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BRIEF COMMUNICATION

Survival of Microencapsulated Islets at 400 Days Posttransplantation in the Omental Pouch of NOD Mice

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The long-term durability of agarose microencapsulated islets against autoimmunity was evaluated in NOD mice. Islets were isolated from 6–8-week-old prediabetic male NOD mice and microencapsulated in 5% agarose hydrogel. Microencapsulated or nonencapsulated islets were transplanted into the omental pouch of spontaneously diabetic NOD mice. Although the diabetic NOD mice that received nonencapsulated islets experienced a temporary reversal of their hyperglycemic condition, all 10 of these mice returned to hyperglycemia within 3 weeks. In contrast, 9 of 10 mice transplanted with microencapsulated islets maintained normoglycemia for more than 100 days. Islet grafts were removed at 100, 150, 200, 300, and 400 days posttransplantation. A prompt return to hyperglycemia was observed in the mice after graft removal, indicating that the encapsulated islet grafts were responsible for maintaining euglycemia. Histological examination revealed viable islets in the capsules at all time points of graft removal. In addition, β-cells within the capsules remained well granulated as revealed by the immunohistochemical detection of insulin. No immune cells were detected inside the microcapsules and no morphological irregularities of the microcapsules were observed at any time point, suggesting that the microcapsules successfully protected the islets from cellular immunity. Sufficient vascularization was evident close to the microcapsules. Considerable numbers of islets showed central necrosis at 400 days posttransplantation, although the necrotic islets made up only a small percentage of the islet grafts. Islets with central necrosis also showed abundant insulin production throughout the entire islets, except for the necrotic part. These results demonstrate the long-term durability of agarose microcapsules against autoimmunity in a syngeneic islet transplantation model in NOD mice.

Key words: Islet transplantation; Autoimmunity; Microencapsulation; Agarose

INTRODUCTION

Graft rejection can be prevented by placing the donor tissue inside immunoisolation devices constructed of semipermeable membranes. Microcapsules keep the donor tissues separate from the recipient immune system and thus negate the need for systemic immune suppression. Isolated pancreatic islets transplanted for the treatment of type 1 diabetes are subject to both allogeneic rejection and autoimmune destruction by the recipient's immune system. Therefore, to achieve "immunosuppressant-free islet transplantation," microcapsules are required to protect the islet graft from both allogeneic rejection and recurring autoimmunity. Although previous studies have already demonstrated the ability of agarose microcapsules to protect against rejection (9,11,18,19), there have been insufficient studies to conclusively demonstrate the protection of microencapsulated islet grafts against autoimmunity. With this in mind, we previously studied the ability of agarose microcapsules to protect islets from autoimmune destruction (13). However, the question of how long these microcapsules can function and maintain their shape in vivo was not assessed in our previous study. Thus, the long-term durability of agarose micro-

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capsules in an autoimmune environment remains unclear.

The most commonly used microcapsules are composed of alginate. Graft survival of alginate encapsulated islets is remarkably prolonged compared to that of nonencapsulated islets, but the duration of euglycemia is unfortunately limited to periods varying from several months in rats (4) to 6 months in dogs (17). Although long-term graft survival of alginate microencapsulated islets was reported in NOD mice (5), others reported the limited graft survival of alginate microencapsulated islets even in an islet isograft model (2–4,16). De Groot et al. (2) maintained that a lack of biocompatibility and hypoxia may be responsible for the limited graft survival of alginate microencapsulated islets. We focused on agarose microcapsules, which possibly differ from alginate with regard to biocompatibility, and tried to overcome the hypoxia issue by transplantation into the omental pouch, which exhibits angiogenic potential by promoting neovascularization in ischemic tissues. In this study, microencapsulated NOD islets were transplanted into the omental pouch in NOD mice and subsequently followed for long-term graft function, which was confirmed histologically at 400 days posttransplantation.

MATERIALS AND METHODS

Animals

NOD/Shi mice were purchased from Clea Japan (Tokyo, Japan). In these animals, the cumulative incidence of diabetes is 23% and 80% in females and 0% and 30% in males at 20 and 30 weeks of age, respectively (data provided by Clea Japan). Diabetic female mice were used as recipients and young (6–8 weeks of age) nondiabetic male mice were used as islet donors. Prediabetic female mice were screened weekly for diabetes by measuring the nonfasting blood glucose level between 0800 and 1000 h, and spontaneous diabetes was diagnosed when the blood glucose level in two consecutive measurements exceeded 20.0 mmol/L. Diabetic NOD mice were transplanted with islets 5–10 days after onset of diabetes.

