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Transient Beneficial Effects of Exendin-4 Treatment on the Function of Microencapsulated Mouse Pancreatic Islets

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Transplantation of microencapsulated islets may reduce hyperglycemia in the absence of immunosuppression. However, the efficiency of microencapsulated islet transplantation is low, requiring more islets to achieve normoglycemia than in vascularized islet transplantation. Exendin-4 (a glucagon-like receptor agonist) has been previously shown to improve islet transplantation outcome in rodents. We investigated whether this treatment would enhance the function of microencapsulated islets in vitro and in vivo. Encapsulated or naked islets were cultured with or without exendin-4 for 72 h. To test in vitro function, insulin release and glucose oxidation rates were measured in the absence or presence of exendin-4. In addition, in vivo function of a minimal mass of 350 microencapsulated islets was assessed by syngeneic transplantation into the peritoneal cavity of alloxan-diabetic mice. Glucose oxidation rates of microencapsulated islets were improved by 72-h pretreatment with exendin-4. Insulin release was increased both acutely after glucose stimulation and over a 40-h culture period by the presence of exendin-4. Transplantation outcome of microencapsulated islets cultured with exendin-4 was initially improved, but by day 7 there were no differences compared with control cultured microencapsulated islets. Culture of microencapsulated islets with exendin-4 increases glucose oxidation and insulin release rates, but the increased function seen in vitro was not enough to improve the long term outcome in a transplantation model.

Key words: Diabetes; Islet transplantation; Microencapsulation; GLP-1; Exendin-4; Alginate

INTRODUCTION

Transplantation of islets of Langerhans could provide a good alternative to insulin treatment for type 1 diabetes patients (28). However, immunosuppression is required to avoid immune rejection and/or recurrence of the autoimmune disease. The side effects of these drugs are extensive and are so severe that the risks outweigh the benefits of islet transplantation in most patients (26). Microencapsulation in simple alginate beads is a technique that could allow the transplantation of islets in the absence of immunosuppression (9). It is based on physical separation of the islets from the immune system. The large immune cells are excluded whereas small molecules such as nutrients and insulin can easily pass through the alginate gel barrier.

However, results from experiments with transplantation of microencapsulated islets to the peritoneal cavity have not been encouraging, with large numbers of islets being required to reverse hyperglycemia (6,9,16). It would therefore be of great interest if the outcome could be improved by, for example, treatment of the encapsulated islets prior to transplantation. Exendin-4, a glucagon-like peptide-1 (GLP-1) receptor agonist, has been shown to have many positive effects on pancreatic beta cells (10,11,20,25,31,32). Activation of the GLP-1 receptor (a G protein-coupled receptor) leads to increases in intracellular cyclic AMP levels in the beta cell (8,13). There is subsequent activation of the cAMP response element binding protein (CREB), which can lead to the expression of genes that play a role in beta cell function and survival (7,14,30). In addition, activation of the GLP-1 receptor activates other signaling pathways, including the phosphatidylinositol 3-kinase/Akt pathway, and induces genes that are implicated in beta cell survival (2,3,7,24). Previous studies using nonencapsulated islets showed that preculture of the islet graft with this peptide improves transplantation outcome in mice (17,29).

We therefore aimed to study the effect of exendin-4 on encapsulated mouse islets, both chronically during a 3-day culture period and acutely during insulin release and glucose oxidation measurements. Encapsulated mouse

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islets were also syngeneically transplanted to establish whether they were more effective after treatment with exendin-4.

MATERIALS AND METHODS

Animals

Inbred male C57BL/6J mice, weighing 25–30 g (M&B, Shensved, Denmark) were used as islet donors and recipients. Recipient animals were made diabetic by an IV alloxan injection (75 mg/kg body weight, Sigma, Irvine, UK). Mice with a nonfasting blood glucose concentration greater than 16.7 mM were considered diabetic and used as transplant recipients. During the experiments, the animals had free access to tap water and pelleted food. The animal experiments were approved by the local animal ethics committee for Uppsala University.

