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EFFICACY OF A PREVASCULARIZED EXPANDED POLYTETRAFLUOROETHYLENE SOLID SUPPORT SYSTEM AS A TRANSPLANTATION SITE FOR PANCREATIC ISLETS 1

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Abstract

An intraperitoneally located and prevascularized expanded polytetrafluoroethylene solid support is potentially a suitable transplantation site for encapsulated pancreatic islets, because it allows for both the implantation of a large volume islet graft in the immediate vicinity of blood vessels, and its complete removal. The present study investigates the efficacy of such solid supports for the implantation of nonencapsulated islet isografts in streptozotocin diabetic rat recipients. These solid supports were always coated with acidic fibroblast growth factor, because we found that this growth factor enhances the neovascularization. The success rates of 5-μl (group A) and 10-μl (group B) islet isografts in solid supports were compared with the success rates of 5-μl (group C) and 10-μl (group D) islet isografts implanted in the unmodified peritoneal cavity. Four of seven rats in group A and all seven rats in group B became normoglycemic for at least 6 months. Only two of eight rats in group C and four of eleven rats in group D showed normoglycemia. The normoglycemia lasted for at least 6 months in zero of two animals in group C and in three of four animals in group D. Because of the low success rates in groups C and D, intravenous and oral glucose testing were restricted to the successful recipients in groups A and B. Glucose tolerance was found to be proportional to the grafted islet volume but, expectedly, in both groups the glucose tolerance and the insulin responses were somewhat lower than in controls. Thus, the prevascularized expanded polytetrafluoroethylene solid support, rather than the unmodified peritoneal cavity, is an efficacious transplantation site, potentially suitable for encapsulated islets.

None of the currently reported sites allowing for successful islet transplantation, i.e., the liver (1-4), the spleen (4-7), beneath the renal capsule (4, 8-11), and the peritoneal cavity (12-16), combine the capacity to bear high numbers of islets and retrievability of the islet graft. A site with both features may be mandatory for clinical transplantation of encapsulated islets, because such grafts still have their functional limitations and, therefore, may require repeated replacement (17).

Recently, the concept has been introduced of an intraperitoneally implanted solid support as a transplantation site for genetically engineered cells (18, 19). Such a solid support may serve as a transplantation site for pancreatic islets as, theoretically, it allows for implantation of high numbers of islets that can be readily retrieved. Additional requirements are that the material is nontoxic for islets and does not interfere with their function, and that it allows for rapid vascularization of the islets, to limit the period of ischemia.

Solid supports made of expanded polytetrafluoroethylene (ePTFE *) are probably the most suitable for islet grafts, as they have been shown to be biocompatible (20) and to become neovascularized after implantation in the peritoneal cavity (20-24). These solid supports consist of ePTFE angel hair-like fibers coated with collagen type IV (20-24) and are, in some studies, additionally coated with acidic fibroblast growth factor (a-FGF) (20, 21, 23).

We tested the efficacy of ePTFE solid supports as a transplantation site for pancreatic islets. To this end, we compared the results of two different volumes of islet isografts implanted in solid supports to those implanted in the unmodified peritoneal cavity, by comparing success rates and by determining glucose tolerance and insulin responses to intravenous and oral glucose challenge. Before the experiments, we determined whether there was any additional neovascularization of the supports when a-FGF was included in the coating procedure, because the role of a-FGF in the neovascularizing of ePTFE solid supports is the subject of some controversy (20-24).

MATERIALS AND METHODS

Design of the study. Diabetic Albino Oxford (AO) rats were intraperitoneally implanted with ePTFE-solid supports, either coated or not coated with a-FGF. After 4 weeks of implantation, the solid supports were examined macroscopically for the presence of blood vessels, and subsequently processed for microscopical assessment of the degree of neovascularization by immunocytochemistry. These experiments were performed to determine whether coated or non-coated ePTFE would be used in our subsequent islet transplantation studies.

For the islet transplantation studies, we used isogenic transplants, to exclude the effects of graft rejection. Graft endocrine volumes studied were 5 µl and 10 µl, which correspond to 50% and 100% of the endocrine volume of the normal rat pancreas, respectively (4, 25). Islets were transplanted either into a solid support, which had been implanted in the peritoneal cavity 4 weeks previously, or into the unmodified peritoneal cavity of diabetic rats. Transplantation was considered successful when nonfasting blood glucose concentrations reached levels below 8.4 mmol/L, within 4 weeks after transplantation. If successful, graft function was tested by an intravenous glucose tolerance test and by a meal test (25, 26). Normal rats served as controls.