Islet Isolation

Donor islets were prepared using a modification (7) of the method described by Lacy et al. (14). Briefly, under sodium pentobarbital anesthesia, pancreata were distended with 2.5 ml of cold Hanks' balanced salt solution (HBSS) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 1.5 mg/ml of collagenase (type V; Sigma, St. Louis, MO). The distended pancreata were then removed and digested for 12 min at 37°C. Islets were purified by centrifugation through discontinuous dextran gradients.

Microencapsulation

Islets were microencapsulated in agarose according to previously reported methods with minor modifications (10). Briefly, purified islets were suspended in 0.2 ml of HBSS and then mixed with 5% agarose solution (Agarose-LGT, Nacalai Tesque Inc, Kyoto, Japan, 150 mg agarose in 3 ml of HBSS). The agarose solution containing the islets was emulsified by adding paraffin oil in 37°C and then immediately solidified by manual shaking in an ice bath for 5 min. Agarose microcapsules were washed with HBSS and then cultured at 37°C overnight. Because a considerable number of empty capsules were made during the encapsulation process, the microcapsules containing islets were hand-picked under a microscope prior to transplantation. This encapsulation procedure results in the formation of morphologically consistent microcapsules. The diameter of each capsules with an entrapped islet ranged from 100 to 400 μm. Typically, each capsule did not contain more than one islet.

Transplantation

Microencapsulated islets were transplanted into the omental pouch of diabetic mice under sodium pentobarbital anesthesia. A median abdominal incision was made and the greater omentum was spread out onto wet gauze. Grafts consisting of 1500 microencapsulated islets (approximately 500 μl) were placed onto the exposed omentum. Next, the omentum was folded up and attached to the stomach using 5-0 nylon sutures to make a pouch. Controls included mice that had 1500 nonencapsulated islets transplanted into the omental pouch.

Assessment of Graft Function

Islet recipients were monitored daily for the first 2 weeks posttransplantation and twice a week thereafter for their nonfasting blood glucose level. Diabetes recurrence was diagnosed when the blood glucose level was greater than 11.1 mmol/L on any single measurement.

Histological Examination

Omental pouches were removed at 100, 150, 200, 300, and 400 days posttransplantation from recipients that had maintained normoglycemia. The graft-bearing omentum was fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4.5 μm. The sections were stained with an anti-insulin antibody (Oriental Yeast Co. Ltd., Osaka, Japan) using an immunoperoxidase technique and counterstained with hematoxylin and eosin. The omentum was also evaluated histologically by aldehyde-fuchsin staining as previously described (15).

RESULTS

Graft Function

Although diabetic NOD mice that received 1500 nonencapsulated islets experienced a temporary reversal of their hyperglycemic condition within 2 days after transplantation, all of these mice returned to hyperglycemia at 12.2 ± 4.0 days (range, 6–21 days) posttransplantation (Table 1, Fig. 1A). In contrast, mice that received 1500 microencapsulated islets experienced normoglycemia for extended periods of time after transplantation (Fig. 1B). Their blood glucose level dropped from a pretransplantation level of 25.6 ± 3.0 mmol/L to 4.0 ± 0.9 mmol/L at 2 days posttransplantation. All these mice maintained normoglycemia for more than 100 days, except for one mouse that returned to hyperglycemia at 65 days posttransplantation. Omental pouches were removed at 100, 150, 200, 300, and 400 days posttransplantation from recipients that had maintained normoglycemia. A prompt return to hyperglycemia in these mice was observed after graft removal, indicating that the encapsulated islet grafts were responsible for maintaining the euglycemia.

Histological Examination

Transplanted microencapsulated islets firmly adhered to the omentum. Histological examination of the graft-bearing omentum revealed viable islets in the capsules without mononuclear cellular infiltration at all time points of graft removal (Fig. 2 shows the histological findings at 400 days posttransplantation). In addition, β-cells within the capsules remained intact and well granulated, indicating the presence of insulin (Fig. 2A–C). Morphological irregularities of the microcapsules were not observed. No immune cells were detected inside the microcapsules at any time point, suggesting that the microcapsules successfully protected the islets from cellular immunity. Sufficient vascularization was evident close to the microcapsule (Fig. 2A, C). However, as shown in Figure 2D–F, a considerable number of islets showed central necrosis at 400 days after transplantation, although the necrotic islets only made up a small percentage of the islet grafts. Islets with central necrosis also had intact capsules and showed sufficient insulin production, except for the necrotic part, as evaluated by immunohistochemistry of the entire islet (Fig. 2E).