Islet Isolation

Pancreatic islets were isolated using collagenase digestion and density gradient purification. Briefly, the animals were anesthetized with an IP injection of 100 mg/kg pentobarbital sodium. Thereafter, 2–3 ml cold collagenase solution (from *Clostridium histolyticum*, Roche Diagnostics, Mannheim, Germany; 2.5 mg/ml Hanks' balanced salt solution, Statens Veterinärmedicinska Anstalt, Uppsala, Sweden) was injected into the pancreas via the common bile duct. After removal of the pancreas, enzymatic digestion was carried out in a 37°C water bath for 18 min. Density gradient separation was then performed on the digested tissue, by means of Histopaque-1077 solution (Sigma-Aldrich). Pelleted digested tissue was resuspended in 15 ml of Histopaque, and approximately 10 ml of RPMI-1640 (Sigma-Aldrich) was added carefully, forming a sharp interface. The samples were centrifuged at 900 × *g* for 22 min. Islets were collected from the interface, washed, and then suspended in culture medium.

Microencapsulation

Directly after isolation, islets were microencapsulated in alginate (1.8% w/v), 73% guluronic acid, and 27% mannuronic acid (Ultra Pure low viscosity high guluronic acid alginate, FMC Corporation, Drammen, Norway), which contained 0.3 M mannitol as an osmolyte. Microcapsules were formed using an electrostatic bead generator [previously described by King et al. (18)], where a mixture of islets and alginate solution was pushed through a needle into a solution of 50 mM $CaCl_2$ and 1 mM $BaCl_2$. The capsules were then washed in saline. Capsule diameter was approximately 700 μm.

Islet Culture

Directly after microencapsulation, islets were placed in culture in groups of 150 free-floating islets for 3 days, at 37°C (95% air and 5% CO_2), in culture medium RPMI-1640 supplemented with 1% (v/v) L-glutamine (200 mM; Sigma-Aldrich), benzylpenicillin (100 U/ml; Roche Diagnostics Scandinavia, Bromma, Sweden), streptomycin (100 μg/ml; Sigma-Aldrich), and 10% (v/v) fetal calf serum (Sigma-Aldrich). Exendin-4 (1 nM) was added to half the number of culture dishes. The microencapsulated islets were cultured for 3 days with a culture medium change after day 1.

Insulin Release

Triplicate groups of 10 encapsulated or naked islets were placed in glass vials containing 250 μl Krebs-Ringer bicarbonate buffer supplemented with 10 mM HEPES (Sigma-Aldrich) (hereafter referred to as KRBH buffer) supplemented with 2 mg/ml bovine serum albumin (fraction V; MP Biomedicals Inc, Aurora, OH, USA) and 1.7 mM or 16.7 mM glucose, for the first and second hour of incubation, respectively. Half of the islets, either naked or encapsulated, were exposed to exendin-4 during the insulin release experiments. After the incubations, the islets were pooled in groups of 30 and insulin was extracted overnight at 4°C. Insulin contents in the incubation media and homogenates were determined by insulin ELISA (Mercodia Rat Insulin ELISA; Mercodia AB, Uppsala, Sweden).

Glucose Oxidation Rate

Islet glucose oxidation rates were determined according to a previously described method (27). Triplicate groups of 10 encapsulated or naked islets were incubated in glass vials containing 100 μl KRBH supplemented with D-[U-^{14}C] glucose (0.3 mCi/mmol, Amersham, UK) and nonradioactive D-glucose to give final glucose concentrations of 1.7 and 16.7 mM. The $^{14}CO_2$ formed by cell metabolism was measured by liquid scintillation counting.

Islet Transplantation

Mice were anesthetized by inhalation of isoflurane. The inhalation gas was administered continuously by an isoflurane pump (Univentor 400 Anaesthesia Unit, AgnTho's AB, Lidingö, Sweden) and contained 2.2–2.3% isoflurane (Forene; Abbot Scandinavia AB, Solna, Sweden) in a mixture of 40% oxygen and 60% nitrogen. A small incision was made in the skin and then in the linea alba through which 350 microencapsulated islets were delivered into the peritoneal cavity using a 1-ml pipette.

In Vivo Experimental Groups

Mice were transplanted with encapsulated islets, which had either been cultured under normal conditions or with exendin-4. In the mice that had received exen-

din-4 cultured islets, half the mice were injected intraperitoneally with exendin-4 (1 nmol/kg) immediately after the surgery and then daily for a period of 14 days. The other mice were injected in the same way but with saline (200 µl). These injections were carried out in the late afternoon. Blood glucose was measured from tail vein samples on days 0, 1, 3, 7, 14, 21, and 28.