Recipients of islet grafts were followed and monitored for blood glucose levels for at least 6 months, after which they were killed for histological evaluation of the native pancreas and the solid supports.

Animals and induction of diabetes. Male inbred AO/G rats were obtained from the Central Animal Laboratory of Groningen. AO/G rats, weighing 300-350 g, served as donors. AO/G rats, weighing 290-320 g, were used as recipients of islet grafts. Diabetes was induced in these rats by injection of 75-90 mg/kg of streptozotocin (Zanosar, Upjohn Co., Kalamazoo, MI) via the tail vein. A second injection of 70-90 mg/kg of streptozotocin was administered to those animals in which the blood glucose level was still below 20 mmol/L at 10-14 days after the first injection. Solid supports were implanted only in animals with severe weight loss, polyuria, polydipsia, and fasting blood glucose levels exceeding 20 mmol/L over a period of at least 4 weeks. Four weeks after implantation of the solid supports and, thus, 8 weeks after induction of diabetes, the animals were used as recipients of islet grafts. Animals receiving an islet graft in the unmodified peritoneal cavity had also been hyperglycemic for 8 weeks. The absence of Beta cells in the native pancreas, defined as less than 5% of normal controls, was always histologically confirmed at the time of death.

Solid supports. Solid supports consisted of multifilament, angel-hair fibers of ePTFE; the fibers were obtained through the courtesy of W.L. Gore & Associates Inc. (Flagstaff, AZ). The solid supports were prepared from the ePTFE-fibers by a modification of the method described by Thompson et al. (20). Briefly, 125 mg of ePTFE-fibers were sterilized by [gamma]-irradiation (^{137}Cs, 40 Gy) in tubes that were siliconized. All further procedures were performed in these siliconized tubes under sterile conditions. The sterile fibers were incubated with 50 µg of collagen IV (native collagen IV isolated from Engelbreth Holm Swarm Sarcoma, dissolved in phosphate-buffered saline [PBS]; Sigma, St. Louis, MO) for 2 hr at 37°C, followed by overnight drying at 42°C in a sterile incubator. Next, the fibers were aggregated into solid supports, not coated with a-FGF. In six separate procedures, we prepared six of these noncoated solid supports. Two were implanted into the unmodified peritoneal cavity of rats. The other four were subjected to a subsequent coating procedure with a-FGF. These were incubated with hormonally defined medium (27) for 16 hr at 37°C under severe agitation, followed by three washing steps with PBS. Then the supports were coated with 1 µg of a-FGF (bovine FGF acidic, R&D Systems, Abingdon, UK) for 1 hr at 4°C, washed three times with PBS, and implanted into the unmodified peritoneal cavity of rats.

Islet isolation. Islets were isolated as previously described (28). Briefly, under ether anesthesia, the abdomen was opened and the common bile duct was cannulated under nonsterile conditions. The donor pancreas was distended with 10 ml of sterile Krebs-Ringer-HEPES supplemented with 25 mmol/L HEPES buffer and containing 10% bovine serum albumin.

The pancreas was chopped, digested using a two-stage incubation of 20 min at 37°C with 1.0 and 0.7 mg/ml collagenase successively (Sigma type XI, Sigma). Islets were separated from exocrine tissue, using a discontinuous dextran gradient (29, 30), and were further purified by handpicking.

The total islet volume obtained by the isolation procedure was determined by measuring the diameters (25) of islets in a 4% aliquot of the islet suspension. Subsequently, the total volume was calculated, assuming the islets to be perfect spheres. In each experiment, we used four to five donors, from which we isolated a total of 3500-4200 islets, which corresponds to 10-15 µl of islet tissue.

The diameter of the islets was measured with a dissection microscope with a fluorescent illuminator (Bausch and Lomb BVB-125 and 31-33-66), equipped with an ocular micrometer with an accuracy of 25 µm.

Surgery. For transplantation in the unmodified peritoneal cavity, islets were injected into the peritoneal cavity with a butterfly needle, via a small incision (3 mm) in the linea alba.

Solid supports were placed under the left liver lobuli without any fixation, via laparotomy, 4 weeks before transplantation of islets. For transplantation of islets, relaparotomy was performed, and the islets were injected through a 25-gauge needle that was introduced into multiple tunnels in the solid support, which had been previously made by puncturing the solid support with a larger, 20-gauge needle, in several directions.

At 10 to 14 weeks after successful transplantation, the rats were provided with two cardiac catheters implanted via the right and left jugular vein for, respectively, blood sampling and infusion of glucose in unanesthetized and freely moving rats (26, 31).