DISCUSSION

In this study, we achieved reversal of diabetes in a syngeneic NOD mouse model and demonstrated the long-term durability of agarose microcapsules in vivo. To our knowledge, this is the first report to present histological data for functioning microencapsulated islets at 400 days posttransplantation.

It is interesting to note that a considerable number of the microencapsulated islets had central necrosis, as indicated by the histology. This result led us to speculate about the mechanism of graft failure for microencapsulated islets. Microcapsules allow the passage of small molecules such as insulin and glucose, but prevent the entry of immune cells. The exact mechanism for microencapsulated islet graft failure (e.g., cytokines, nitric oxide, perforin, poor nutrition, or hypoxia) is currently unknown. Based on the histological finding of central necrosis in the islets, we speculate that chronic hypoxia and poor nutrition are the most likely major causes of islet cell death in microcapsules. If cytotoxic mediators, such as cytokines, were responsible for the graft necrosis, the islets would be damaged around the periphery with the center remaining intact and viable. However, the observations in the current study reveals that the opposite is in fact the case, with islets appearing necrotic in the center and remaining viable and insulin-rich around the periphery. These results therefore suggest that microencapsulated islets lose function due to the detrimental effects of chronic hypoxia and poor nutrition, because the central part of the islets probably suffers more severe hypoxia and poorer nutrition than the periphery.

Alginate is commonly used as the capsule material for microencapsulated islet transplantation. However, limited graft survival has been reported, even with islet isografts (2–4,16), whereas long-term graft function was reported in NOD mice (5). In this study we achieved long-term normoglycemia with agarose microcapsules up to 400 days posttransplantation. Some possible reasons for the improved graft survival observed in the

Table 1. Graft Survival of Nonencapsulated and Microencapsulated Islets in NOD Mice

	n	Graft Survival (Days)	Mean ± SD
Nonencapsulated islets	10	6, 9, 10, 10, 12, 12, 13, 14, 15, 21	12.2 ± 4.0
Microencapsulated islets	10	65, 100*, 100*, 150*, 150*, 200*, 200*, 300*, 300*, 400*	—

*Time of removal of microencapsulated islets in days.