Statistical Analysis

Values are expressed as mean ± SEM. When two groups were compared, Student's *t*-test was used. When more than two groups were compared, one-way repeated measures analysis of variance (RM ANOVA) was used. In cases where several treatments were being compared a two-way RM ANOVA was used. If there was a statistically significant difference between groups in the ANOVA, individual groups were compared using the Bonferroni post hoc test. For all comparisons, values of $p < 0.05$ were considered statistically significant. All statistics were carried out using Sigmastat 3.1 (Systat Software, Erkrath, Germany).

RESULTS

Effects of Exendin-4 on In Vitro Function of Naked and Encapsulated Islets

Culture media were harvested from day 2–3 of culture. Both in the case of naked and encapsulated islets, the insulin accumulation to the culture media was increased twofold by the presence of exendin-4 (Fig. 1) ($p = 0.024$ and $p = 0.038$, respectively, paired Student's *t*-test).

Independently of the culture condition during the 3 days, both encapsulated and naked islets had a 3- to 10-fold increased insulin secretion in response to 16.7 mM glucose in the short-term experiments (Fig. 2). Moreover, exendin-4 increased the glucose-induced insulin release, compared to control islets, especially when exendin-4 was present both during the culture period and in the insulin release incubation media [$p = 0.021$ for naked islets cultured and incubated with exendin-4 (Fig. 2a), and $p = 0.002$ for encapsulated islets incubated with exendin-4 and $p = 0.002$ for encapsulated islets both cultured and incubated with exendin-4 (Fig. 2b), RM ANOVA with Bonferroni post hoc test].

There was no difference in islet insulin content after naked or encapsulated islets were cultured with exendin-4 ($p = 0.075$, two-way RM ANOVA) (Fig. 3). However, the islet insulin content after 3 days of culture and after the short-term insulin release experiments was reduced in the encapsulated islets ($p = 0.004$, two-way RM ANOVA) (Fig. 3).

Presence of exendin-4 in the media during the 3 days of culture increased the glucose oxidation rate in the encapsulated islets ($p < 0.001$, paired Student's *t*-test), but did not affect that of the naked islets (Fig. 4). However, no further acute effects on glucose oxidation rates were observed when exendin-4 was added to the incubation media (data not shown).

Effects of Exendin-4 on In Vivo Function of Intraperitoneally Transplanted Encapsulated Islets

Transplanted mice had an increase in body weight from day 0 to day 28 posttransplantation; however, no statistically significant difference due to exendin-4 treat-

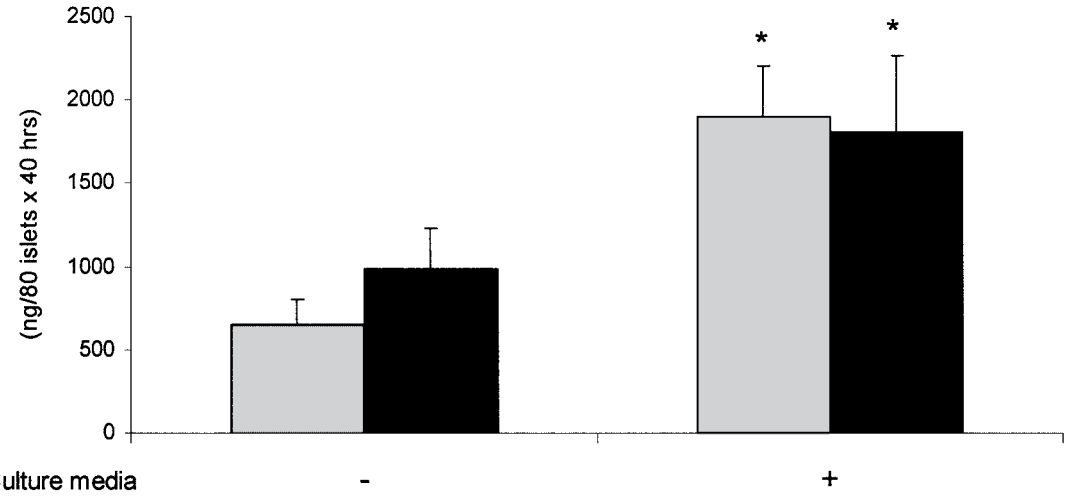

Figure 1. Insulin accumulation from 80 naked (gray columns) or microencapsulated (black columns) islets cultured for 40 h with (+) or without (–) 1 nM exendin-4 added to the culture media. *$p < 0.05$ when compared to corresponding control cultured islets, paired Student's *t*-test ($n = 4$).