All procedures were performed under halothane anesthesia.

Glucose tolerance tests. Intravenous glucose tolerance tests were performed by infusion of 200 mg of glucose at a rate of 10 mg/min. The meal tests were carried out by offering the animal 2 g of rat chow (containing 53% carbohydrates, 20% protein, 5% fat, and 22% other constituents [minerals, cellulose, water]), mixed with 2 ml of water. The animals had been habituated to consume the meal within 5 min.

In both tests, blood samples were taken at 10 min and immediately before the start of the tests, and at 1, 3, 5, 7, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, and 120 min after the start of the tests. Blood lost during sampling was replaced by transfusing blood from normal donor rats, after every blood sample.

In some recipients, glucose tolerance could not be completely tested or could not be tested at all, as a consequence of technical complications, such as death related to cannulation surgery or to embolism introduced during the tests, occlusion of the catheters, or refusal to eat more than 70% of the meal.

Chemical determinations and calculations. Glucose concentrations during the glucose tolerance testing were determined in whole blood by a ferricyanide method, with a Technicon autoanalyzer. The glucose concentration in blood obtained at two weekly time intervals was determined with glucose test tapes (Reflolux, Boeringer Mannheim, Germany). Plasma insulin was measured by a radioimmunoassay (rat insulin RIA kit; Linco, St. Charles, MO), with rat insulin as a standard.

The amount of plasma insulin responsible for the plasma insulin elevation during the 20-min period of infusion of glucose was quantified by calculating the area under the curve from the first measurement above basal value until the end of the infusion. The amount of insulin responsible for the total plasma insulin elevation during the meal test was quantified by calculating the area under the curve from the first measurement above basal value until basal values were reached again.

Histology and immunohistochemistry of recovered material. Solid supports used for investigating the effect of a-FGF on neovascularization were fixed in precooled 2% paraformaldehyde, buffered with 0.05 M phosphate, and processed for glycol methacrylate embedding (32, 33). Sections were prepared at 2 μm and processed for immunohistochemical staining of the endothelial cell marker von Willebrand factor (anti-VWF, Dako, Glostrup, Denmark), to study the degree of neovascularization. Sections were incubated with the polyclonal antibody, and then with normal goat serum. After several washing steps, the sections were incubated with appropriate goat anti-rabbit and subsequently with horseradish peroxidase-conjugated second-step reagents. Peroxidase activity

was demonstrated by applying 3,3"-diaminobenzidine tetra HCl, containing hydrogen peroxidase. In control sections, we used PBS, instead of the first-stage monoclonal antibody.

The solid supports implanted with islets were always divided in two parts. One half was fixed in precooled 2% paraformaldehyde, and processed for methacrylate embedding and VWF immunohistochemical staining as specified above. The second half of the islet-containing solid support system was fixed in Bouin's solution for paraffin processing, sectioned at 5 μm, and stained with aldehyde fuchsin to determine the presence or absence of viable Beta cells.

Statistical analysis. Comparison of the transplant success rate was performed by applying Fisher's exact probability test. All further statistical evaluations were performed using the Mann-Whitney *U* test. Only $P<0.05$ was considered to indicate a statistically significant difference. Values are expressed as mean ± SEM.

RESULTS

Effect of coating with a-FGF. On macroscopical examination 1 month after implantation, solid supports coated with a-FGF were found to be covered and penetrated by large numbers of blood vessels (Fig. 1). This high degree of neovascularization was only observed in the presence of a-FGF. This different degree of neovascularization was confirmed in our subsequent microscopical study, in which only a few blood vessels were found in the noncoated solid supports, whereas large numbers were found in the a-FGF-coated solid supports (Fig. 2). Consequently, we chose to apply a-FGF-coated solid supports in our subsequent transplantation studies.

Figure 1. An a-FGF-coated ePTFE-solid support, 4 weeks after implantation in a diabetic AO rat. Blood vessels indicated by the arrows are running over and into the solid support.

