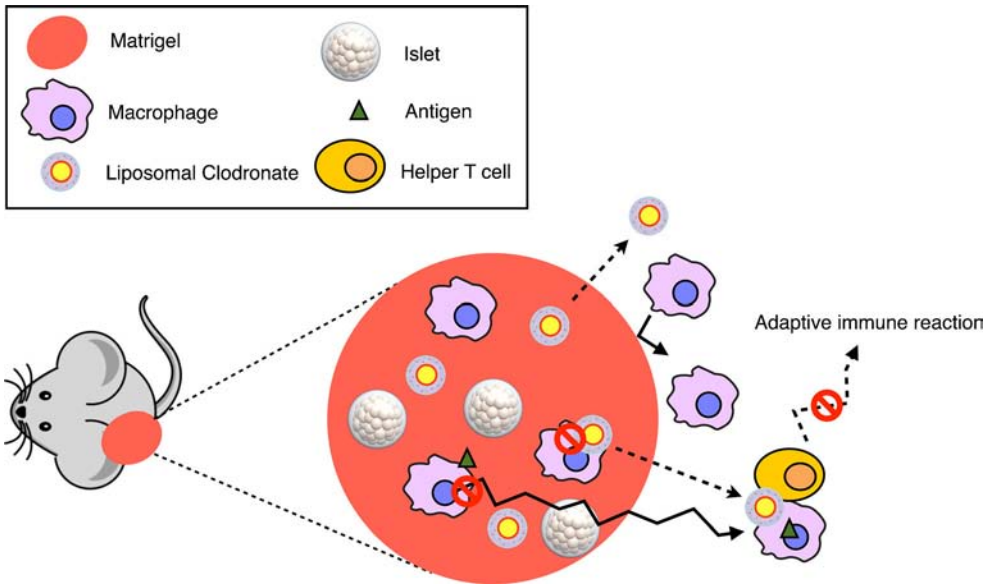

Fig. 6 Illustration of immunoprotection of locally co-delivered liposomal clodronate for the successful local delivery of islets

Cellular immune rejection is considered dominant when xenogenic islets are transplanted into the body of another (19,20). Islet graft rejection is initiated when the host macrophages trigger immune responses to any foreign proteins produced by xenogeneic organs, where antigen-presenting cells (APC) are recognized by the T cells (21). There are two different ways to activate immune reaction: the direct pathway where the donor antigens are presented to donor APC and the indirect pathway where the donor antigens are presented to recipient APC. These two immune reaction pathways were initiated by APC in the transplanted area. When single dose of liposomal clodronate was systemically injected into recipients, the drug concentration at the transplanted area was very low therefore unsuccessfully blocked the macrophage activation pathways. However, our local drug delivery system simultaneously depleted the host and recipient's macrophages in the local islet-injected area, thereby effectively inhibited the direct and indirect immune reaction (22,23). Since the macrophage is activated by the transplanted islets in the matrigel, the local effect of clodrosome surrounding the matrigel would be important. Therefore, if the release rate of clorodsome from the matrigel was sustained better by controlling the porosity of the matrigel or enhancing the interaction of the liposome with the matrigel, the immunoprotective effect of clodrosome would be further improved. The general side effects of systemically administered clodrosome are abdominal pain and diarrhea, nausea, vomiting, tingling sensation in lips or tongue, change in kidney function, osteonecrosis etc. However, these side effects were not detected in this study. The incidence of these side effects might be significantly reduced in matrigel system since the lower dose of clodronate in the local site was administered and it was slowly released out. Once it is uptaken by the macrophages, the liposome layers are disrupted under the influence of lysosomal phospholipases. The released clodronate inside the macrophage cell does not escape by crossing the cell membrane but accumulates within the cell, eventually killing the cell. Clodronate released from the dead macrophages cannot penetrate the cell membrane of other cell and has extremely short half-life, which explains its low side effect. On the other hand, systemically administered clodrosome may cause unwanted destruction of macrophages in an undesired part of the body, which may make the recipient severely immune deficient.

The normal blood glucose level of recipients was achieved up to 60 days after islet delivery, and macrophage, T cell, B cell and pro-inflammatory cytokine accumulation was significantly decreased in the transplantation site. The results obtained from the *in vivo* experiment reveal that initial macrophage depletion by liposomal clodronate plays an important role in improving the overall graft survival time. In addition, although the dose of liposomal clodronate in matrigel was equivalent to one-fifth of the clinical dose, the survival time of grafted islets was remarkably increased.

Therefore, locally co-delivered liposomal clodronate would require lower dose of liposomal clodronate to achieve therapeutic effect than the total dose of repeatedly systemically administered liposomal clodronate (16,24). Subsequently, locally delivered liposomal clodronate could reduce the undesired side effects associated with systemic drug delivery. In this study, since the liposomal clodronate is physically embedded in the matrigel, a prolonged macrophage depleting effect was observed, and locally delivered liposomal clodronate in matrigel effectively improved the grafted survival time of islets.

CONCLUSION

We established new immunoprevention protocol for improving the survival time of subcutaneously injected islets with

matrigel containing liposomal clodronate. Locally delivered liposomal clodronate acted to depletion of macrophages for a longer periods (Fig. 6). At the same time, pro-inflammatory cytokines secreted from macrophages were significantly decreased in Islets-Matrigel-Clodrosome group. These findings have an excellent potential for the treatment of type 1 diabetes that diminished acute-immune reaction and prolonged graft survival of islets.

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