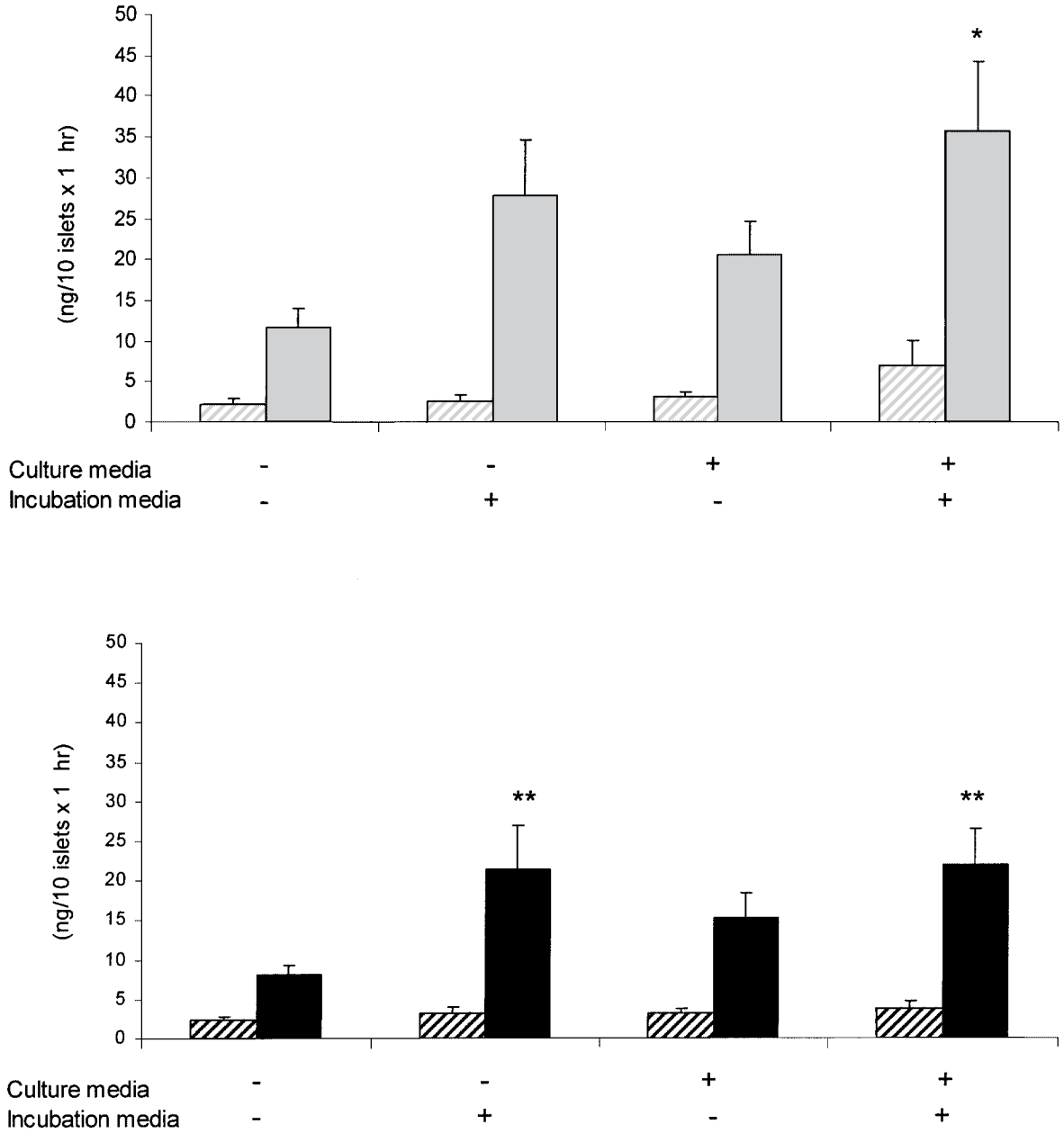

Figure 2. Insulin release, at 1.7 mM glucose (hatched columns) followed by 1 h at 16.7 mM (filled columns), of triplets of 10 naked (a) or encapsulated (b) islets cultured for 3 days with (+) or without (–) 1 nM exendin-4 added to the culture media, and with (+) or without (–) 1 nM exendin-4 added to the short-term incubation media. *$p < 0.05$ and **$p < 0.01$ when compared to corresponding control cultured and control incubated islets, RM ANOVA with Bonferroni post hoc test ($n = 5$).

ment was observed at any time point (data not shown). In general, the alloxan-diabetic mice transplanted with 350 encapsulated islets had decreased blood glucose levels already the first day after transplantation (from 24.4 ± 0.6 mM day 0 to 10.5 ± 0.7 mM glucose, $n = 29$) (Fig. 5).