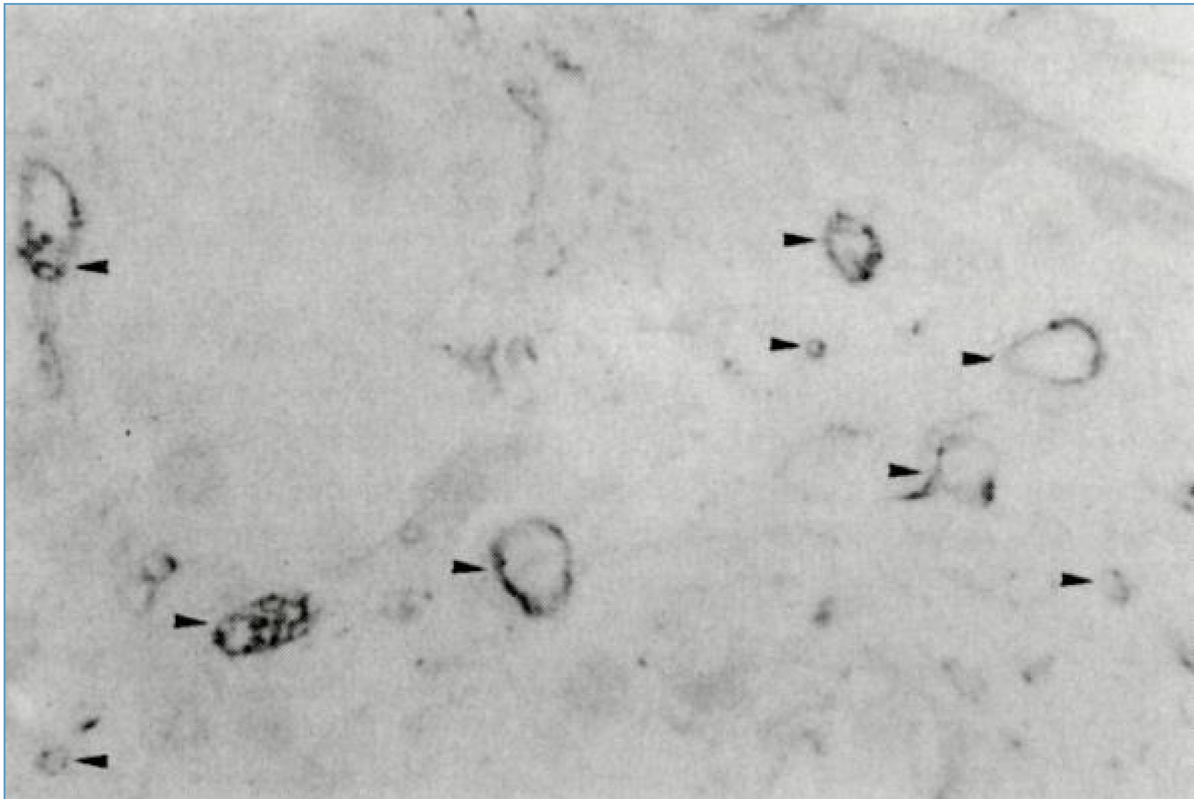

Figure 2. Blood vessels in a solid support coated with a-FGF. The arrows indicate VWF-positive groups of endothelial cells. (glycolmethacrylate embedded histological section, stained for VWF, original magnification ×200.)

Transplant success. In all animals with 10-μl islet grafts and in 57% of the animals with 5-μl islet grafts placed in the solid support, normoglycemia was restored within the first week after transplantation. Success rates were better with the solid supports than with the unmodified peritoneal cavity. This difference, however, was statistically significant only with the 10-μl islet grafts (P<0.01) (Table 1). All animals with successful islet grafts in a solid support remained normoglycemic for the duration of the study, which was a period of 6 months; among the recipients of successful islet grafts in the unmodified peritoneal cavity, one with a 10-μl islet graft and all with a 5-μl islet graft returned to hyperglycemia within this period of time.

Transplant site	Success rate[a]	
	5-μl graft	10-μl graft
Solid support	4/7 (57%)	7/7 (100%)[b]
Unmodified peritoneal cavity	2/8 (25%)	4/11 (36%)

[a] Successful grafts/total number of grafts (percentage in parentheses).

[b] Significantly higher success rate than in recipients of 10-μl islet grafts in the unmodified peritoneal cavity (P<0.01).

Table 1. Transplant success rate of two different volumes of islet isografts implanted in solid supports or in the unmodified peritoneal cavity

At the time of death, large numbers of viable islets were found in solid supports retrieved from the peritoneal cavity (Fig. 3), but we were unable to retrieve islets from recipients of islet grafts in the unmodified peritoneal cavity.