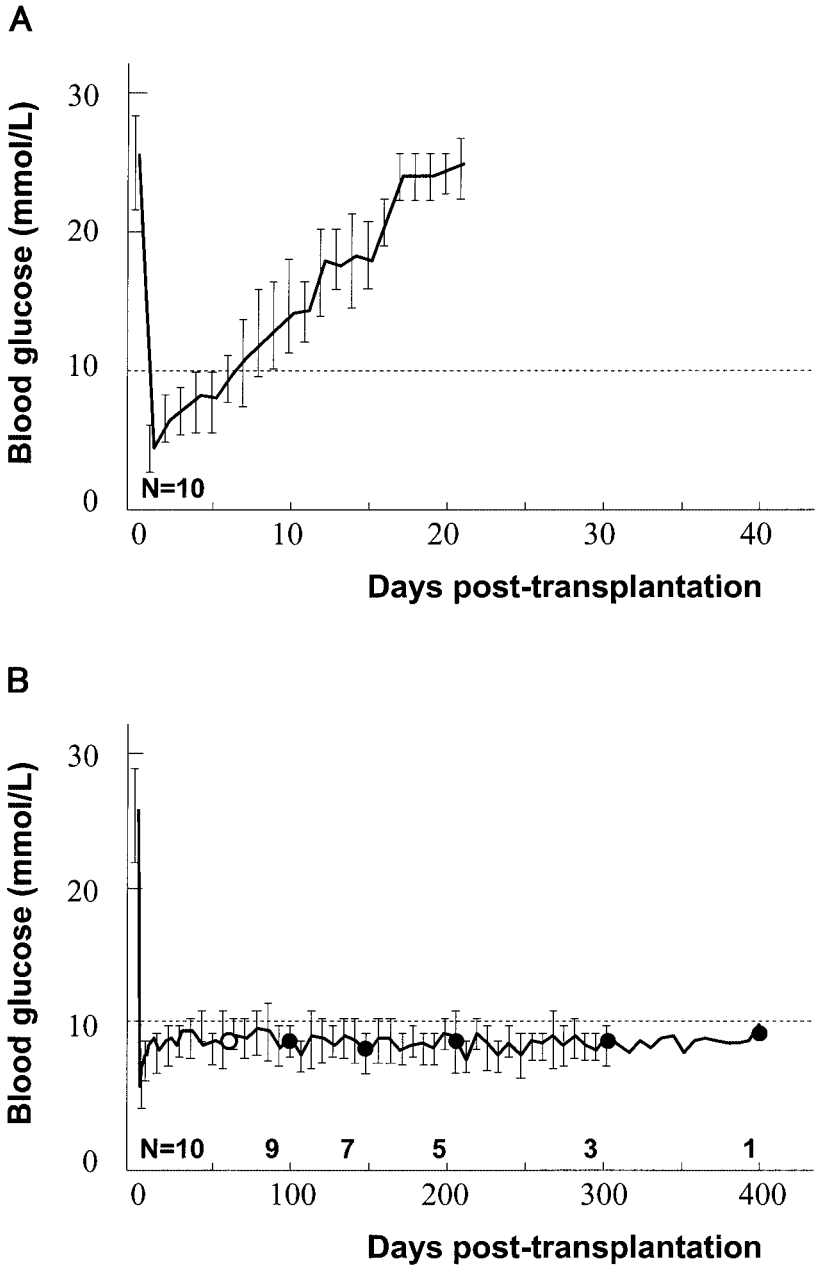

Figure 1. Diabetic NOD mice that received 1500 nonencapsulated islets experienced a temporary reversal of their hyperglycemic condition within 2 days after transplantation, but later returned to hyperglycemia within 3 weeks (A). In contrast, mice that received 1500 microencapsulated islets experienced normoglycemia for extended periods of time after transplantation (B). One recipient returned to hyperglycemia at 65 days posttransplantation (open circle). Omental pouches were removed for histology at 100, 150, 200, 300, and 400 days posttransplantation from recipients that had maintained normoglycemia (filled circles).

present study can be considered as follows. The first is that the biocompatibility of agarose may differ from that of alginate. When empty microcapsules are transplanted into the peritoneal cavity, the status of the capsules greatly depends on the capsule material used. Most alginate microcapsules remain free floating in the peritoneal cavity without vascularization (Kobayashi et al., submitted manuscript 2005), whereas agarose microcapsules