Mice transplanted with exendin-4 precultured encapsulated islets had lower blood glucose levels than the control mice on day 3 after transplantation ($p = 0.037$, Student's *t*-test). At all other time points tested their blood glucose levels did not differ statistically significantly from those of the control mice. Exendin-4 treatment of the recipients by IP injections for 14 days had no further beneficial effect (data not shown).

DISCUSSION

Beneficial effects of GLP-1 and exendin-4 have been reported both in vitro and in vivo (10,11,20,25,31,32). With regard to islet transplantation, the most interesting

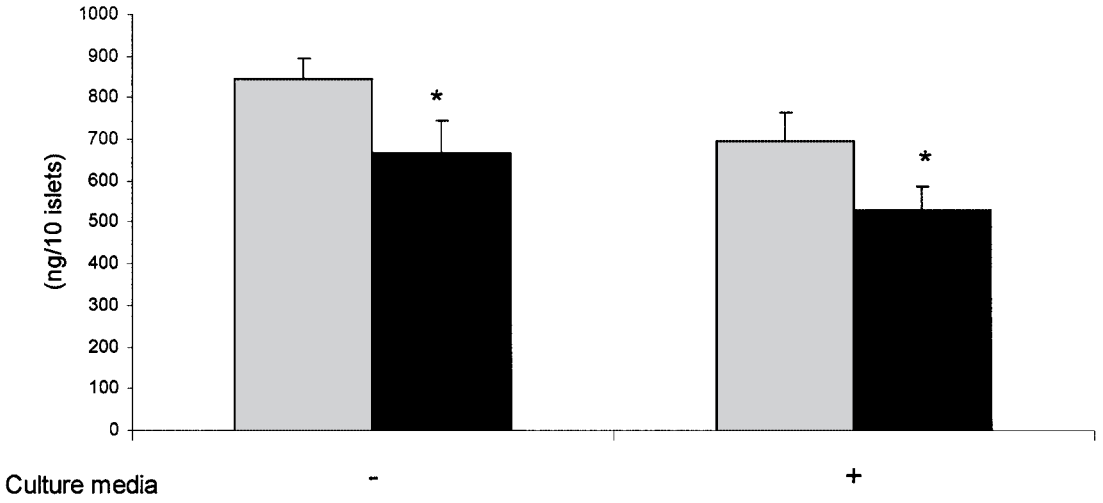

Figure 3. Insulin content of 30 naked (gray columns) or microencapsulated (black columns) islets after 3 days of culture and after a short-term stimulated insulin release experiment. The islets were cultured either with (+) or without (–) 1 nM exendin-4 added to the culture media ($n = 8$). *$p < 0.05$ for microencapsulated control or exendin-4 cultured islets compared to corresponding group of naked islets, two-way RM ANOVA ($n = 8$).

effects of exendin-4 may be its effects on beta cell proliferation and antiapoptotic effects (10,20,32). An increase in beta cell replication or decrease in beta cell apoptosis rate could potentially increase the beta cell mass, which is a critical parameter in islet transplantation. Indeed, it has been estimated that in diabetic rodents approximately 50% of islets do not survive the first few days of transplantation (5), especially if a suboptimal number of islets are transplanted (22). It is therefore essential to improve islet transplantation efficiency.

Pretreatment of islets prior to transplantation could be an effective way to improve transplantation outcome. Although it has previously been shown that fresh islet transplantation is more effective in rodents (17,23), culture of islets may be desirable in human islet transplantation (12). One advantage of treatment of islets in culture is that the patient does not have to be administered with the drug systemically and thus it is a safer option. A variety of compounds have been used in islet culture in the hope of improving transplantation outcome, including nicotinamide (19), nerve growth factor (21), and interleukin 6 (4). Exendin-4 is an ideal compound, as it has a wide variety of positive effects on beta cells, as