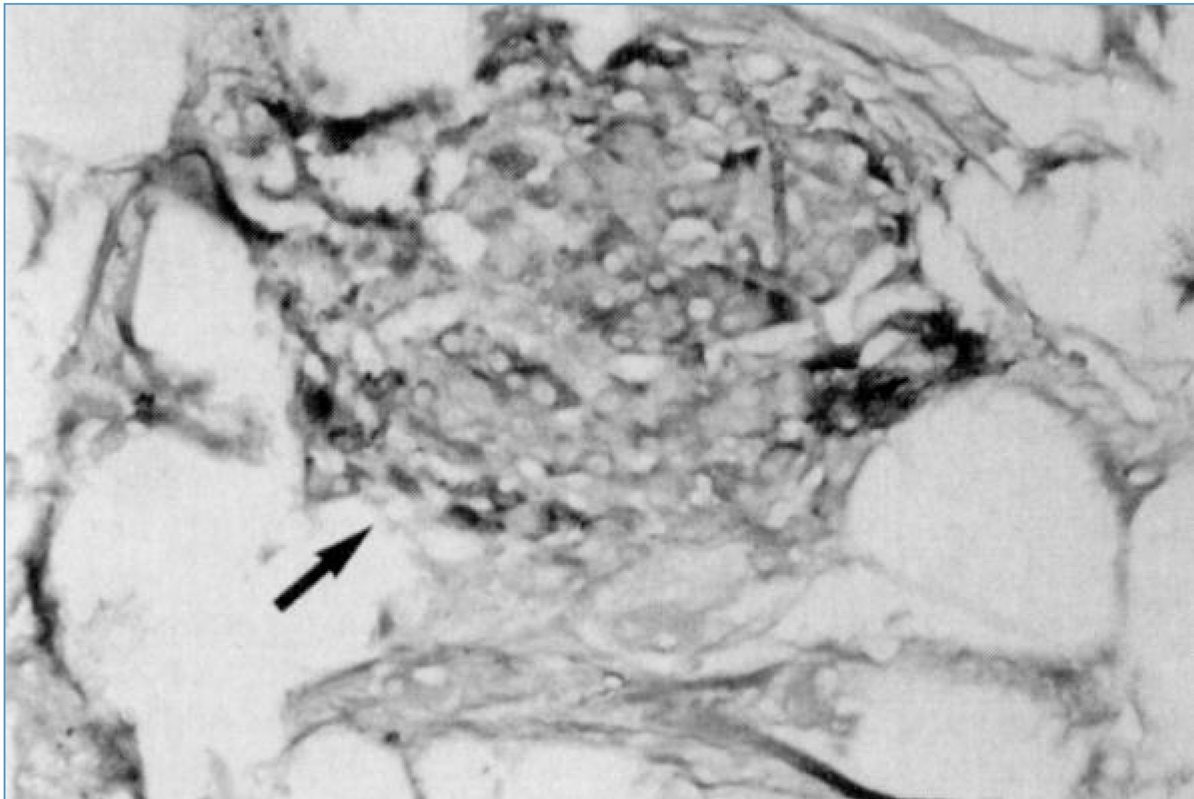

Figure 3. Well granulated, viable islets in a prevascularized solid support, 6 months after grafting. (Paraffin-embedded histological section, aldehyde fuchsin stained, original magnification ×450.)

Glucose tolerance tests. In view of the small number of animals involved, we have not analyzed the glucose tolerance of animals with successful grafts in the unmodified peritoneal cavity.

Basal blood glucose levels in animals with 5-μl and 10-μl islet grafts in solid supports did not differ significantly from normal control animals (Figs. 4 and 5), but basal insulin levels were lower in recipients than in controls.