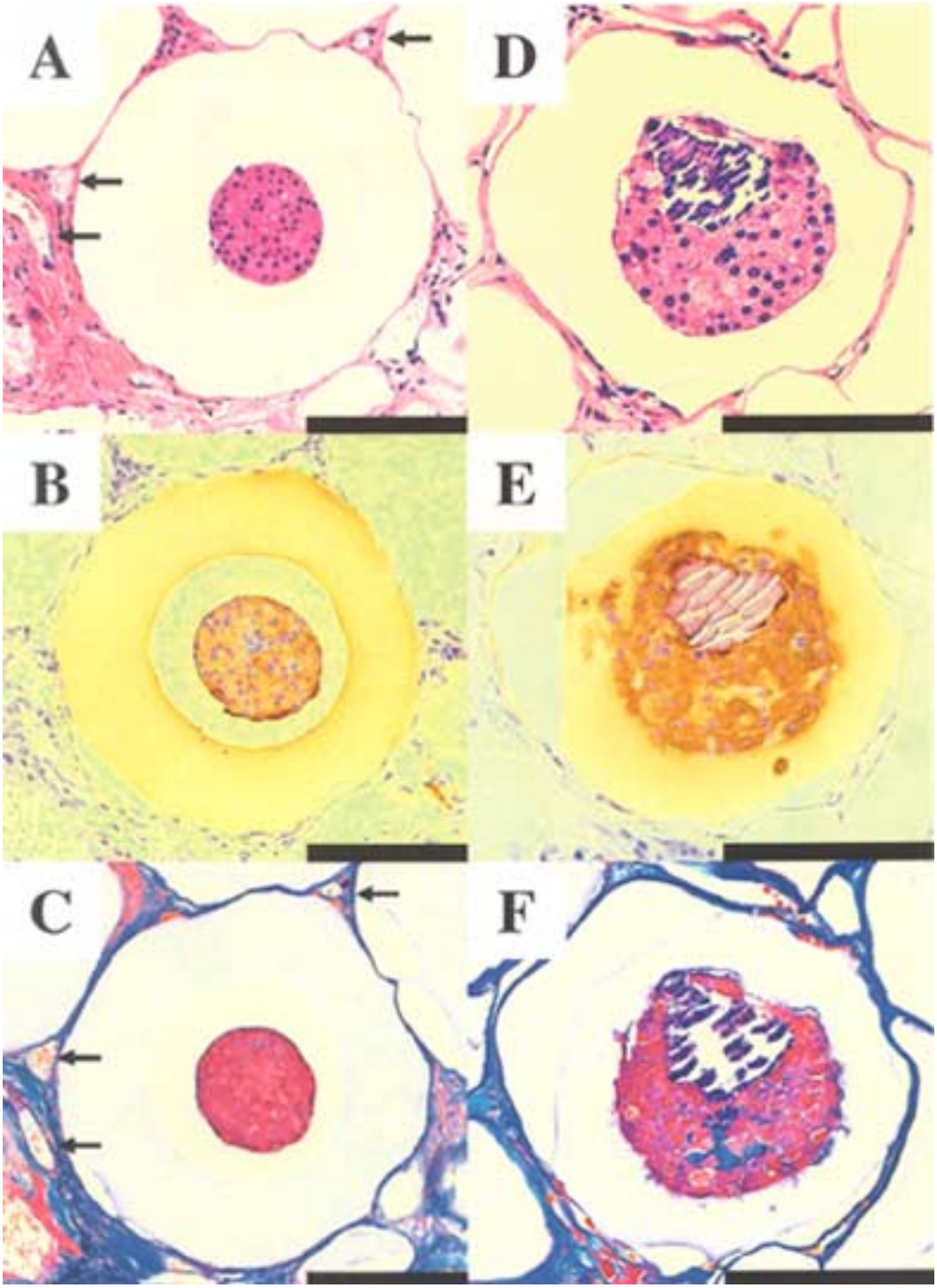

Figure 2. Histological findings of encapsulated islet grafts obtained from the omental pouch at 400 days posttransplantation. Hematoxylin and eosin staining (A) revealed viable islets in the capsule. Morphological irregularities of the microcapsules were not observed. Immunohistochemical insulin staining (B) shows sufficient insulin production in well-granulated β-cells. Aldehyde-fuchsin staining (C) reveals many viable β-cells that are stained violet. Sufficient vascularization is evident close to the microcapsule (A and C, arrows). (A), (B), and (C) are consecutive sections. However, as shown in (D), (E), and (F), some islets show central necrosis at 400 days posttransplantation, although the necrotic islets only make up a small percentage of the islet grafts. Islets with central necrosis also have intact capsules (D) and show sufficient insulin production, except for the necrotic part, as evaluated by immunohistochemistry (E) of the entire islet. Aldehyde-fuchsin staining (F) reveals many viable β cells that are stained violet. (D), (E), and (F) are consecutive sections. Scale bars: 100 μm.

adhere to the peritoneum and become embedded in the host tissue (13). Moreover, histological examination has revealed that capillaries develop around agarose capsules (13). Vascular formation around capsules may facilitate oxygen and nutrient delivery to the islets. It also may lead to a more rapid response of insulin secretion to hyperglycemia. On the other hand, it may cause an inflammation around capsules. Although it is unclear how beneficial vascularization is for islet survival at this time, these results suggest that agarose microcapsules have a different biocompatibility from alginate microcapsules at least in terms of vascular formation around capsules.

The second reason for improved graft survival is the site of transplantation. Many previous studies have reported that the omentum exhibits angiogenic potential in promoting neovascularization in ischemic tissues (6,8, 20), which may be beneficial for islet engraftment and function (1,12). Moreover, islet grafts placed in the omental pouch also provide hepatic portal delivery of the secreted insulin, thereby offering a greater physiological advantage over systemic delivery.

The third is that agarose may be harder and more stable than alginate. Alginate microcapsules are created by Ca^{2+} or Ba^{2+} cross-linked gel formation. These ions may gradually dissolve over time and there is the theoretical possibility that the capsules will disintegrate in the long-term following transplantation. In fact, De Vos et al. (3) observed that empty alginate capsules contained defects at 1 month posttransplantation and were overgrown by macrophages. On the other hand, agarose microcapsules are created by gel formation using low temperatures. Agarose solutions begin to gel below 25°C, but agarose gel does not begin to melt until 60°C. In other words, the agarose gel melting temperature is very different from the gelling temperature. Therefore, agarose microcapsules will not melt as long as the body temperature is maintained under 60°C. Consequently, agarose microcapsules may be advantageous with regard to stability and durability as an immunoisolation membrane in vivo.

In this study, long-term durability of agarose microcapsules against autoimmunity for up to 400 days posttransplantation was demonstrated in a syngeneic islet transplantation model in NOD mice. Further studies are needed to improve the capsule quality, including the biocompatibility, durability, and immunoprotective properties, in order to achieve the ultimate goal of "immunosuppressant-free islet transplantation" using microcapsules.

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