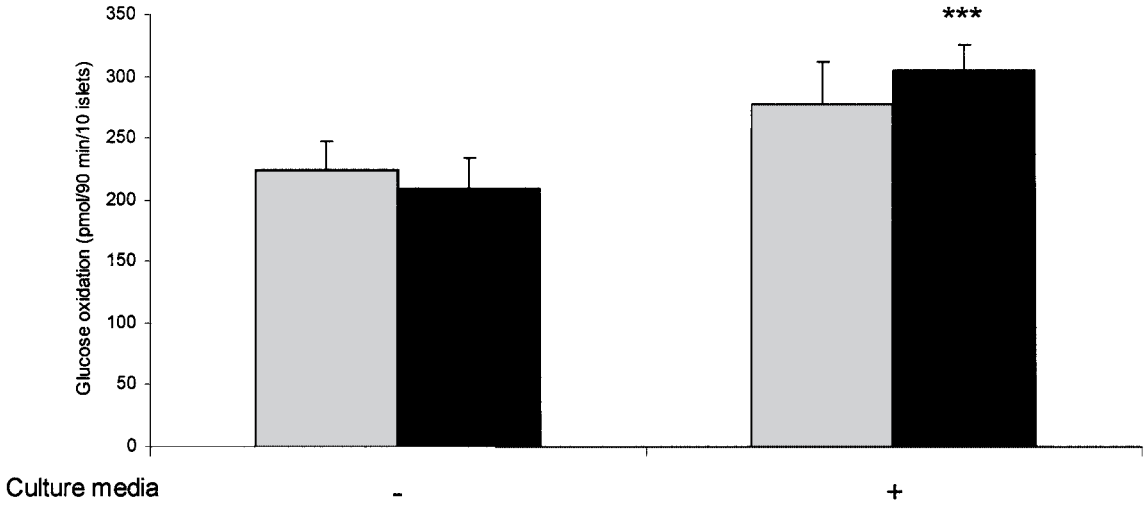

Figure 4. Rates of glucose oxidation at 16.7 mM glucose of triplets of 10 naked (gray columns) or encapsulated (black columns) islets cultured for 3 days with (+) or without (–) 1 nM exendin-4 added to the culture media. ***$p < 0.001$ when compared to corresponding control cultured islets, paired Student's t-test ($n = 10$).

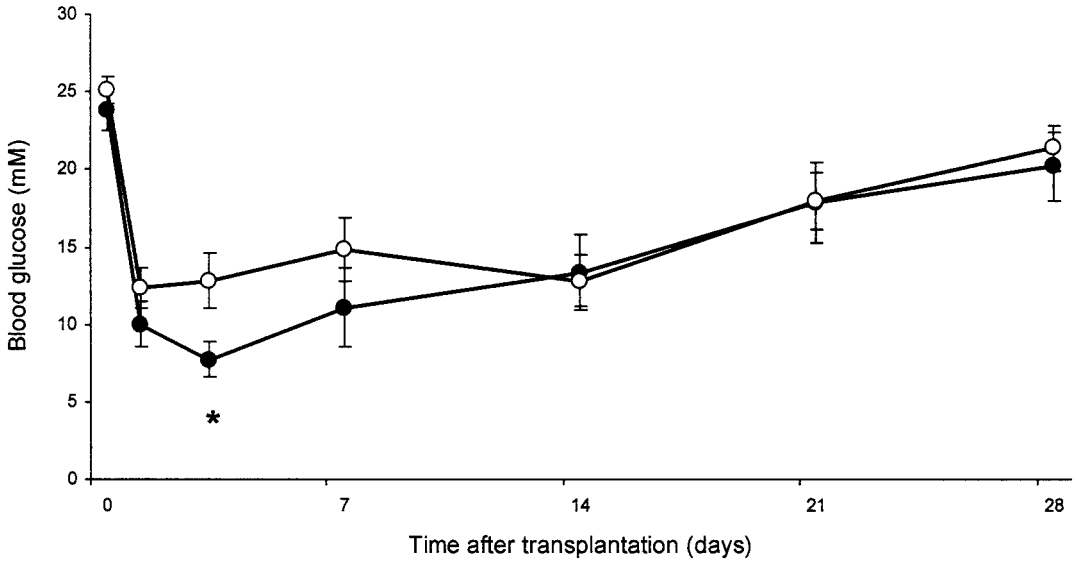

Figure 5. Blood glucose concentrations of alloxan-diabetic mice transplanted with 350 encapsulated islets. The islets had been cultured for 3 days prior to transplantation in the absence (open circles) or presence (filled circles) of 1 nM exendin-4. $*p < 0.05$ for mice grafted with islets cultured in the presence of exendin-4, when comparing with control mice ($n = 9–10$).

mentioned above. This compound has been used in nonencapsulated islet transplantation and shown very promising results (17,29). Pretreatment of encapsulated islets could potentially lead to a completely drug-free islet transplantation.