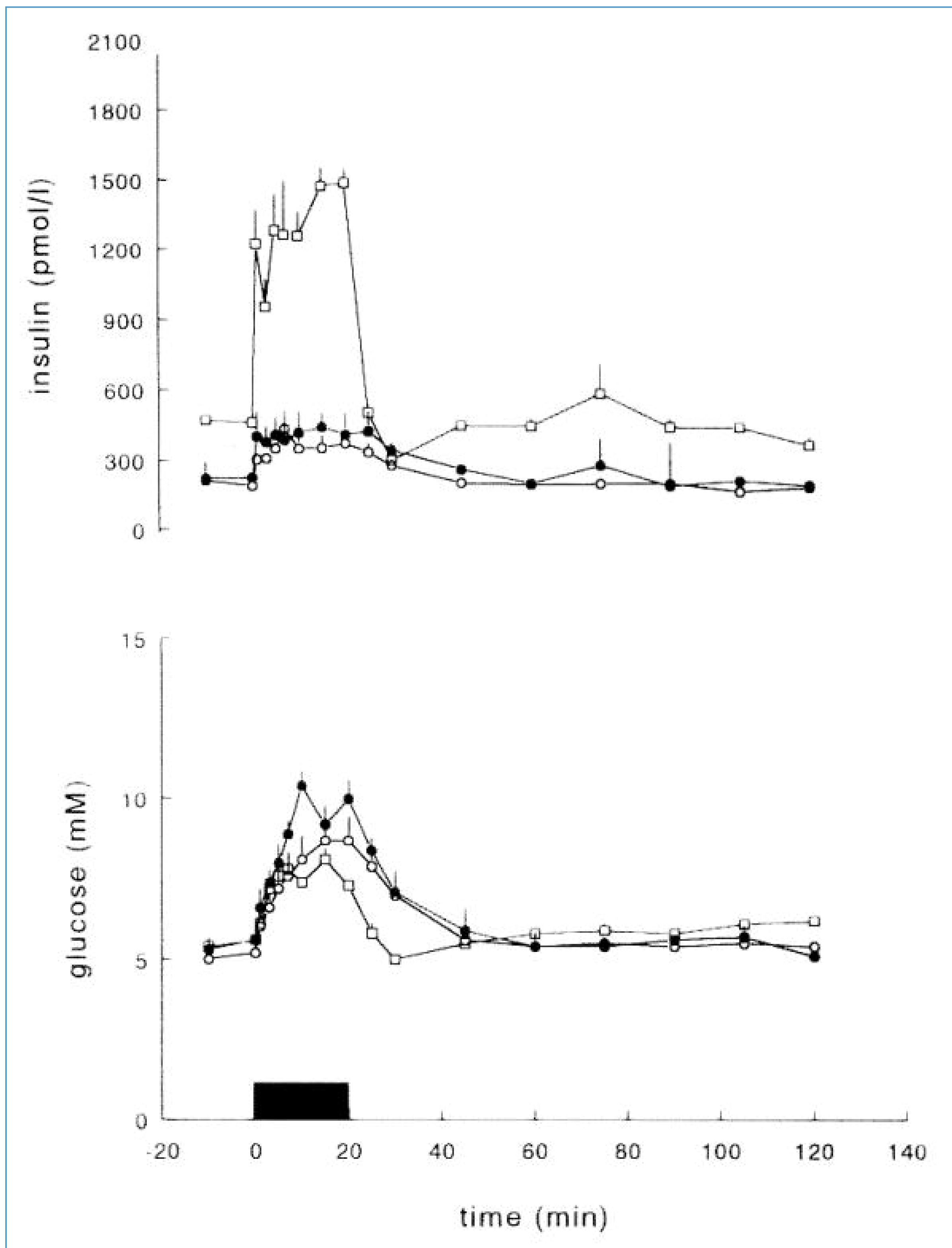

Figure 4. Blood glucose and plasma insulin levels after intravenous glucose infusion (10 mg/min) in AO recipients of 5-μl (•) (n=3) and 10-μl ([white circle]) (n=5) islet isografts in solid supports and in normal control rats ([white square]) (n=4).

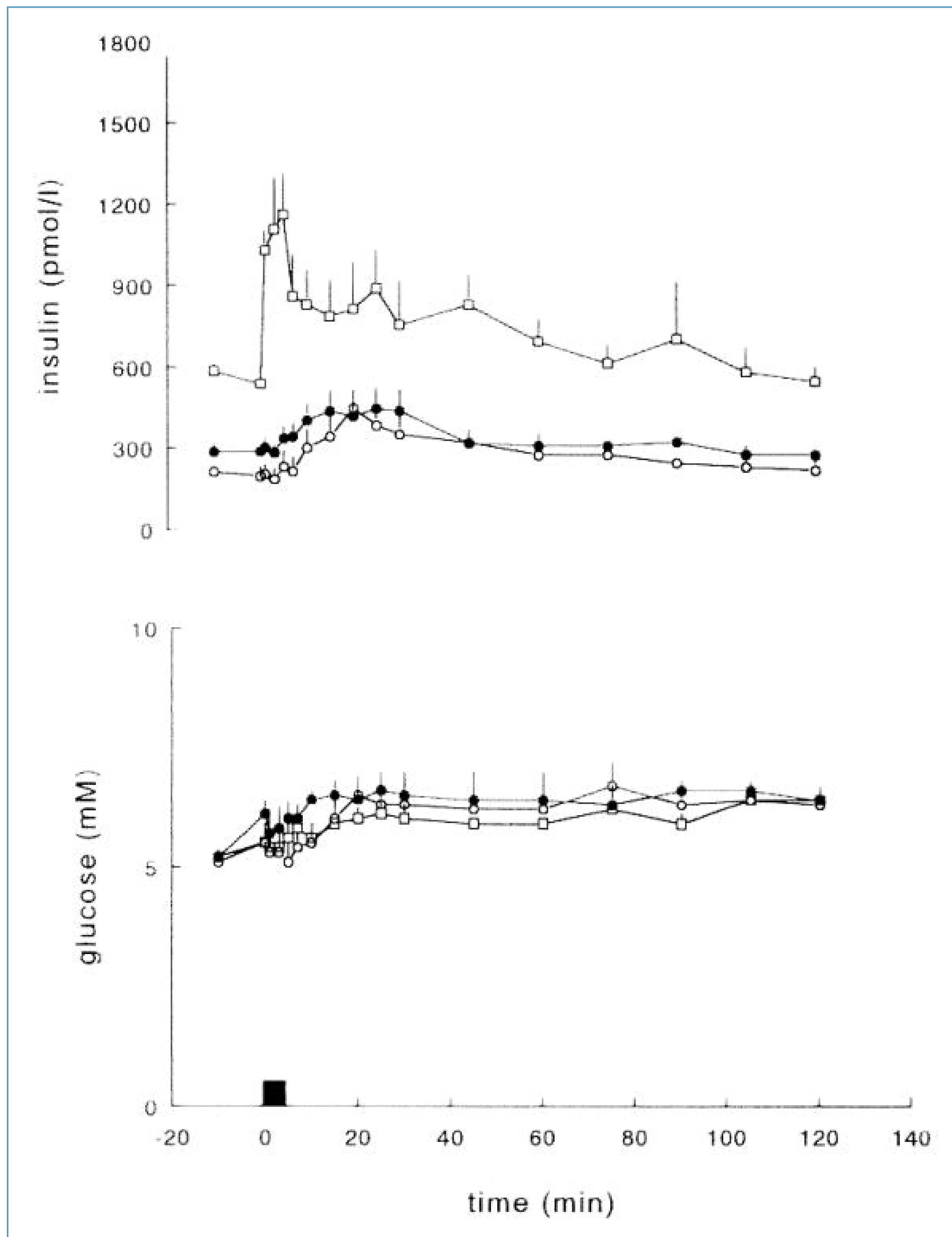

Figure 5. Blood glucose and plasma insulin levels after spontaneous ingestion of a meal in AO recipients of 5-μl (•) (n=4) and 10-μl ([white circle]) (n=5) islet isografts in solid supports and in normal control rats ([white square]) (n=4).

Glucose levels during infusion of glucose in animals bearing the islet grafts in the solid supports reached somewhat higher levels than in normal controls (Fig. 4). These higher glucose levels corresponded well with the concomitant lower insulin secretion by the grafts, as estimated by the insulin secretion during the infusion of glucose (Table 2). Although the insulin responses were quantitatively lower than in normal controls, both groups of recipients showed a biphasic insulin response.

	AUCivgtt(ins20) (pmol/L · min)
Control	16,622±1,917
10-μl isograft	3,609±632[b]
5-μl isograft	4,003±1,184[b]

[a] Recipients were tested at 11–15 weeks after transplantation. Insulin secretion is expressed as area under the curve from 0–20 min (AUCivgtt(ins20)). Values are mean ± SEM.
[b] Significantly different versus controls ($P<0.05$).

Table 2. Insulin secretion in response to intravenous glucose infusion in normal rats (control) and in rats with isografts in a solid support[a]

With the meal tests, we also observed the maximum glucose increments in recipients to be higher than in controls (Fig. 5) and the insulin secretion to be concomitantly lower by the grafts (Table 3). The insulin responses to the meal, in recipients and controls, were not only quantitatively, but also qualitatively, quite different. In normal controls, we observed a clear preabsorptive insulin secretion, i.e., a significant increase of the plasma insulin level preceding an elevation of the glucose level, during the first minute of food ingestion. This preabsorptive insulin secretion was absent in recipients, which showed a first significant rise of insulin secretion only after blood glucose elevation.

	AUCmeal(instotal) (pmol/L · min)
Control	32,228±12,840
10-μl isograft	10,890±2,256[b]
5-μl isograft	8,321±3,816[b]

[a] Recipients were tested at 11–15 weeks after transplantation. Insulin secretion is expressed as area under the curve from the first measurements above basal value until basal values were reached again (AUCmeal(instotal)). Values are mean ± SEM.
[b] Significantly different versus controls ($P<0.05$).

Table 3. Insulin secretion in response to the ingestion of a meal in normal rats (control) and in rats with isografts in a solid support[a]

DISCUSSION

This study shows that islet grafts can be successfully transplanted into an ePTFE solid support. It also shows these solid supports to be efficacious transplantation sites, when compared with the unmodified peritoneal cavity. Furthermore, this study demonstrates that a-FGF coating promotes neovascularization of ePTFE solid supports.