In our study, we could detect some of the previously reported positive effects of exendin-4 on beta cell function (25). Insulin secretion was increased both acutely in a 1-h incubation and over a 40-h period. This was seen in both encapsulated and nonencapsulated islets, indicating that exendin-4 can easily pass through the capsule. We, however, saw no change in insulin content, with a trend towards decreased insulin content in the encapsulated islets. This indicates that the insulin biosynthesis could not keep up with the demand for the increased insulin secretion. It has previously been shown that GLP-1 increases intracellular insulin content in beta cells after a 24-h incubation period (11). However, another study showed that this increase was not in line with the increase in insulin secretion (31). Thus, the exposure time used in the present study (72 h) may explain the lack of increase in insulin content, which has also been seen in a previous study using the same incubation period (17). The insulin content in encapsulated islets was decreased compared to that of naked islets. It is unclear whether this is due to real differences in insulin content of the microencapsulated islets or whether it was due to the technical difficulties of extracting all the insulin out of the encapsulated islets.

To our knowledge, the effect of exendin-4 on glucose oxidation has not previously been studied in islets. Glucose oxidation was unchanged in an acute experiment, whereas after 3 days of culture with exendin-4, glucose oxidation is increased. This indicates an indirect effect due to increased beta cell survival, rather than an effect on glucose oxidation per se.

It is thus clear that exendin-4 has got positive effects on microencapsulated islets in vitro. One interesting approach to improve the function of encapsulated islets is to conjugate GLP-1 to the capsule (15). This technique has shown promising results, although the system has not yet been evaluated in vivo.

In our in vivo studies, we used a suboptimal mass of 350 encapsulated islets transplanted intraperitoneally. We have previously shown that 1000 encapsulated islets are required to consistently reduce hyperglycemia in mice (16). However, more recent studies in our laboratory have indicated that 700 encapsulated islets produced with the present encapsulation technology can reduce hyperglycemia both in syngeneic (1) and allogeneic settings (unpublished observations). The use of a minimal mass of islets, however, allows differences in treatment groups to be determined; if all mice were to be cured, it would be difficult to determine whether the treatment is beneficial. We found that 350 encapsulated islets was indeed a minimal mass, with most mice not retaining normoglycemia. In the present study, no effect of systemic administration of exendin-4 was detected. This is in line with a previous study (17). It was envisaged that exendin-4 may have had more effect in the present study as the microencapsulated islets were located in the peritoneal cavity, which was the site of ex-

endin-4 injection. Using a similar dose as in the present study, Sharma et al. were able to show a positive effect of recipient treatment with exendin-4 on islet graft function (29). The reason for this disparity in results is unclear, although it should be pointed out that in their study they implanted rat islets that had been in culture for a shorter time period.

Although treatment of encapsulated islets in culture had positive effects on islet function in vitro, the effect after transplantation lasted less than 7 days. The reason for this very transient effect is unclear. One major difference in our model, compared to previous studies using nonencapsulated islets, is the kinetics of glycemia. In vascularized minimal mass islet grafts, the animals tend to gain normoglycemia slowly (17). In this encapsulated islet model, all animals normalized their blood glucose levels initially. However, most animals were not able to retain the normoglycemia. The mice increased their weight during this time period, indicating that the low blood glucose levels were not due to a lack of food intake. The reason for the graft failure could be due to an inability of the encapsulated islets to compensate for the increased insulin demand. Indeed, it may be that the islets have been stimulated too much in relation to their ability to synthesize insulin. It is likely that by increasing the number of encapsulated islets in the grafts we would have maintained long-term normoglycemia, but in the present model where the encapsulated islets were placed in a metabolically demanding environment, exendin-4 could not prevent loss of graft function. It can be concluded that exendin-4 does indeed increase the function of encapsulated islets, but, in contrast to nonencapsulated islet grafts, the increased function seen in vitro was not enough to improve the outcome in a transplantation model where approximately half the required number of islets was transplanted.

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