Our findings regarding the promoting effect of a-FGF on vascularization seem to contradict those of Borel Rinkes et al. (22-24), but they corroborate those of Thompson et al. (20, 21). The contradictory findings should largely be explained by differences in the technique of coating the ePTFE fibers. The drying temperature of

ePTFE-fibers after incubation with collagen IV was 42°C in our procedure, whereas it was room temperature in the procedure of Borel Rinkes et al. (23, 24). Application of this high-temperature drying step is essential, as this temperature induces denaturation of collagen IV (34), which may promote adhesion of molecules and, therefore, also promote the adhesion of a-FGF. Furthermore, the procedure of Borel Rinkes et al. does not contain an incubation step with hormonally defined medium, as in our procedure and that of Thompson et al. (20). This hormonally defined medium (27) contains growth factors such as insulin, epidermal growth factor, and somatotropin, which are known to stimulate mitosis (35-39) and also migration (39-42) of cells and, therefore, may contribute to the neovascularizing effect of a-FGF (43-45).

Blood vessels vascularizing the solid supports originate from the adjacent organs (20). By transplanting the solid supports under the liver lobuli and adjacent to the native pancreas, we have tried to favor portal drainage of insulin secreted by the islets in the solid supports and, by that, to mimic the physiological route of insulin delivery. As reported in one of our previous studies (4, 25), portal drainage of insulin secreted by the islet graft is associated with normal basal insulin concentrations. Indeed, as observed in our previous studies (4, 25), basal insulin levels were similar, not only to those of normal controls, but also to the levels in AO rat recipients of 5 µl and 10 µl of islet tissue in the spleen, i.e., of portally draining islet grafts. Our present controls, however, had basal insulin levels twice as high (4, 16, 25), for which we have no explanation.

Although there were good survival rates of islet grafts transplanted in solid supports, glucose tolerance remained somewhat disturbed, even with 10-µl islet grafts, i.e., 100% of the endocrine volume of the normal rat pancreas. This, however, was to be expected, because we found in a previous study (4) that grafting of 10-µl islet tissue into other efficacious islet transplantation sites like the liver, the spleen, and the renal capsule, was also associated with disturbed glucose tolerance. Factors contributing to this impaired glucose tolerance are probably (i) a reduction of the functioning Beta-cell mass as a consequence of ischemia, during the period before revascularization (46) and (ii) an inadequate reinnervation of the islets (25, 47). This inadequate reinnervation of islets transplanted in solid supports was illustrated by the absence of a preabsorptive insulin secretion, which is assumed to be accomplished by vagal stimulation (4).

Our present findings on the efficacy of the peritoneal cavity as a transplantation site for pancreatic islets seem to contradict our previous study, in which we found that the peritoneal cavity is an efficacious transplantation site for islet isografts, as 100% of the recipients of both 5-µl and 10-µl islet isografts became normoglycemic (16). The experimental protocol of our previous study, however, differs principally in two ways from the present protocol. First, the recipients in the present study were hyperglycemic for a longer period of time, i.e., at least 8 weeks as opposed to 2 weeks in the previous study. The longer time period is found to be associated with functional impairment (48, 49) and reduced survival of grafted islets (50, 51). Second, in the present study, islets were introduced into the unmodified peritoneal cavity by laparotomy, instead of by direct injection through the abdominal wall. A laparotomy is more traumatic and associated with wound healing and, therefore, with the release of cytokines (52), which may have a deleterious effect on the islets (53-58). It was, nevertheless, used for the sake of a sound comparison to recipients with solid supports, because the introduction of a solid support necessitates a laparotomy.

The principle of grafting in solid supports is not only applicable for nonencapsulated islets, but is also an attractive implantation site for encapsulated islets. As a consequence of its large volume, such an encapsulated islet graft can only be transplanted in the peritoneal cavity, where it remains nonvascularized as a freely floating graft. The absence of vascularization interferes with insulin (59) and glucose responses (60) and with graft survival (61-62).† Thus, encapsulated islets should preferably be transplanted in a well-vascularized site, such as a prevascularized solid support. ePTFE seems to be a suitable material for construction of these solid supports, as ePTFE is biocompatible (20, 63), can be conveniently coated with various components of the extracellular matrix (20, 21, 64, 65), and can be engineered to meet any required configuration (20, 66-68).

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Footnotes

This work was supported by a grant from the Diabetes Research Fund of The Netherlands. [Context Link] [Context Link]

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